Immuno-Double-Diffusion Serological Relationships Among *Spiroplasma* Isolates

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Spiroplasma citri strains Maroc R8A2^T (T = type strain), C189, and C3B, corn stunt *Spiroplasma* sp. strain E275, and honeybee *Spiroplasma* sp. strain BC3 all cross-reacted in reciprocal serological tests when an agar double-diffusion system was used. These strains, which are members of *Spiroplasma* group I, did not show any cross-reactions with flower *Spiroplasma* strains BNR1 and PPS1, (*Spiroplasma floricola* and "*Spiroplasma apis*") or with the tick-derived organism *Spiroplasma mirum*. Tick *Spiroplasma* sp. strain 277F and honeybee *Spiroplasma* sp. strain AS576 reacted only with antisera prepared against other members of group I. Group III flower spiroplasmas *S. floricola* 23-6^T and BNR1 and group IV strains PPS1 and SR3 did not react with antisera prepared against isolates outside their own groups. The members of each major group showed close antigenic relatedness to each other but not to *S. mirum* strain SMCA^T, which showed minimal one-way cross-reactions with members of group I. Previous serogrouping results for the *Spiroplasma* strains tested were confirmed by the serological reactions of the organisms in the double-diffusion system.

The list of spiroplasmas that have been isolated and cultivated has become extensive enough to establish criteria that are useful for strain and species identification within the genus *Spiroplasma*. The following techniques can be used to characterize and compare the diverse isolates of *Spiroplasma*: serological methods, such as growth inhibition (3, 16, 18-21, 23; R. E. Davis et al., Abstr. Annu. Meet. Am. Phytopathol. Soc. 1978, abstr. no. 415, p. 215; B. C. Raju and T.-A. Chen, Abstr. Div. Meet. Am. Phytopathol. Soc. 1977, abstr. no. NE-71, p. 195) and complement fixation (19); organism deformation (16, 23, 25; A. H. McIntosh et al., Abstr. Div. Meet. Am. Phytopathol. Soc. 1977, abstr. no. NE-57, p. 193; Raju and Chen, Abstr. Div. Meet. Am. Phytopathol. Soc. 1977); metabolic inhibition (3, 7; R. E. Davis, Abstr. Div. Meet. Am. Phytopathol. Soc. 1978, abstr. no. PO-7, Phytopathol. News, Vol. 12; Raju and Chen, Abstr. Div. Meet. Am. Phytopathol. Soc. 1977); ring interface precipitin tests (19; R. E. Davis et al., Abstr. Annu. Meet. Am. Phytopathol. Soc. 1978; Raju and Chen, Abstr. Div. Meet. Am. Phytopathol. Soc. 1977); and an enzyme-linked immunosorbent assay (1). To our knowledge, immuno-double-diffusion has not been used as a technique to characterize and compare isolates of *Spiroplasma*. Within the genus *Mycoplasma*, it has been reported that some strains which are distinguishable by other serological methods appear to be closely related on the basis of immuno-double-diffusion tests (11, 12, 17) and that these tests provide a means of clarifying the status of strains or variants of a given species (8, 12). Thus, the use of immuno-double-diffusion as a method for demonstrating group antigens might provide a means for serologically defining strains of *Spiroplasma* species.

The present study was undertaken (as part of a cooperative effort of the *Spiroplasma* Working Team of the International Research Programme of Comparative Mycoplasmaology) to determine the antigenic relationships among 12 *Spiroplasma* strains by using immuno-double-diffusion tests to identify common or group antigens. The 12 strains were chosen to represent the four major *Spiroplasma* groups and

the subgroups of group I described by Davis et al. (7) and Williamson et al. (22). After this study was completed, revisions of the serological classification added several new groups (20; J. M. Bové et al., Yale J. Biol. Med., in press).

MATERIALS AND METHODS

Organisms. The *Spiroplasma* strains used in this study and the sources from which they were derived are shown in Table 1. *Spiroplasma citri* strains C3B, C189, and Maroc R8A2^T (T = type strain) were from cloned cultures maintained in our laboratory. Cloned cultures of strains E275 (corn stunt strain), 277F (tick strain), and BC3 (honeybee strain), *Spiroplasma floricola* BNR1, "*Spiroplasma apis*" PPS1, and *Spiroplasma mirum* SMCA^T (Georgia), were supplied by R. F. Whitcomb, Plant Protection Institute, U.S. Department of Agriculture, Beltsville, Md. Cultures of strain AS576, *S. floricola* 23-6^T, and strain SR3 were supplied by R. E. Davis, Plant Virus Laboratory, Plant Protection Institute, U.S. Department of Agriculture, Beltsville, Md.

Cultivation of organisms and preparation of antigens. All strains except strain SMCA^T were propagated and maintained in M1A liquid medium (9). Strain SMCA^T was propagated and maintained in SP-4 liquid medium (19).

To eliminate nonspecific cross-reactions in the diffusion tests resulting from serum antigens, the medium used for cultivation of the immunizing antigen was supplemented with *Mycoplasma*-free bovine serum (GIBCO Laboratories, Grand Island, N.Y.), whereas horse serum (GIBCO) was used for growing the serological test antigens. A 2-ml sample from a logarithmic growth phase culture was used as the inoculum for each 600 ml of liquid medium. All cultures were incubated aerobically at 30 to 32°C until we observed a decrease in the pH, as indicated by a color change in the medium from red to yellow. At this stage the cultures were centrifuged at 14,000 × g for 20 min to sediment the organisms. The pelleted organisms were washed three times with phosphate-buffered saline and then suspended in 1/300 of the original culture volume of phosphate-buffered saline. The total protein contents of all preparations were measured by the procedure of Lowry et al. (13), using crystalline bovine albumin as the standard. For immunizing antigens,

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TABLE 1. Sources of strains of *Spiroplasma* species

Taxon	Strain	Host origin	ATCC no.	Reference(s)
<i>S. citri</i>	Maroc R8A2 ^T	Citrus (stubborn disease)	27556 ^T	15
<i>S. citri</i>	C189	Citrus (stubborn disease)	27665	
<i>S. citri</i>	C3B	<i>Circulifer tenellus</i>		Lee ^a
<i>Spiroplasma</i> sp.	E275	Corn (stunt disease)	28329	24
<i>Spiroplasma</i> sp.	277F	Rabbit tick	29761	2
<i>Spiroplasma</i> sp.	BC3	Honeybee	33219	4
<i>Spiroplasma</i> sp.	AS576	Honeybee	29416	7
<i>S. floricola</i>	BNR1	Tulip tree flowers	33220	20
<i>S. floricola</i>	23-6 ^T	Tulip tree flowers	29989 ^T	5
" <i>S. apis</i> "	PPS1	Powderpuff flowers	33450	14
<i>Spiroplasma</i> sp.	SR3	Tulip tree flowers	33095	5, 6
<i>S. mirum</i>	SMCA ^T	Rabbit tick	29335 ^T	19

^a I.-M. Lee, Ph.D. thesis, University of California, Riverside, 1977.

samples containing 15 to 25 mg of total protein were placed in small vials and stored at -60°C if they were not used immediately. Test antigens were divided into 1-ml portions containing 40 mg of total protein, lyophilized, and stored at -60°C until they were used. To observe nonspecific reactions with the medium components, a control antigen was prepared from uninoculated M1A and SP-4 broth media processed in an identical manner.

Preparation of antisera. Antisera against *S. citri* strains C3B, C189, and Maroc R8A2^T, corn stunt strain E275, honeybee strain BC3, flower strains BNR1 and PPS1, and the tick-derived organism *S. mirum* SMCA^T were prepared in rabbits. Before immunizations were started, 5 ml of pre-immunization serum was collected from each rabbit. Immunizing antigens were prepared by mixing 1.0 to 1.5 ml of antigen (15 to 25 mg of protein) with an equal volume of Freund complete adjuvant. Each animal received four intramuscular injections at 10-day intervals. At 10 days after the fourth injection, each animal was test-bled for titer determinations. When the serum titer, as determined by microprecipitin tests and immunodiffusion tests, reached 1:1,024 or above, the animals were bled every 3 or 4 days until the titer declined. The antisera were divided into 1-ml portions and stored at -20°C until they were used.

Gel diffusion tests. Because many *S. citri* antigens seem to be associated with membranes (7), the methods used for disrupting the cells of this organism are important in the satisfactory preparation of a test antigen for immunodiffusion systems (7). Cells of *S. citri* were treated in various ways, including osmotic shock with distilled water, treatment with surface-active agents and solvents, such as Igepon T-73 (sodium *N*-methyl-*N*-oleoyltaurate), sodium dodecyl sulfate, Leonil SA (sodium dibutyl-naphthalenesulfonate), Triton X-100, and polyvinylpyrrolidone, alternate cycles of freezing and thawing, and ultrasonic disruption. The effects of these treatments were evaluated by immunodiffusion. Igepon T-73 added at a final concentration of 0.1% to the serological test antigen 10 min before use resulted in the greatest number of precipitin lines in our immunodiffusion system.

The serological test antigens used in this study were diluted so they had comparable protein contents. Based on preliminary studies, two dilutions of antigen (40 and 10 mg of protein per ml) and two dilutions of antiserum (undiluted and diluted 1:3) were used in the four possible combinations. One test consisted of five replicates for each of the four combinations. All tests were repeated three times, with similar results. The tests were performed on immunodiffusion microslides containing an agar gel supplemented with

0.1% (wt/vol) sodium azide (Hyland Diagnostics, Bannockburn, Ill.). Each slide had three sets of wells, and each set comprised a central well and five peripheral wells that were 2 mm in diameter. The distance between the centers of the central well and the peripheral wells was 7 mm. The antigen wells were filled with 20 µl of antigen, and the slides were incubated at room temperature in a humid chamber for 2 to 5 days.

RESULTS

The pre-immunization rabbit sera did not react with antigens prepared in agamma horse serum, whereas the hyper-immune sera produced precipitation lines with the homologous antigens but not with concentrates of uninoculated broth media (control antigen). The antigens of 12 *Spiroplasma* strains were cross-tested with antisera against 8 of these strains (Table 2). As a result of these studies, the *Spiroplasma* strains listed in Table 2 were placed in the groups and subgroups that were proposed by Davis et al. (7) and Junca et al. (10) and later revised by Whitcomb et al. (20).

One example of the relationships among the *Spiroplasma* strains is illustrated in Fig. 1. Antiserum prepared against *S. citri* C189 formed three precipitation lines, designated lines a, b, and d (Fig. 1, wells 1, 4, 6, and 9), with the homologous antigen. At least one of the precipitation lines (line a) produced by the strain C189 antiserum and its homologous antigen fused with a corresponding line formed with *S. citri* Maroc R8A2^T and C3B and *Spiroplasma* sp. strains E275, 277F, BC3, and AS576, indicating that there was at least one identical antigenic component among these strains. The strains Maroc R8A2^T and C3B shared another common antigen (precipitin line b), which also fused with the corresponding line in the homologous reaction (Fig. 1, wells 1, 2, 4, and 5). In addition to this common antigen, a line corresponding to line b of the homologous reaction was also produced by the strain C189 antiserum and the antigens of strains E275, BC3, and 277F. These precipitin lines formed spurs at the junctions of the homologous reactions (Fig. 1, wells 2, 7, and 10), indicating that they are related to precipitin line b of the homologous systems but not identical. All strains except strains Maroc R8A2^T and C3B lacked precipitin line c. Furthermore, both strain C189 and strain C3B showed one additional line (designated line d for strain C189 and line e for strain C3B) (Fig. 1, wells 1 and 2). The strain PPS1, SR3, BNR1, 23-6^T, and SMCA^T antigens did not show any precipitation lines, suggesting that they did not have any antigenic components which were identical or related to strain C189 components.

TABLE 2. Comparison of 12 *Spiroplasma* isolates by double immunodiffusion tests

Group	Sub-group	Antigen from:	No. of precipitin lines with antiserum from strain:							
			Maroc R8A2 ^T	C189	C3B	E275	BC3	BNR1	PPS1	SMCA ^T
I	1	<i>S. citri</i> Maroc R8A2 ^T	3 (a,b,c) ^{a,b}	3 (a,b,c)	3 (a,b,c)	1 (a)	2 (a,d-p)	0	0	1 (a)
	1	<i>S. citri</i> C189	3 (a,b,d)	3 (a,b,d) ^b	3 (a,b,d)	1 (a)	3 (a,b,d-p)	0	0	1 (a)
	1	<i>S. citri</i> C3B	4 (a,b,c,e)	4 (a,b,c,e)	3 (a,b,c) ^b	1 (a)	3 (a,b,d-p)	0	0	1 (a)
	2	Honey bee strain BC3	2 (a,b-p)	2 (b-p)	1 (b-p)	1 (a)	4 (a,b,d,h) ^b	0	0	1 (a)
II		Honey bee strain AS576	1 (a)	1 (a)	0	0	2 (a,b-p)	0	0	1 (a)
	3	Corn stunt strain E275	2 (a,b-p)	2 (a,b-p)	2 (a,b-p)	2 (a,f,g) ^b	2 (a,d-p)	0	0	1 (a)
	4	Tick strain 277F	2 (a,b-p)	2 (a,b-p)	1 (b-p)	1 (a)	0	0	0	1 (a)
		<i>S. floricola</i> BNR1	0	0	0	0	0	3 (i,j,k) ^b	0	0
III		<i>S. floricola</i> 23-6 ^T	0	0	0	0	0	2 (i,j)	0	0
		Strain PPS1	0	0	0	0	0	0	2 (l,m) ^b	0
IV		Strain SR3	0	0	0	0	0	0	1 (l)	0
		<i>S. mirum</i> SMCA ^T	0	0	0	0	0	0	0	3 (m,n,o) ^b

^a The letters in parentheses are precipitin line designations; the suffix -p indicates partial identity (spurred precipitin lines).

^b Homologous reaction.

The results of our tests can be summarized as follows: (i) strains Maroc R8A2^T, C189, and C3B share at least two common antigenic components; (ii) strains Maroc R8A2^T and C3B share three common antigenic components; (iii) at least one antigenic component is common to strains Maroc R8A2^T, C189, C3B, E275, BC3, and AS576; (iv) strains Maroc R8A2^T, C189, C3B, E275, 277F, and BC3 have an additional partially related component; (v) strains BNR1, 23-6^T, PPS1 and SR3 are antigenically distinct from the strains mentioned above; and (vi) antiserum prepared against strain SMCA^T contained antibodies to one antigenic component of strains Maroc R8A2^T, C189, C3B, E275, 277F, BC3, and

AS576, but the reciprocal tests did not produce in similar reactions.

DISCUSSION

The data in Table 2 show very clear antigenic relationships among *S. citri* strains Maroc R8A2^T, C189, and C3B and, to a lesser extent, relationships with the other strains in group I (strains BC3, AS576, E275, and 277F). The *Spiroplasma* strains in groups III and IV showed no cross-reactions with the strains belonging to other groups. *S. mirum* SMCA^T, the only member of group V tested, did not show any cross-reactions with the strains in groups III and IV. However, it did have one common antigen with the group I strains. This common component was present in very low concentrations, because no precipitation line formed after antigen or antiserum dilution. This low-level one-way reaction may confirm previous reports that indicated a possible antigenic relationship between *S. mirum* SMCA^T and the corn stunt strain (18, 19). The one-way cross-reactions between the honeybee strains and antiserum to strain BNR1 that were revealed by metabolic inhibition and deformation tests (22) were not evident in this study. This is not surprising since the results of immuno-double-diffusion identification of mycoplasmas often differ from the results obtained by other methods (11, 12, 17).

Although common antigenic components among all strains of the same major group were not found with the techniques used in this study, they may exist but may not be recognized immunologically by rabbits, or they may be present in low concentrations and not detected by our assays. Despite our inability to demonstrate similar strong common antigens, the consistent presence of reciprocal cross-reactions among group I strains suggests that at least some members of this group possess common antigens which may be regarded as group antigens.

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FIG. 1. Photographs and line drawings of immuno-double-diffusion plates showing serological relationships among seven of the isolates tested. Well As, *S. citri* C189 antiserum; well 1, *S. citri* C189; well 2, *S. citri* C3B; well 3, *Spiroplasma* sp. strain E275; well 4, *S. citri* C189; well 5, *S. citri* Maroc R8A2^T; well 6, *S. citri* C189; well 7, *Spiroplasma* sp. strain BC3; well 8, *Spiroplasma* sp. strain AS576; well 9, *S. citri* C189; well 10, *Spiroplasma* sp. strain 277F.

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