Species-Specific and Cross-Reactive Monoclonal Antibodies to the Plant-Pathogenic Spiroplasmas *Spiroplasma citri* and *S. kunkelii*

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We gratefully acknowledge Mary Ann Guaragna for technical assistance and Peggy Hall for typing the manuscript. We also thank Hei-ti Hsu, Florist and Nursery Crops Laboratory (formerly of the American Type Culture Collection, Rockville, MD 20852), and Robert F. Whitcomb, Insect Pathology Laboratory, USDA-ARS, Beltsville, MD 20705, for providing some of the spiroplasma strains used in this study.

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Accepted for publication 27 March 1989 (submitted for electronic processing).

ABSTRACT

Jordan, R. L., Konai, M., Lee, I.-M., and Davis, R. E. 1989. Species-specific and cross-reactive monoclonal antibodies to the plant-pathogenic spiroplasmas *Spiroplasma citri* and *S. kunkelii*. Phytopathology 79:880-887.

A panel of 46 murine monoclonal antibody-secreting hybridomas was developed from mice immunized with a mixture of strains of *Spiroplasma citri* (M24OH) and *S. kunkelii* (I-747, F32, and PU8-17). Monoclonal antibodies with a range of specificities were selected in enzyme-linked immunosorbent assay (ELISA) tests by using intact and disrupted spiroplasma cells and purified spiroplasma cell membrane preparations. Forty of the monoclonal antibodies recognized antigenic sites found on Ig-trapped intact spiroplasmas and spiroplasma membrane preparations, whereas the remaining six monoclonal antibodies reacted only with disrupted spiroplasma cells in indirect ELISA. When used with 36 strains of spiroplasmas representing group I and groups III–XI spiroplasmas, 17 monoclonal antibodies reacted specifically with strains of *S. citri* (subgroup I-1), and an additional 17 monoclonal antibodies reacted only with *S. kunkelii* (subgroup I-3) strains. The remaining 12 monoclonal antibodies reacted with antigenic sites common in three to eight of the eight group I subgroups, including honeybee spiroplasmas, *S. melliferum* (I-2), 277F spiroplasma (I-4), green leaf bug spiroplasmas (I-5), Maryland flower spiroplasma (I-6), *Cocos* spiroplasmas (I-7), and the periwinkle spiroplasma, *S. phoeniceum* (I-8). None of the monoclonal antibodies reacted with any non-group I spiroplasmas tested. At least 17 different antigenic sites were defined by the 46 monoclonal antibodies. These discriminatory serological reagents will be useful for the detection and identification of spiroplasmas in plants and insects and for providing information on the antigenic relationships among other spiroplasma strains and newly recognized spiroplasmas.

Additional keywords: citrus stubborn, corn stunt, differentiation, identification, serologic relationships, serology, spiroplasma serogroups.

Spiroplasmas are a group of motile, helical, wall-free prokaryotes. Several spiroplasma species are plant or insect pathogens. They include *Spiroplasma citri*, the causal agent for citrus stubborn disease (31) and horseradish brittleroot disease (14); *S. kunkelii*, the etiological agent of corn stunt disease (33,39); *S. phoeniceum*, the causal agent of a yellowing disease of periwinkle (32); and *S. melliferum*, which is lethal to the honeybee (7). Spiroplasmas have also been isolated from other insects (5,10,26) and from the surfaces of flowers of several plant species (10,13,36).

Twenty-three spiroplasma groups and eight subgroups have been proposed, based on serological, biochemical, genomic, and cultural comparisons (38). Group I, referred to as the *S. citri* complex (3), contains eight subgroups with complex patterns of serological and genomic interrelationships. These spiroplasmas show various degrees of heterologous reactions in several serological tests, including growth inhibition, cell deformation, metabolic inhibition, and ELISA (2,7,10,31–35). DNA-DNA hybridization, *Eco*RI restriction mapping of spiroplasma DNAs, and spiroplasma protein patterns support the serological results (4,7,23,31–33). Groups II through XXII are serologically distinct from group I and from each other (3,4,9,34,35,38).

Serology has been a most useful tool in the classification, identification, and detection of spiroplasmas. The specificity of serological techniques has been enhanced with the introduction of hybridoma technology (21). The advent of monoclonal antibody production has provided homogenous and biochemically defined immunological reagents with unique specificities and extreme discriminatory abilities. Monoclonal antibodies have an advantage over polyclonal antisera in serological tests because they react with a single antigenic determinant (epitope) rather than with many different epitopes and/or antigens. In addition, monoclonal antibodies specific to unique epitopes can be generated even when nontarget antigens or "mixed" immunogens are used for immunization, as long as a target-specific screening assay is used to select the desired monoclonal antibodies (17,18). Monoclonal antibodies specific for various plant pathogens, including viruses, bacteria, and mycoplasmalike organisms, have been produced and used in diagnostic and taxonomic studies (15,17).

Two separate panels of monoclonal antibodies produced by Lin and Chen to *S. citri* (27) and *S. kunkelii* (28) were species-specific and did not cross-react with any other spiroplasmas tested. All of the *S. citri*-specific monoclonal antibodies reacted with all of the nine U.S. strains and one North African strain tested but did not react with four strains isolated in Algeria, Iran, and Israel (27).

The aims of the work presented here were to produce *S. citri*-specific, *S. kunkelii*-specific, and cross-reactive monoclonal antibodies to be able to identify and differentiate strains of *S. citri* and *S. kunkelii* and to differentiate both *S. citri* and *S. kunkelii* from other serovar subgroups and species of *Spiroplasma*, and to investigate the serologic relationships among spiroplasma strains within group I. We report here the isolation and characterization of species-specific and group-specific monoclonal

antibodies using *S. citri* and *S. kunkelii* strains in a mixture as an immunogen and separately as screening antigens. Preliminary results have been reported (19,20).

MATERIALS AND METHODS

Spiroplasma strains. Thirty-six spiroplasma strains were used in this study, including 26 isolates of spiroplasma group I (7,31–33) and 10 isolates of spiroplasma groups III through XI (3,10,34,38). The source, geographical origin, and host origin of the 26 group I isolates are listed in Table 1. The M200H and M240H strains of *S. citri* are single clones from a 200th and 240th passage, respectively, of R8A2 (R. E. Davis et al, *unpublished*). The non-group I spiroplasma strains used in this study were: *S. floricola* strain 23-6 (ATCC 29989, group III), *S. apis* strain B-31 (ATCC 33834, group IV), *S. mirum* strain SMCA (ATCC 29335) and strain TP-2 (ATCC 33503, group V), *Ixodes* spiroplasma strain Y32 (ATCC 33835, group VI), *Monobia* spiroplasma strain MQ-1 (ATCC 33825, group VII), *Syrphid* spiroplasma strain EA-1 (ATCC 33826, group VIII), *Cotinus* spiroplasma strain CN-5 (ATCC 33827, group IX), *S. culicicola* strain AES-1 (ATCC 35112, group X), and *Monobia* spiroplasma strain MQ-4 (ATCC 35262, group XI). Strains B-31, SMCA, TP-2, Y32, AES-1, and MQ-4 were obtained from R. F. Whitcomb, and strains 23-6, MQ-1, EA-1, and CN-5 were obtained from the American Type Culture Collection, Rockville, MD.

Antigen preparation. Spiroplasmas used as immunogens and as screening antigens were grown in 50- to 500-ml volumes of LD-8S (24) or serum-free LD-59 medium (25) at 30 C, pelleted at 12,000 *g*, washed and resuspended in phosphate-buffered saline containing 10% sucrose, counted, and stored at −70 C. Cultures received from R. F. Whitcomb were tested directly from aliquots of asceptically resuspended lyophilized spiroplasma culture and from subcultures of the resuspended cells. Spiroplasma cell membranes were prepared as described by Razin et al (30) from cultures in early log-phase increase, resuspended in 20-mM Tris-buffered saline, pH 7.5 (TBS), and stored at −20 C. The protein concentration of cells and membrane preparations was determined by the method of Lowry (29).

Immunization and hybridoma production. Each of two BALB/c mice were immunized by five intraperitoneal (ip) injections 9–14 days apart with a total of 100 μg of spiroplasma antigen. The first ip injection consisted of 5×10^6 cells each of *S. kunkelii* strains I-747 and F32 in TBS (emulsified in Freund's complete adjuvant). The second injection was a mixture of *S. kunkelii* strain PU8-17 (8×10^6 cells) and *S. citri* strain M240H (2×10^6 cells) in TBS (emulsified in Freund's incomplete adjuvant [FICA]). The third and fourth injections were emulsified in FICA and both consisted of a mixture of 2.5×10^6 cells of each of the four strains.

The final injection was identical to injections 3 and 4 but was not emulsified in adjuvant. Four days later, one mouse was sacrificed, the spleen removed, and the splenocytes were fused with P3/NS1/1-AG4-1 myeloma cells essentially as described (16,17). The fused cells were washed and resuspended in growth medium consisting of RPMI 1640 containing 15% fetal bovine serum, 1 mM pyruvate, 1 mM L-glutamine, 0.1 mg of gentamycin sulfate per milliliter, 0.1 mM hypoxanthine, 0.4 μM aminopterin, 0.016 mM thymidine, and 10–20% "myeloma-conditioned medium," and dispensed into 18 96-well culture plates. ("Myeloma-conditioned medium" was 0.2 μM-filtered

TABLE 1. Group I *Spiroplasma* strains used in serological comparisons

Serovar subgroup[a]	Binomial and/or common name	Strain[b]	Host	Geographic origin	Source (reference)
I-1	*Spiroplasma citri* (Citrus Stubborn spiroplasma)	R8A2^T (27556)	Citrus	Morocco	R. E. Davis (30)
		Maroc G	Citrus	Morocco	R. F. Whitcomb
		M200H	Citrus	Morocco	R. E. Davis
		M240H	Citrus	Morocco	R. E. Davis
		C189(27665)	Citrus	California	R. E. Davis (30)
		B106	Citrus	California	R. F. Whitcomb
		Israel	Citrus	Israel	R. F. Whitcomb
		ASP-1	Citrus	Israel	R. F. Whitcomb
		Iran	Citrus	Iran	R. F. Whitcomb
		BR-3	Horseradish	Maryland	R. E. Davis (14)
I-2	*S. melliferum* (Honey bee spiroplasma)	BC-3^T(33219)	Honeybee	Maryland	R. F. Whitcomb (7)
		AS 576(29416)	Honeybee	Maryland	R. E. Davis (9)
		R667	Flowers	Jamaica	R. F. Whitcomb
I-3	*S. kunkelii* (Corn stunt spiroplasma)	E275^T(29320)	Corn	Texas	R. F. Whitcomb (33)
		I-747(29051)	Corn	Texas	R. E. Davis (6)
		PU8-17	Corn	Peru	R. E. Davis (9)
		F32	Corn	Mississippi	R. E. Davis
		Miss E	Corn	Mississippi	R. F. Whitcomb
		B655(33289)	Corn	Jamaica	R. F. Whitcomb (12)
		CSEE2	*Exitianus*	Texas	R. F. Whitcomb
I-4	277F spiroplasma	277F(29761)	Rabbit tick	Montana	ATCC (5)
I-5	Green leaf bug spiroplasma	LB-12(33649)	Green leaf bug	Taiwan	ATCC (26)
I-6	Maryland flower	M55(33502)	Flowers	Maryland	ATCC (34)
I-7	*Cocos* spiroplasma	N525(33287)	*Cocos* palm	Jamaica	ATCC (13)
		N628	*Cocos* palm	Jamaica	R. F. Whitcomb
I-8	*S. phoeniceum* (Periwinkle spiroplasma)	P-40^T(43115)	Periwinkle	Syria	R. F. Whitcomb (32)

[a] Serogroups according to Williamson et al (38) and Bove (3).
[b] American Type Culture Collection accession number in parentheses.

supernatant from 24–48 hr myeloma cultures grown in RPMI 1640 growth medium without hypoxanthine, aminopterin, and thymidine.)

Hybridoma cultures were fed every 3–4 days by replacing 50% of the medium in each well with growth medium. After 14 days and thereafter, the cells were fed with growth medium without aminopterin.

Hybridoma selection and initial screening assays. Hybridomas producing antibodies specific for spiroplasma proteins were detected by indirect and modified indirect ELISA (1) beginning 10 days after fusion. In the indirect ELISA, flexible polyvinyl chloride (PVC) microtiter plates (Dynatech) were coated (100 μl per well, 16 hr, 6 C) at 1 μg/ml (about 4×10^7 total cells per milliliter) with a 1:1 mixture of 0.2% sodium dodecyl sulfate (SDS)-treated and untreated spiroplasma cell antigens, which consisted of an admixture of *S. citri* strain M240H and *S. kunkelii* strains I-747, F32, and PU8-17 at a ratio of 1:1:1:1. (For SDS treatment, spiroplasma cells at about 2×10^9 cells per milliliter were adjusted to 0.2% SDS and incubated at 56 C for 15 min before dilution in TBS to 4×10^7 cells per milliliter and a final SDS concentration of 0.004%.) In the modified indirect ELISA, PVC plates were first incubated with rabbit polyclonal anti-*S. citri* M200H or anti-*S. kunkelii* I-747 Ig (100 μl per well) at 1 μg/ml in TBS (2–3 hr, 23 C). After washing the wells with a mixture of TBS and 0.05% Tween-20 (TBS-T), an untreated admixture of spiroplasma strains M240H, I-747, F32, and PU8-17 in TBS at 1.25 μg/ml (about 6.4×10^7 total cells per milliliter) was added and incubated as above (16 hr, 6 C). In both assays, plates were subsequently washed with TBS-T before the addition of 2% bovine serum albumin (BSA) in TBS (1 hr, 23 C) to saturate the remaining binding sites on the plastic. This was replaced with a 1:4 dilution (in TBS) of hybridoma culture supernatant. After 2 hr at 23 C, the wells were washed as above and antigen-specific antibodies were detected with a goat anti-mouse IgG, IgA, IgM alkaline phosphatase conjugate (Kirkgaard and Perry, Gaithersburg, MD) at 125 ng/ml in TBS-0.1% BSA (2–4 hr, 23 C). Substrate (1 mg of *p*-nitrophenyl phosphate per liter in 0.1 M diethanolamine and 1 mM Mg_2Cl, pH 9.8) was incubated at room temperature for 1 hr before being read at 405 nm in a microplate ELISA reader. Growth medium and cell culture medium from NS1 myeloma cells were used as negative controls in the assays, while immune serum from the mouse used for cell fusion was used as a positive control.

Spiroplasma antigen-specific antibody-producing hybridoma cell lines were cloned to single cells by limiting dilution (8). Monoclonal antibodies were precipitated from culture supernatant with 0.9 volumes of saturated $(NH_4)_2SO_4$ (adjusted to pH 7.5 with NH_4OH), dissolved in one fifth of the original volume with TBS, and dialyzed against TBS.

The class and subclass of the monoclonal antibodies were identified by Ouchterlony immunodiffusion and indirect ELISA with rabbit antisera specific for mouse heavy and light chains (Litton Bionetics, Kensington, MD).

Polyclonal rabbit antibodies produced to *S. citri* M200H or *S. kunkelii* I-747 (10) were also precipitated with ammonium sulphate as described.

Antigen specificity and cross-reactivity assays. The specificity of the monoclonal antibodies was tested in indirect or modified indirect ELISA as described above. The antigens for these assays were the individual spiroplasma immunogen strains, both as intact cells and as 0.2% SDS-disrupted cells (at 1 μg/ml), and 0.2% SDS-treated spiroplasma membrane preparations from each of the immunogen strains (at 1 μg/ml).

The cross-reactivities of the monoclonal antibodies were determined in indirect ELISA by using 0.2% SDS-treated group I and non-group I spiroplasmas. Substrate reactions were measured after 1 hr at 23 C and again after overnight incubation at 5 C. *S. citri* strain M240H and *S. kunkelii* strain PU8-17 were used in all assays as "homologous reference" antigens. All of the selected monoclonal antibodies were tested (in duplicate) at the same time against each test antigen in a minimum of two independent assays.

RESULTS

Production and general properties of the monoclonal antibodies. A total of 1,280 hybridomas were produced from a single fusion, with 54 (about 4%) cell lines producing antibodies that were spiroplasma antigen-specific. Forty-six of these cell lines remained stable through propagation, liquid nitrogen storage, and subsequent cloning. All 46 monoclonal antibodies, designated "SCC" (for *Spiroplasma citri* complex), were tested against the individual immunogen spiroplasmas in indirect ELISA, using a mixture of untreated and 0.2% SDS-treated cells as the coating antigen. The results of isotype analysis of each of the 46 SCC monoclonal antibodies and their reactivities to the immunogen spiroplasmas are presented in Table 2. The most frequent isotype was IgG1 (35 monoclonal antibodies), followed by IgG2a (3), IgG2b (3), IgG3 (1), and IgA (1). We were not able to determine the isotype of three of the monoclonal antibodies. The SCC monoclonal antibodies could be differentiated into four primary screening classes. Nine monoclonal antibodies reacted with all four immunogens, 19 monoclonal antibodies reacted only with *S. citri* strain M240H, and 15 monoclonal antibodies reacted specifically with all three strains of *S. kunkelii*. The remaining three monoclonal antibodies reacted only with *S. kunkelii* strain PU8-17. Indirect ELISA antibody titer of hybridoma culture supernatants ranged from 1:128 for monoclonal antibody SCC 44 for the immunogen PU8-17 to 1:524,000 for monoclonal antibody SCC 22 for M240H.

Antigen specificity assays. The ability of the SCC monoclonal antibodies to react with antigenic sites found on intact spiroplasmas, detergent-disrupted spiroplasma cells, or membrane preparations from the immunogen strains was tested in indirect and modified indirect ELISA. Selected results of testing the monoclonal antibodies with the different spiroplasma antigen preparations are presented in Table 3. Examples were selected from each of the four primary screening classes (Table 2).

Forty monoclonal antibodies bound to intact spiroplasmas trapped on antispiroplasma rabbit Ig-coated plates. These same monoclonal antibodies also reacted with spiroplasma cells and purified membrane preparations that were coated directly on microtiter plates. Generally, these monoclonal antibodies had equal or greater reactivity with SDS-denatured spiroplasmas and purified membrane preparations as they did with intact spiroplasma cells (e.g., monoclonal antibodies SCC 4, 10, 29; Table 3). The remaining six monoclonal antibodies (SCC 1–3, 5–7) did not react with Ig-trapped intact spiroplasmas nor with the membrane preparations (Table 3; monoclonal antibodies SCC 1, 3, and 6 are the selected examples). However, these monoclonal antibodies did react with spiroplasmas coated directly on microtiter wells. Generally, the stronger reaction was with antigen that had been detergent-disrupted.

Based on these data, most of the SCC monoclonal antibodies react with antigenic sites found within or on the spiroplasma cell membrane, whereas six monoclonal antibodies react with nonmembrane protein sites. These, presumably cytoplasmic, antigenic sites become available for antibody-binding only after detergent-disruption of cells, or by repeated freeze-thawing, and/or by direct antigen coating of ELISA plates. Binding of antigen directly to microtiter wells has been shown to "expose" antigenic sites that are masked or are not available for antibody-binding when antigen is trapped on antibody-precoated plates (1,17,18). The conditions used in this report to coat the spiroplasmas onto microtiter plates probably disrupt the cells sufficiently to allow internal nonmembrane proteins to be available for binding to the plate and, subsequently, by antibody.

Monoclonal antibody species-specificity and cross-reactivity assays. To assess the strain- and *Spiroplasma* species-specificity of the monoclonal antibodies, each was tested against the individual immunogens, other group I spiroplasmas, and several non-group I spiroplasmas in indirect ELISA using 0.2% SDS-disrupted spiroplasmas as antigen. Twenty-six spiroplasma strains representing all eight group I subgroups (38), as well as 10 non-group I spiroplasmas, were tested. The results are expressed as

TABLE 2. General ELISA reactivities and isotypes of the 46 *Spiroplasma citri* complex (SCC) antibodies to the *S. citri* (M240H) and *S. kunkelii* (PU8-17, I-747, or F32) immunogens

Primary screening class	SCC monoclonal antibody (hybridoma cell line)	Isotype	Immunogen specificity[a]			
			M240H	PU8-17	I-747	F32
1	1(12G4), 2(13E11), 3(15F12) 4(10C10), 6(7A3), 7(7B2)	IgG1	0.3–1.8[b]	0.8–2.0	0.4–2.0	0.3–2.0
	5(17A5)	unk[c]	0.7	0.8	0.8	0.4
	8(5H4)	IgA	1.5	1.8	1.6	1.6
	9(17C5)	IgG2a	0.2	1.0	0.8	0.9
2	10(12F7), 11(12F8), 14(17C3), 15(15G5), 16(15G6), 17(16H4), 18(16H5), 19(15A5), 20(15A9), 21(10A7), 22(10A8), 23(8E11), 24(10F11), 25(12H12), 26(11D2), 27(14B10), 28(14B11), 29(7H6)	IgG1	0.9–2.0	0	0	0
	13(8C3)	IgG2b	2.0	0	0	0
3	30(9D11), 32(1E10), 34(18F5) 35(3H6), 36(4G4), 37(9D12) 40(8F10), 41(9D1), 42(11H1)	IgG1	0	1.3–2.0	1.3–2.0	1,3–2.0
	12(11B2), 31(12F12)	IgG2a	0	0.4–2.0	0.3–2.0	0.3–2.0
	44(7E4)	IgG2b	0	0.4	0.4	0.4
	38(8H4)	IgG3	0	1.2	1.0	1.0
	33(7G5), 39(5H11)	unk	0	0.7–0.9	0.7–0.9	0.7–0.9
4	43(2E10), 45(2C11), 46(6G1)	IgG1	0	0.3–0.9	0	0

[a]The individual antigens tested in indirect ELISA consisted of a 1:1 admixture of untreated cells and 0.2% SDS-disrupted spiroplasma cells, diluted in Tris-buffered saline to 0.004% SDS, 4×10^7 cells per milliliter (about 1 μg/ml, 100 μl per well).
[b]Range of ELISA values (A_{405}) of the listed monoclonal antibodies, of that isotype and primary screening class, with the tested immunogen.
[c]unk = unknown isotype; unable to determine.

TABLE 3. Reactivity of selected *Spiroplasma citri* complex (SCC) monoclonal antibodies and mouse polyclonal antibodies with differently treated spiroplasma antigen preparations in modified indirect and indirect ELISA tests, using either rabbit polyclonal antibody precoated plates or antigen-coated plates, respectively

Primary screening class	SCC monoclonal antibody	Rabbit IgG[a] without treatment[b]		Antigen					
				Untreated frozen[c]		0.2% SDS, 56 C[d]		Membrane[e]	
		M240H	PU8-17	M240H	PU8-17	M240H	PU8-17	M240H	PU8-17
1	1	−[f]	−	+	+	++	+++	−	−
	3	−	−	++	++	++++	++++	−	−
	4	+	+++	+	+++	++	++++	++	++++
	6	−	−	++	++	++	++	−	−
	8	+++	+++	+++	+++	++++	++++	++++	++++
2	10	++	−	+++	−	++++	−	++++	−
	13	+++	−	++++	−	++++	−	++++	−
	22	++++	−	++++	−	++++	−	++++	−
	27	++	−	++++	−	++++	−	++++	−
	29	++	−	+++	−	++++	−	+++	−
3	30	−	+++	−	+++	−	+++	−	+++
	42	−	+++	−	+++	−	+++	−	+++
	44	−	++	−	++	−	++	−	++
4	43	−	+	−	+	−	+	−	+
	MS[g]	++	+++	+++	++++	+++	++++	++	+++

[a]Homologous polyclonal rabbit anti-*S. citri* or anti-*S. kunkelii* antibodies used at 1 μg/ml.
[b]Intact, live spiroplasmas (directly from culture) used at 1×10^7 cells per milliliter.
[c]Spiroplasma cells with less than two freeze/thaw cycles used at 4×10^7 cells per milliliter (about 1 μg/ml).
[d]Spiroplasma cells as per (c), treated at 56 C, 15 min in 0.2% sodium dodecyl sulfate (SDS) before being coated at 0.004% SDS, 4×10^7 cells per milliliter (about 1 μg/ml).
[e]Purified spiroplasma cell membrane preparation treated as in (d) and used at 1 μg/ml, 0.004% SDS.
[f]ELISA absorbance (405 nm) values: −, less than 0.1; +, 0.1–0.4; ++, 0.4–0.8; +++, 0.8–1.2; ++++, greater than 1.2.
[g]MS = mouse serum polyclonal antibodies from mouse from which spleen cells were obtained for hybridoma production; used at 1:16,000 dilution.

relative activity values. For those SCC monoclonal antibodies that reacted with only one of the two "homologous reference" antigens (*S. citri* strain M240H or *S. kunkelii* strain PU8-17; i.e., primary screening classes 2, 3, or 4, Table 2), relative activity was estimated by the equation, $(He/Ho) \times 10$, where *He* and *Ho* are the absorbances at 405 nm (minus the blank value) for the heterologous and homologous reactions, respectively. For those SCC monoclonal antibodies that reacted to both "homologous reference" antigens (i.e., primary screening class 1, Table 2), the relative activity was determined by the equation, $[(2 \times He)/(Ho_1 + Ho_2)] \times 10$, where Ho_1 and Ho_2 are the absorbances at 405 nm for M240H and PU8-17 reactions, respectively.

Monoclonal antibodies SCC 1 through 12 cross-reacted to various degrees with spiroplasmas within three or more of subgroups I-1 through I-8 (Table 4). Monoclonal antibodies SCC 1–3 reacted with all 26 group I spiroplasmas tested. Monoclonal antibodies SCC 4–9 reacted with three or more strains of *S. citri, S. melliferum, S. kunkelii*, and *S. phoeniceum*. Monoclonal antibodies SCC 10 and 11 reacted with all *S. citri, S. melliferum*, and *S. phoeniceum* strains tested, and monoclonal antibody SCC 12 reacted to all *S. melliferum, S. kunkelii*, and *S. phoeniceum* strains analyzed.

Seventeen monoclonal antibodies (monoclonal antibodies SCC 13–29) were *S. citri* species-specific in that they reacted differentially only with strains of *S. citri* (Table 5). Most of these monoclonal antibodies reacted to some degree with all of the isolates, regardless of the isolate's geographical origin. Quantitative differences were observed in the binding of monoclonal antibodies SCC 21–24 with the Israeli isolates compared to that with the isolates from the United States and Morocco. Monoclonal antibodies SCC 27–29 showed reduced or no reactivity with the California B106 isolate and the Iranian and Israeli isolates. B106 could also be differentiated from all isolates tested based on the reproducible dissimilar reactivities of monoclonal antibodies SCC 19 and 20 with it compared to those with all the other isolates. The horseradish brittleroot strain of *S. citri* could not be distinguished from the citrus stubborn isolates with this panel of monoclonal antibodies.

The remaining 17 SCC monoclonal antibodies (Nos. 30–46) react differentially only with strains of *S. kunkelii* (Table 6). SCC monoclonal antibodies 30–38 reacted with all isolates tested. The corn stunt type strain E275 could be distinguished from the other strains by the reduced or lack of binding of monoclonal antibodies SCC 39, 41, and 42. The Peruvian isolate PU8-17 could be differentiated from the isolates from the United States and Jamaica by the unique reactivity pattern of monoclonal antibody SCC 46.

None of the SCC monoclonal antibodies reacted with any of the group III–XI spiroplasmas tested (data not shown).

Antigen site specificity. Antibodies that exhibit similar or identical reactivity patterns with a panel of test antigens can be grouped together and, further, can be differentiated from those groups of antibodies that show markedly different reactivity patterns. The different patterns of reactivity of the different SCC monoclonal antibodies (Tables 4–6), and their reactivities with

TABLE 4. Cross-reactivity of *Spiroplasma citri* complex (SCC) monoclonal antibodies and mouse polyclonal antibodies with Group I spiroplasmas in indirect ELISA

Serovar subgroup	Strain[a]	SCC monoclonal antibody												Polyclonal antibodies[b]
		1	2	3	4	5	6	7	8	9	10	11	12	
I-1	M240H	7[c]	8	8	4	10	9	8	10	2	10	10	–	10
	M200H	13	14	17	5	10	11	–	10	2	10	11	–	10
	R8A2	6	19	7	6	7	7	8	10	2	9	10	–	10
	C189	6	12	4	5	6	5	5	10	1	10	10	–	10
	BR-3	12	11	6	10	9	8	9	9	–	10	10	–	10
	Maroc G	5	3	3	2	6	6	8	9	–	10	10	–	10
	B106	6	2	3	12	6	5	5	9	–	10	10	–	10
	Iran	9	13	6	8	10	9	9	10	4	10	10	–	7
	Israel	4	15	7	12	7	7	7	10	3	10	10	–	6
	ASP-1	11	10	9	4	13	13	13	9	–	10	10	–	6
I-2	BC-3	5	5	3	30	5	7	7	12	22	10	10	14	4
	AS 576	5	10	5	30	6	5	5	12	22	12	12	12	3
	R667	6	11	4	21	5	6	7	12	29	16	18	17	3
I-3	PU8-17	13	13	11	16	10	11	11	10	17	–	–	10	10
	I-747	15	16	14	16	10	11	11	10	14	–	–	6	10
	F32	5	10	8	16	5	5	6	10	16	–	–	7	10
	Miss E	11	12	3	11	1	1	1	5	11	–	–	9	10
	CSEE2	2	14	3	21	1	1	1	3	10	–	–	7	10
	B655	7	2	4	25	2	4	–	4	6	–	–	4	10
	E275	3	3	2	29	1	1	–	–	3	–	–	4	9
I-4	277F	8	58	9	–	–	–	–	–	–	–	–	–	3
I-5	LB-12	21	3	8	–	–	–	–	–	–	–	–	–	3
I-6	M55	14	17	9	–	–	–	–	–	–	–	–	–	3
I-7	N628	7	3	9	–	–	–	–	–	–	–	–	–	3
	N525	5	6	7	–	–	–	–	–	–	–	–	–	2
I-8	P40	4	8	6	30	4	4	4	12	12	4	4	3	6

[a] Spiroplasma strain acronyms and sources as in Table 1. Spiroplasma cells treated at 56 C for 15 min in 0.2% sodium dodecyl sulfate (SDS) before coating of ELISA plate wells at 0.004% SDS, 4×10^7 cells per milliliter (about 1 μg/ml).

[b] Mouse serum polyclonal antibodies from mouse from which spleen cells were obtained for hybridoma production; used at 1:16,000 dilution.

[c] Relative activity values were determined by the equation: $[(2 \times He)/(Ho_1 + Ho_2)] \times 10$, where *He*, Ho_1, and Ho_2 are the absorbances (405 nm, minus the blank value) for the heterologous and two homologous reactions, respectively, with M240H and PU8-17 as the two "homologous reference" antigens Ho_1 and Ho_2, respectively; (– = less than 0.5).

a purified membrane preparation, indicate that most of the antigenic sites they detect are distinct.

At least 17 different antigenic sites can be detected using these 46 monoclonal antibodies (Table 7). The 12 cross-reacting monoclonal antibodies define at least eight different common antigenic sites (A–H, Table 7). Three monoclonal antibodies, SCC 1–3, detect a nonmembrane common antigen found in all 26 group I spiroplasma strains that were tested (Tables 3 and 4). The sites recognized by these monoclonal antibodies could be distinguished by the degree of reactions of the three monoclonal antibodies with certain subgroup I-3, -4, and -5 spiroplasma strains (specifically Miss E, CSEE2, 277F and LB-12; Tables 4 and 7). Monoclonal antibodies SCC 4–9 detect different common antigenic sites (B–F, Table 7) of subgroups I-1, -2, -3, and -8 (*S. citri, S. melliferum, S. kunkelii*, and *S. phoeniceum*, respectively) (Tables 4 and 7). Sites B, E, and F occur on a cell membrane, whereas sites C and D are nonmembrane sites (Table 3). An antigenic site common in strains of *S. citri, S. melliferum*, and *S. phoeniceum* is detected by monoclonal antibodies SCC 10 and 11, whereas monoclonal antibody SCC 12 detects an antigenic site common in strains of *S. melliferum, S. kunkelii*, and *S. phoeniceum* (Tables 4 and 7). The 17 *S. citri*-specific monoclonal antibodies (Table 5) define at least three different antigenic sites (I, J, and K; Table 7). One of these sites (I, Table 7) could be further delineated based on the degree of reactions of specific monoclonal antibodies with certain strains of *S. citri* (Tables 5 and 7). Six different antigenic sites could be defined by the *S. kunkelii*-specific monoclonal antibodies SCC 30–46 (L–Q, Table 7). Site L (Table 7) could be further delineated based on reactions of SCC 39 and 40 with strains B655 and E275 (Table 6).

DISCUSSION

We have described the production, isolation, and preliminary characterization of a panel of 46 monoclonal antibodies from hybridomas obtained after immunization of mice with a mixture of four distinct spiroplasma strains. This approach enabled us to obtain 17 monoclonal antibodies that react specifically (only) with strains of *S. citri*, an additional 17 monoclonal antibodies that react specifically with strains of *S. kunkelii*, and 12 other hybridomas secreting monoclonal antibodies that cross-react with 10 to 26 group I spiroplasmas (Tables 4–6). This panel of monoclonal antibodies can be used to identify and discriminate strains and species within the group I spiroplasma complex.

Seventeen different antigenic sites can be defined by these monoclonal antibodies. These include nine species-specific determinants (three *S. citri*-specific and six *S. kunkelii*-specific), seven determinants common in three to four spiroplasma species (*S. citri, S. melliferum, S. kunkelii*, and *S. phoeniceum*), and one common determinant found in all group I spiroplasmas tested.

The monoclonal antibody reactivity patterns also indicate the general patterns of serological relationships among the tested spiroplasma strains. Overall, the results presented in Tables 4–6 indicate a large degree of serological interrelatedness among the group I spiroplasmas. In DNA-DNA hybridization reactions with group I strains, *S. citri* (R8A2, subgroup I-1) probes showed 65–70% homology with *S. melliferum* (BC-3, subgroup I-2), 50–57% homology with *S. kunkelii* (E275, subgroup I-3), 30% homology with 277F (subgroup I-4), 27% homology with LB-12 (subgroup I-5), 26% homology with M55 (subgroup I-6), 24% homology with N525 (subgroup I-7), and 60% homology with *S. phoeniceum* (P40, subgroup I-8) (23,32,38). Results of studies

TABLE 5. Reactivity of the *Spiroplasma citri*-specific *S. citri* complex (SCC) monoclonal antibodies with different strains of *S. citri*

Strain[a]	SCC monoclonal antibody																	Polyclonal antibodies[b]
	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
M240H	10[c]	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
M200H	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
R8A2	10	10	10	10	10	10	11	10	10	10	10	12	10	10	10	11	7	10
C189	10	10	10	10	10	10	11	10	10	10	10	12	10	10	11	11	8	10
BR-3	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Maroc G	10	10	10	10	10	10	10	10	10	10	10	10	9	10	8	8	5	10
B106	10	10	8	7	13	13	2	2	10	10	8	9	9	5	2	2	—	10
Iran	10	10	10	10	10	10	10	10	9	9	7	5	3	3	2	2	—	7
Israel	10	10	7	6	10	10	10	10	5	5	2	2	2	1	—	—	—	6
ASP-1	10	10	7	7	10	10	10	10	4	4	2	2	1	1	—	—	—	6

[a]Spiroplasma strain acronyms and sources as in Table 1. Spiroplasma cells treated at 56 C for 15 min in 0.2% sodium dodecyl sulfate (SDS) before coating of ELISA plate wells at 0.004% SDS, 4×10^7 cells per milliliter (about 1 μg/ml).
[b]Mouse serum polyclonal antibodies from mouse from which spleen cells were obtained for hybridoma production; used at 1:16,000 dilution.
[c]Relative activity values were determined by the equation: $(He/Ho) \times 10$, where *He* and *Ho* are the absorbances (405 nm, minus the blank value) for heterologous and homologous reactions, respectively, with M240H as "homologous reference" antigen; (— = less than 0.5).

TABLE 6. Reactivity of the *Spiroplasma kunkelii*-specific *S. citri* complex (SCC) monoclonal antibodies with different strains of *S. kunkelii*

Strain[a]	SCC monoclonal antibody																	Polyclonal antibodies[b]
	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	
PU8-17	10[c]	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
I-747	10	10	10	10	10	10	9	9	9	10	11	9	9	—	9	—	—	10
F32	10	10	10	11	10	10	10	10	8	10	9	9	10	—	10	—	—	10
Miss E	10	10	12	11	10	10	10	10	8	14	4	5	6	9	10	—	—	10
CSEE2	9	10	10	12	9	9	9	9	5	11	3	3	5	10	—	5	—	10
B655	9	13	12	12	8	10	10	10	7	14	1	4	3	10	—	6	2	10
E275	10	10	12	11	10	8	15	13	6	2	1	—	—	8	—	5	—	9

[a]Spiroplasma strain acronyms and sources as in Table 1. Spiroplasma cells treated at 56 C for 15 min in 0.2% sodium dodecyl sulfate (SDS) before coating of ELISA plate wells at 0.004% SDS, 4×10^7 cells per milliliter (about 1 μg/ml).
[b]Mouse serum polyclonal antibodies from mouse from which spleen cells were obtained for hybridoma production; used at 1:16,000 dilution.
[c]Relative activity values were determined by the equation: $(He/Ho) \times 10$, where *He* and *Ho* are the absorbances (405 nm, minus the blank value) for heterologous and homologous reactions, respectively, with PU8-17 as "homologous reference" antigen; (— = less than 0.5).

TABLE 7. Antigenic site specificity of the *Spiroplasma citri* complex (SCC) monoclonal antibodies based on their patterns of reactivity with different spiroplasma strains and antigen preparations

Antigenic site	SCC monoclonal antibody	Distinguishing reactivities[a]	
		Subgroup specificity[b]	Differentiating antigen(s)[c]
A1	1	I-1 to I-8	CSEE2, 277F, LB-12; (NR)[d]
A2	2	I-1 to I-8	B655, 277F, LB-12; (NR)
A3	3	I-1 to I-8	Miss E, CSEE2, 277F, LB-12; (NR)
B	4	I-1, -2, -3, -8	E275, BC-3, P40
C	5,6	I-1, -2, -3, -8	E275, P40; (NR)
D	7	I-1, -2, -3, -8	M200H, E275, P40; (NR)
E	8	I-1, -2, -3, -8	E275, P40
F	9	I-1, -2, -3, -8	M240H, ASP-1, BC-3, P40
G	10, 11	I-1, -2, -8	M240H, BC-3, PU8-17
H	12	I-2, -3, -8	M240H, BC-3, PU8-117
I1	13–18	I-1	All 10 *S. citri* strains
I2	19, 20	I-1	B106
I3	21, 22	I-1	Israel, ASP-1
I4	23, 24	I-1	Israel, ASP-1
I5	25, 26	I-1	Iran, Israel, ASP-1
J	27, 28	I-1	B106, Iran, Israel, ASP-1
K	29	I-1	B106, Iran, Israel, ASP-1
L1	30–38	I-3	All seven *S. kunkelii* strains
L2	39	I-3	E275
L3	40	I-3	CSEE2, B655, E275
M	41, 42	I-3	CSEE2, B655, E275
N	43	I-3	I-747, F32, E275
O	44	I-3	CSEE2, B655, E275
P	45	I-3	I-747, Miss E, E275
Q	46	I-3	I-747, Miss E, E275

[a] Monoclonal antibody reactivity patterns (from Tables 4–6) were used to identify specific antigenic sites.
[b] Serovar subgroup(s) (Table 1) in which identified SCC monoclonal antibodies had positive reactions in indirect ELISA.
[c] Spiroplasma strain(s) within the identified subgroup that could be used to differentiate the monoclonal antibodies (based on degree or lack of reaction) and, therefore, the antigenic sites they recognize.
[d] NR = no reaction. The lack of reaction of the specified monoclonal antibody with intact spiroplasmas and spiroplasma cell membrane preparations (Table 3) was also used to differentiate antigenic sites.

comparing cell proteins of representative strains by one- and two-dimensional polyacrylamide gel electrophoresis closely paralleled the genomic hybridization analyses (4,7,31–33). The data presented here, using species-specific and group-specific monoclonal antibodies generated against a mixture of subgroup I-1 and I-3 strains, confirm the distinctiveness of the strains representing subgroups I-1 through I-8.

The reactions of mouse polyclonal antibodies and monoclonal antibodies SCC 1–3 indicate that the 26 group I spiroplasma strains tested are serologically related (Table 4). They are distinct from the group III–XI strains tested as shown by the lack of reaction of these same antibodies with the non-group I antigens. The serological uniqueness of the strains representing subgroups I-4 through I-7 is evident, based on the differential degrees of reactions of monoclonal antibodies SCC 1–3 with these spiroplasmas (Table 4). The profile of serological cross-reactions between strains of subgroups I-1, I-2, I-3, and I-8 with monoclonal antibodies SCC 4–12 indicates very close serological relationships among these plant- and insect-pathogenic spiroplasmas (Table 4). These data are in agreement with the 50–70% DNA-DNA homologies between these spiroplasma species (32). The reactivities of monoclonal antibodies SCC 1–12 (especially the quantitative differential binding activities of monoclonal antibodies SCC 10–12), suggest that the periwinkle yellows spiroplasma *S. phoeniceum* may share more antigenic determinants with the honeybee spiroplasmas *S. melliferum* than with the citrus stubborn or corn stunt spiroplasmas, *S. citri* and *S. kunkelii*, respectively (Tables 4 and 7). The distinctiveness of subgroups I-1 and I-3 is also demonstrated by the serological reactions of the *S. citri*-specific and *S. kunkelii*-specific monoclonal antibodies (Tables 5 and 6).

Several of the monoclonal antibodies have been shown to be useful as diagnostic reagents in the detection of *S. kunkelii* from plants infected with corn stunt (11) and of *S. citri* in plants and insects (R. Jordan, M. Pandey, and R. E. Davis, *unpublished*), and in immunofluorescent microscopy studies of spiroplasma cell structure (22; and *unpublished*). All of the SCC monoclonal antibodies are being tested further in other investigations to obtain further information on epitope discrimination, topological relationships between epitopes, and serological identification of the specific proteins recognized by the monoclonal antibodies. Preliminary evidence from Western-blot analysis of polyacrylamide gel-separated spiroplasma cell proteins indicates that the membrane-specific monoclonal antibodies recognize a protein of 26,000 molecular weight, most likely spiralin (37), whereas the other monoclonal antibodies recognize a 56,000-molecular-weight protein present only in total cell extracts (R. Jordan, *unpublished*).

These highly discriminatory serological reagents should be very useful as sensitive immunological probes for the detection and identification of spiroplasmas in plants and insects, in providing additional information on the antigenic relationships among other strains and newly recognized spiroplasmas, and as molecular probes in identifying and locating specific proteins, for example those proteins that may be involved in motility, helicity, or membrane integrity or substructure.

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