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Patterns of Reactivity between a Panel of Monoclonal Antibodies and Forage *Rhizobium* Strains

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A panel of 11 monoclonal antibodies raised against vegetative cells of *Rhizobium leguminosarum* biovar trifolii or *Rhizobium meliloti* was tested by enzyme-linked immunosorbent assay for reactivity with 47 strains of *R. leguminosarum* biovar trifolii and 60 strains of *R. meliloti*. The goal of the study was to define the degree of specificity associated with each antibody and to gain an understanding of the amount of antigenic diversity found among the strains and between the species. Each antibody was tested against each *Rhizobium* strain in four forms: washed steamed cells, washed unsteamed cells, cell-free culture broth, and nodule squash material. Each antibody showed a different pattern of reactivity among the 107 strains. One of each of the antibodies developed against *R. meliloti* and *R. leguminosarum* biovar trifolii reacted in a highly specific manner with cells or antigen from the immunogenic strain only. Nine of the antibodies recognized secreted as well as cellular antigen from many of the strains. Analysis of patterns of reactivity between the 107 strains and the 11 antibodies separated the strains into 28 groups of which 12 were represented by one strain only.

The search for improved *Rhizobium* strains requires that an efficient means be found to identify, monitor, and characterize promising strains both in the laboratory and in the field. Serological tests developed to accomplish these ends have been used for over 50 years (19, 22). The cell agglutination techniques originally used to identify rhizobial strains have largely given way to variations of fluorescent-antibody (2, 4, 18) and enzyme-linked immunosorbent assay (ELISA) techniques (1, 10, 11). Monoclonal antibody (MAb) technology (12) was first reported in connection with *Rhizobium* work in 1984 (6) and shows increasing promise for use in new techniques and studies which will further enhance understanding of rhizobial ecology. Several groups have reported the development and use of antirhizobial MAbs in recent years (3, 7, 17, 21, 26).

The use of MAbs in place of polyclonal antibodies is associated with both advantages and disadvantages for *Rhizobium* strain identification. Advantages include the ability to freeze hybridomas and to replenish identical antibody as needed. Also, MAbs lend themselves to antigenic analysis of bacteria and bacterial products by virtue of the fact that only one antibody specificity is responsible for an observed reaction. The disadvantages of MAb use include the difficulties of production of specific antibodies and the associated degree of laboratory specialization required. Also, recognition of a single epitope may become problematical in tracer studies in which rhizobia are subjected to environmental stress or undergo antigenic changes during bacteroid formation (9, 15, 26).

The major objective of the work described here was to evaluate a variety of MAbs produced against strains of *Rhizobium meliloti* or *Rhizobium leguminosarum* biovar trifolii which, by preliminary results, had indicated various specificities for strains in the respective groups of rhizobia. A total of 16 MAb-producing cell lines were originally screened for inclusion in this study. Five of the 16 cell lines were excluded because they provided very low or inconsistent ELISA results in the standardized assay selected. The wide spectrum of strains and MAbs used was intended to provide an estimate of the antigenic variability among the strains and between the two species. Other objectives were to evaluate the potential usefulness of panels of MAbs for purposes of *Rhizobium* strain identification and to evaluate the effect of heat treatment on rhizobial antigen used in ELISA. The 11 MAbs were tested for reactivity with 107 effective *Rhizobium* strains in the forms of steamed cells, unsteamed cells, sterile-filtered culture broth, and nodule squash material.

MATERIALS AND METHODS

MAbs. The 11 MAb-secreting hybridoma cell lines used in this work were independently produced in two separate laboratories. All of the hybridomas were produced using BALB/c mice and cell line P3-NS1-Ag4-1 as the myeloma parent. Five of the hybridoma cell lines (producers of MAbs 1 to 5) were developed against washed, whole cells of *R. leguminosarum* bv. trifolii strains (Table 1) at the U.S. Department of Agriculture—Agricultural Research Service laboratory in Beckley, W. Va., using the fusion method described by Oi and Herzenberg (14). Immunization protocols were as described by Wright et al. (26). The five anti-*R. leguminosarum* bv. trifolii MAbs were produced by secretion of cloned hybridoma cells into tissue culture media.

Six of the hybridomas (producers of MAbs 6 to 13) used were produced against steamed, washed, whole cells of *R. meliloti* strains at the Agriculture Canada Research Station, Beaverlodge, Alberta, Canada (Table 1) following basic procedures as outlined by Goding (5). The anti-*R. meliloti* MAbs used were produced by ascitic fluid production in BALB/c mice.

Dilutions (Table 1) of the tissue culture supernatant or ascitic fluids were used directly, without purification, in the ELISA testing. All 11 MAbs were tested by ELISA for class and subisotype using a Mouse Typer kit (Bio-Rad Laboratories [cat. no. 172-2055]).

Polyclonal antibodies. Polyclonal antisera against steamed,

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TABLE 1. Characteristics of 11 anti-*Rhizobium* MAb-secreting hybridoma cell lines

MAb no. (cell line)	Secreted antibody type	Immunogenic strain	Legume host	MAb source[a]	MAb origin	ELISA dilution
1 (31B12E)	IgG3	WV22	Clover	TC	Beckley	50
2 (31B4F)	IgG3	WV22	Clover	TC	Beckley	50
3 (2FE11)	IgG1	162X95	Clover	TC	Beckley	50
4 (12-1-G9)	IgG1	162X95	Clover	TC	Beckley	50
5 (22aB2)	IgG3	ANU843	Clover	TC	Beckley	50
6 (F2P18E)	IgG3	Balsac	Alfalfa	AF	Beaverlodge	2,100
7 (F12P92F)	IgM	NRG34	Alfalfa	AF	Beaverlodge	3,000
8 (F4P26B)	IgM	NRG185	Alfalfa	AF	Beaverlodge	3,000
9 (F4P211F)	IgM	Balsac	Alfalfa	AF	Beaverlodge	2,100
10 (F12P64C)	IgG3	NRG34	Alfalfa	AF	Beaverlodge	2,100
11 (F3P49C)	IgG1	NRG185	Alfalfa	AF	Beaverlodge	3,000

[a] TC, tissue culture; AF, ascitic fluid.

washed, whole cells of *R. meliloti* Balsac and NRG185 were produced in rabbits. These antisera were stored frozen at −135°C and have been previously characterized and described (16).

***Rhizobium* strains.** A total of 107 strains were tested; these included 47 strains of *R. leguminosarum* bv. trifolii (Table 2) and 60 strains of *R. meliloti* (Table 3). Most of the strains were obtained from the culture collections of the two collaborating laboratories or from R. S. Smith (LiphaTech Inc. [Nitragin Co.], Milwaukee, Wis.). Six *R. leguminosarum* bv. trifolii strains were obtained from Brian Holl, University of British Columbia. All strains were stored as frozen broth cultures at −135°C (yeast extract mannitol broth plus 10% glycerol) until use. All of the strains produced nodules on appropriate alfalfa (*Medicago sativa* L. var. Peace) or red clover (*Trifolium pratense* L. var. Norlac) host plants in growth pouches under sanitary conditions in a growth chamber.

Antigen Preparation for ELISA. (i) Cells. Cells of each strain were grown at 28°C in 50-ml flasks containing yeast extract mannitol broth (23) for 3 days. These cultures were used for all four types of antigen preparation (steamed cells, unsteamed cells, cell-free culture supernatant, and plant nodules). Harvested cells were washed five times in phosphate-buffered saline (PBS; 7.65 g of NaCl, 1.27 g of Na_2HPO_4, 0.1 g of NaH_2PO_4, and 0.21 g of KH_2PO_4 per liter of H_2O [pH 7.2]) and then divided into two portions. One portion was steamed at 102°C for 15 min at low pressure in an autoclave. Both steamed and unsteamed portions were then washed a further five times with PBS and stored at 4°C in PBS containing 0.02% NaN_3 (PBS-NaN_3). All steamed and unsteamed cell preparations were adjusted to an optical density at 620 nm of 0.15 for use in the ELISAs.

(ii) Culture supernatant. A portion of 3-day broth culture from each strain was centrifuged, and the supernatant was decanted. The supernatant was then diluted 1:10 in PBS and filtered through a 0.22-μm-pore-size filter. Filtered culture broths were further diluted to a total dilution of 1:100 in PBS-NaN_3 and then stored at 4°C for use in the ELISA.

(iii) Nodules. A 1-ml portion of each 3-day culture was aseptically applied to eight seedlings each of alfalfa and clover in separate growth pouches and grown under sanitary conditions in a growth chamber. After 5 weeks, 10 nodules produced by each *Rhizobium* strain were removed and crushed in 6 ml of PBS–NaN_3, steamed at low pressure in an autoclave for 10 min, and stored at 4°C. A few strains produced fewer than 10 nodules per pouch, and in these cases all available nodule material was used. No attempt was made to standardize bacteroid cell concentrations because of the intrinsic variation in quantities of associated plant material.

Positive-control antigens. The 11 MAbs used in this study were each produced against one of six *Rhizobium* strains (Table 1). Whenever a MAb was used in the ELISA testing, cells of the corresponding immunogenic strain were used as the positive control. Steamed cells were used as positive controls in ELISA of steamed cell antigens, culture supernatant antigens, and nodule squash antigens. Unsteamed cells were used as positive controls in ELISA of unsteamed cell antigens.

ELISA procedure. All ELISAs were performed in the indirect format. The wash solution was PBS with 0.05% polyoxyethylene-20-sorbitan monolaurate (Tween 20) (PBST). The blocking reagent was PBST containing 1% fetal bovine serum. The detecting antibody conjugate was goat anti-mouse immunoglobulin G-horseradish peroxidase (blotting grade, affinity purified, and reactive with both heavy and light antibody chains [Bio-Rad Laboratories, Richmond, Calif.]) and was used at 1:6,000 in PBST plus 1% fetal bovine serum. Enzyme substrate was 3,3′,5,5′-tetramethylbenzidine (TMB peroxidase enzyme immunoassay substrate kit; Bio-Rad Laboratories). Substrate from the same lot was used for all of the work described in this paper.

Antigens were added in 100-μl volumes to the inner 60 wells of 96-well microplates (Immulon 4; Dynatech Laboratories). Replicate wells of positive control cells and replicate wells of negative controls (PBS without antigen) were included on each microplate. Antigen sorption was allowed to proceed overnight at room temperature. The plates were then washed with an automated plate washer and blocked with PBST plus 1% fetal bovine serum (150 μl per well) for 1 h. The plates were washed, MAbs were added to the wells (100 μl per well), and the plates were incubated for 90 min. The plates were washed, antibody conjugate (goat anti-mouse immunoglobulin G-horseradish peroxide) was added (100 μl per well), and the plates were incubated for 75 min at room temperature. The plates were washed, freshly prepared enzyme substrate was added to the wells, and color development was measured after 30 min. Absorbance readings were made with an automated plate reader (Dynatech MR600) operating with ELISA + software (Meddata Inc.). All incubations were carefully controlled for duration to have a less-than-20-s variation per step between plates in each ELISA run.

Data analysis. All test sample ELISA absorbance data were transformed to percentages relative to the mean absorbance values of the respective positive controls, which were defined as 100% absorbance values for the respective plates. Reactions were assigned as positive or negative by using as a cutoff value an absorbance value equal to 15% of that of the appropriate positive control (+, >15%; −, <15%). The 15% cutoff value was selected because two of the MAbs used in this study

TABLE 2. Reactivities of anti-*R. leguminosarum* bv. trifolii MAbs with *R. leguminosarum* bv. trifolii strains

Strain	Reactivity of the following MAbs with antigen form[a]									
	1				2				3				4				5			
	S	U	B	N	S	U	B	N	S	U	B	N	S	U	B	N	S	U	B	N
USDA2165	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
USDA2104	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−
USDA2232	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
162Y14	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
162X92	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
WV21	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	+
162P30b	+	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
162C13	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
162X77	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
162X100	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
USDA2137	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
USDA2102	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
USDA2006	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	+	+	+	−
WV7	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
162X97	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
USDA2149	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
USDA2227	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−
162X20	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−
162Y13	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
162X68	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
USDA2043	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
WV3	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
WV23	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
162X84	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
USDA2229	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
USDA2203	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−
USDA2087	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
USDA2080	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
USDA2134	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−
WV12	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−
WV18	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−
WVE5	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−
WV22	+	+	−	+	+	+	−	−	+	+	−	+	−	−	−	−	−	−	−	−
ANU843	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	+	+	+	+
162X95	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
162P30a	+	+	−	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
162P17	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
162S7a	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
162BB1	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
162X7a	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
LEL/06	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−
LEL/05	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
LEL/04	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
LEL/03	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−
LEL/02	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
LEL/01	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
NRG40	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−

[a] S, steamed cells; U, unsteamed cells; B, cell-free culture broth; N, nodule squash.

showed reactivities with the respective positive controls in which A_{630} was slightly less than 0.40. Ascription of a positive reaction on the basis of an absorbance value of less than 15% of this value was not meaningful, and the cutoff level for the whole test was set at 15% to meet these worst-case conditions. Positive and negative reactivity patterns against the 11 MAbs were generated for each strain in the forms of steamed cells, unsteamed cells, and steamed and unsteamed cells considered together. This produced strings of 11 (steamed or unsteamed cells) or 22 (steamed and unsteamed cells) positive or negative reactions which were used to compare the strains. These patterns were used to group strains with no immunologically distinguishable differences. Patterns were developed only for steamed and unsteamed types of antigens, because these forms had been standardized in terms of concentration by adjustment of optical density and, therefore, all steamed and unsteamed cell preparations were similar in terms of the numbers of cells per ml used in the ELISA. Filtered culture broth antigen and nodule squash antigen could not be standardized in terms of antigen concentration, and MAb reactivity pattern analysis with these types of antigen was therefore not attempted.

RESULTS

ELISA results for the entire study had almost no background noise with an average negative-control well value of A_{630} = 0.002. Interwell positive-control values (four replicate positive controls per plate) deviated from the mean by less

TABLE 3. Reactivities of anti-*R. meliloti* MAbs with *R. meliloti* strains

Strain	Reactivity of the following MAbs with antigen form[a]											
	6				7				8			
	S	U	B	N	S	U	B	N	S	U	B	N
Urb166	+	+	−	−	−	−	−	−	+	+	+	−
Urb165	+	+	−	−	−	−	−	−	+	+	+	−
Urb164	+	+	−	−	−	−	−	−	+	+	+	−
NRG352	+	+	−	−	−	−	−	−	+	+	+	−
102F34	+	+	+	−	−	−	−	−	−	−	−	−
102F77	+	+	−	−	−	−	−	−	−	−	−	−
102F66	+	+	−	−	−	−	−	−	−	−	−	−
102F51	+	+	−	−	−	−	−	−	−	−	−	−
104A13	+	+	−	+	−	−	−	−	−	−	−	−
102F28	+	+	−	−	−	−	−	−	−	−	−	−
Balsac	+	+	+	−	−	−	−	−	−	−	−	−
NRG315	+	+	+	−	−	−	−	−	+	+	+	−
NRG312	−	−	−	+	−	−	−	−	+	−	−	−
NRG308	+	+	−	−	−	−	−	−	+	+	+	−
NRG304	+	+	+	−	−	−	−	−	+	+	+	−
NRG302	+	+	+	+	−	−	−	−	−	−	−	−
NRG299	+	+	−	−	−	−	−	−	−	−	−	−
NRG296	+	+	−	−	−	−	−	−	+	+	+	−
NRG289	−	−	−	−	−	−	−	−	+	−	−	−
NRG286	+	+	−	−	−	−	−	−	+	+	+	−
NRG284	+	+	−	−	−	−	−	−	−	−	−	−
NRG282	−	−	−	−	−	−	−	−	+	−	−	−
NRG278	+	+	−	−	−	−	−	−	+	+	−	−
NRG277	+	+	−	−	−	−	−	−	+	+	+	−
NRG275	+	+	−	+	−	−	−	−	+	+	+	−
NRG271	+	+	−	−	−	−	−	−	+	+	+	−
NRG238	+	+	−	−	−	−	−	−	−	−	−	−
NRG232	+	+	−	−	−	−	−	−	−	−	−	−
NRG230	+	+	−	−	−	−	−	−	−	−	−	−
NRG192	+	−	−	+	−	−	−	−	+	−	−	−
NRG185	+	+	−	−	−	−	−	−	+	+	+	−
NRG170	+	+	−	−	−	−	−	−	−	−	−	−
NRG149	+	+	−	−	−	−	−	−	−	−	−	−
NRG147	+	+	−	−	−	−	−	−	−	−	−	−
NRG144	+	+	+	−	−	−	−	−	+	+	−	−
NRG139	+	+	−	−	−	−	−	−	−	−	−	−
NRG133	−	−	−	−	−	−	−	−	+	−	−	−
NRG131	+	+	−	−	−	−	−	−	−	−	−	−
NRG126	+	+	+	−	−	−	−	−	−	−	−	−
NRG124	+	+	−	−	−	−	−	−	−	−	−	−
NRG118	+	+	−	−	−	−	−	−	+	+	−	−
NRG90	+	+	−	−	−	−	−	−	−	−	−	−
NRG86	+	+	−	−	−	−	−	−	−	−	−	−
NRG85	+	+	−	−	−	−	−	−	−	−	−	−
NRG84	+	+	−	−	−	−	−	−	−	−	−	−
NRG79	+	+	−	−	−	−	−	−	−	−	−	−
NRG71	+	+	+	−	−	−	−	−	−	−	−	−
NRG70	+	−	−	−	−	−	−	−	−	−	−	−
NRG61	+	+	−	−	−	−	−	−	−	−	−	−
NRG60	+	+	−	−	−	−	−	−	+	+	−	−
NRG57	+	+	−	−	−	−	−	−	−	−	−	−
NRG55	+	+	−	−	−	−	−	−	−	−	−	−
NRG53	+	+	−	+	−	−	−	−	+	+	+	−
NRG50	+	+	−	−	−	−	−	−	+	+	−	−
NRG47	+	+	−	−	−	−	−	−	−	−	−	−
NRG43	+	+	−	−	−	−	−	−	−	−	−	−
NRG38	+	+	+	+	−	−	−	−	−	−	−	−
NRG34	+	+	+	+	+	+	+	+	−	−	−	−
NRG24	+	−	−	−	−	−	−	−	−	−	−	−
NRG23	+	+	−	−	−	−	−	−	−	−	−	−

Strain	Reactivity of the following MAbs with antigen form[a]											
	9				10				11			
	S	U	B	N	S	U	B	N	S	U	B	N
Urb166	−	−	−	−	+	+	+	+	+	+	−	−
Urb165	+	+	−	−	+	+	+	+	+	+	−	−
Urb164	−	−	−	−	+	+	+	+	+	+	+	+
NRG352	−	−	−	−	+	+	+	+	+	+	+	−
102F34	+	+	+	−	+	+	+	+	−	−	+	−
102F77	−	−	−	−	+	+	+	+	+	+	+	−
102F66	−	−	−	−	+	+	+	+	+	+	−	−
102F51	−	+	−	−	+	+	−	−	−	−	−	−
104A13	−	−	−	−	+	+	+	+	+	+	+	+
102F28	+	+	−	−	+	+	−	+	+	+	−	−
Balsac	+	+	+	+	+	+	+	+	+	+	+	+
NRG315	−	−	−	−	+	+	+	+	+	+	+	−
NRG312	−	−	−	+	−	−	−	+	−	−	−	+
NRG308	−	−	−	−	+	+	+	+	+	+	−	−
NRG304	−	−	−	−	+	+	+	+	+	+	+	+
NRG302	−	−	−	−	+	+	+	+	+	+	+	+
NRG299	−	−	−	−	+	+	+	+	+	+	−	−
NRG296	−	−	−	−	+	+	+	+	+	+	+	−
NRG289	−	−	−	−	−	−	−	+	−	−	−	+
NRG286	−	−	−	−	−	−	−	−	−	−	−	−
NRG284	+	+	−	−	+	+	+	+	+	+	+	−
NRG282	−	−	−	−	−	−	−	+	−	−	−	+
NRG278	−	−	−	−	+	+	−	+	+	+	−	−
NRG277	−	−	−	−	+	+	+	+	+	+	−	+
NRG275	+	−	−	−	+	+	+	+	+	+	+	+
NRG271	+	−	−	−	+	+	+	+	+	+	+	+
NRG238	−	+	−	−	+	+	−	+	+	+	−	+
NRG232	−	−	−	−	+	+	+	+	+	+	+	+
NRG230	+	+	−	−	+	+	+	+	+	+	−	−
NRG192	−	−	−	−	+	−	−	+	−	−	−	−
NRG185	−	−	−	−	+	+	+	+	+	+	+	+
NRG170	+	+	−	−	+	+	+	+	+	+	−	−
NRG149	+	+	−	−	+	+	+	+	+	+	−	−
NRG147	−	+	−	−	+	+	+	+	+	+	−	−
NRG144	−	+	−	−	+	+	+	+	+	+	+	+
NRG139	−	−	−	−	+	+	+	+	+	+	+	+
NRG133	−	−	−	−	−	−	−	+	−	−	−	−
NRG131	+	+	−	−	+	+	+	+	+	+	−	+
NRG126	−	−	−	−	+	+	+	+	+	+	+	+
NRG124	−	−	−	−	+	+	+	+	+	+	−	+
NRG118	+	+	−	−	+	+	−	+	+	+	−	−
NRG90	+	+	−	−	+	+	−	+	+	+	−	−
NRG86	−	−	−	−	+	+	+	+	+	+	−	−
NRG85	−	−	−	−	+	+	+	+	+	+	+	+
NRG84	−	+	−	−	+	+	+	+	+	+	−	−
NRG79	−	−	−	−	+	+	+	+	+	+	+	+
NRG71	−	−	−	−	+	+	+	+	+	+	+	−
NRG70	−	−	−	−	+	+	+	+	+	+	+	+
NRG61	−	+	−	−	+	+	+	+	+	+	−	−
NRG60	+	+	−	−	+	+	−	+	+	+	−	+
NRG57	−	−	−	−	+	+	+	+	+	+	+	+
NRG55	−	−	−	−	+	+	+	+	+	+	−	−
NRG53	−	−	−	−	−	−	−	−	−	−	−	−
NRG50	−	−	−	−	+	+	+	+	+	+	−	−
NRG47	−	−	−	−	+	+	+	+	+	+	+	−
NRG43	+	+	−	−	−	−	−	−	−	−	−	−
NRG38	−	−	−	−	+	+	+	+	+	+	+	+
NRG34	+	−	−	−	+	+	+	+	+	+	+	+
NRG24	−	−	−	−	+	+	+	+	+	+	−	−
NRG23	−	−	−	−	−	−	−	−	−	−	−	−

[a] S, steamed cells; U, unsteamed cells; B, cell-free culture broth; N, nodule squash.

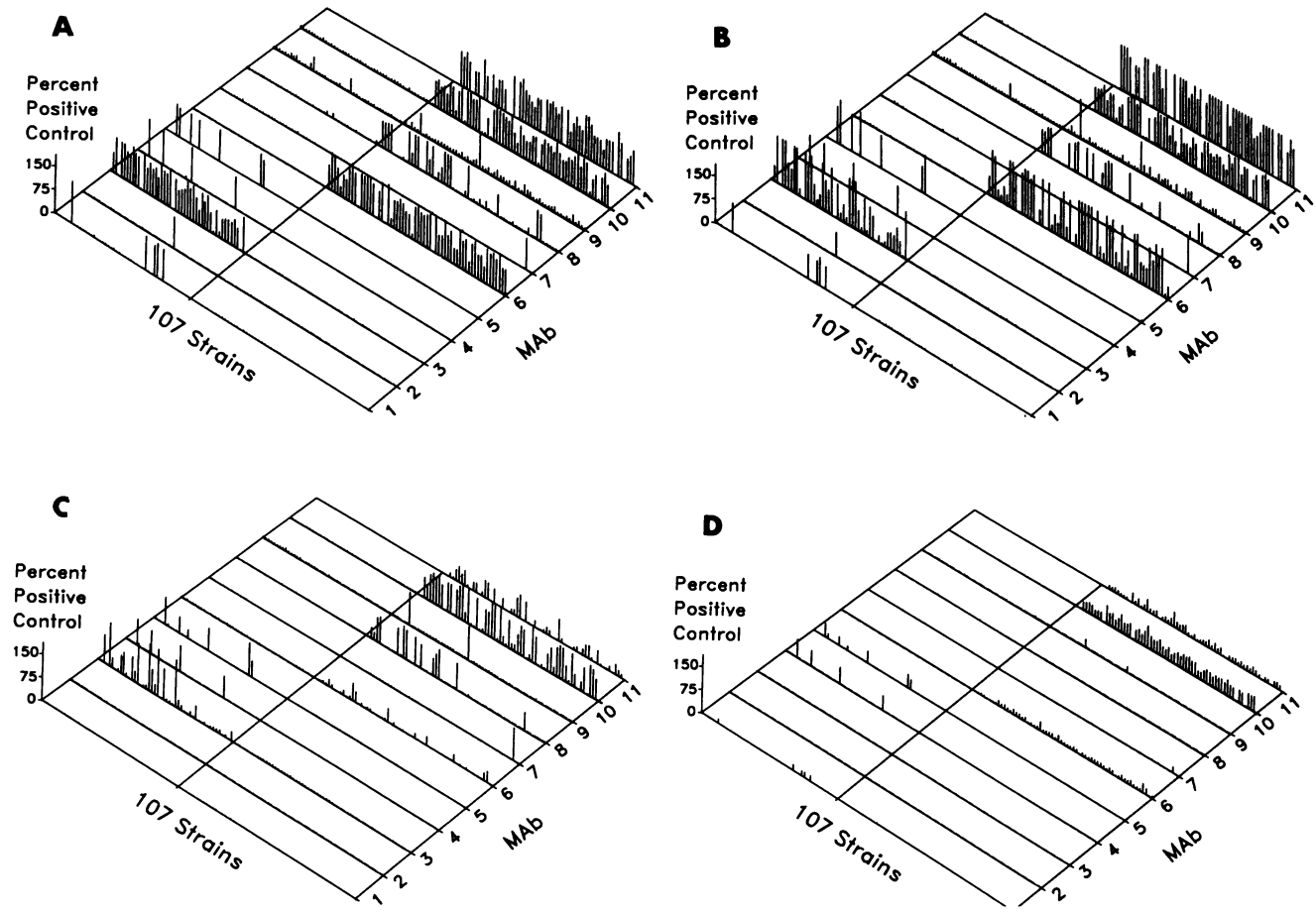

FIG. 1. ELISA reactivities of the 107 strains with the 11 MAbs. The intensities of the ELISA reactions are expressed in terms of the heights of the vertical lines. The positive-control axis shows line heights equivalent to 75 and 150% of the reactivity of the MAbs with immunogen (positive-control) strains. The results for 47 *R. leguminosarum* bv. trifolii strains are indicated to the left of the central dividing line in each diagram, and the results for 60 *R. meliloti* strains are indicated to the right. (A) Steamed cell antigen; (B) unsteamed cell antigen; (C) cell-free culture broth antigen; (D) nodule squash antigen.

than 5%. Positive-control ELISA absorbance values for the 11 MAbs following reaction with their respective immunogens, either as steamed or unsteamed cells, varied from 0.372 to 1.782. These differences reflect differences in the reactivities of the various MAbs with the standardized concentration of the respective positive-control antigens used.

None of the 11 MAbs were identical in patterns of reactivity with the 107 strains, although anti-*R. meliloti* MAbs 6, 10, and 11 showed similar patterns of reactivity in that they reacted with most *R. meliloti* strains (Fig. 1A to D).

One each of the anti-*R. leguminosarum* bv. trifolii (MAb 2) and anti-*R. meliloti* (MAb 7) MAbs reacted only with the strain which had been originally used as immunogen to produce the MAb (Fig. 1A and B). Both of these MAbs reacted specifically with the cells of the immunogenic strain in both steamed and unsteamed cell forms. MAb 2 did not react with either filtered culture broth or nodule antigen from the immunogenic strain, whereas MAb 7 reacted with both (Fig. 1C and D). All 11 MAbs reacted with both steamed and unsteamed cells of at least some strains. Steaming of the cells had little effect on the specificities of reaction with the MAbs but did result in less variation in extremes of reactivity.

On the basis of the 15% cutoff value selected as criterion for positive ELISA reactions with the MAbs and with these results applied as a string of + or − values for each strain with respect to the 11 MAbs, 28 different patterns of reactivity between the 11 MAbs and the 107 strains were found. Twelve of these patterns applied to one strain only (Table 4). The strains with MAb reactivities falling into a given pattern were considered antigenically distinct from strains falling into other patterns. Strains showing identical patterns in the analysis are grouped and listed in Table 4.

Five of the 47 *R. leguminosarum* bv. trifolii strains did not react with any of the MAbs (Table 2). All of the *R. meliloti* strains reacted with one or more of the MAbs (Table 3). The only cross-reactivity found between MAbs raised against one *Rhizobium* species and cells of strains of the other species was associated with anti-*R. meliloti* MAbs 8 and 9. Anti-*R. meliloti* MAb 9 cross-reacted with *R. leguminosarum* bv. trifolii USDA2080 and 162X97 in the steamed-cell form and with USDA2080 alone in the unsteamed-cell form (Fig. 1A and B). All cross-species reactivity results were confirmed by repetition of the work at a later time, including regrowth and preparation of the cells used as antigen. In contrast to the general lack of cross-species reactivity found with the MAbs, polyclonal antisera raised to *R. meliloti* NRG185 and Balsac cross-reacted widely, not only between strains, but between species (Fig. 2A and B).

TABLE 4. Strain groupings based on similarity of reactivities with MAbs[a]

Group	Strain(s) represented
1	162X97, WV7, and 162X77
2	USDA2080
3	WV18
4	WV22
5	ANU843, USDA2006, and WV21
6	162X95, 162X68, 162X100, and 162X92
7	162P30a and 162P30b
8	162P17
9	162BB1
10	162X7a and WVE5
11	LEL/01, WV23, USDA2102, 162Y14, and USDA2165
12	NRG40, LEL/02, LEL/03, LEL/04, LEL/05, LEL/06, 162S7a, WV12, USDA2134, USDA2087, USDA2203, USDA2229, 162X84, WV3, USDA2043, 162Y13, 162X20, USDA2227, USDA2149, USDA2137, 612C13 USDA2232, and USDA2104
13	102F34
14	102F51
15	NRG271 and NRG275
16	NRG192
17	NRG144
18	NRG133, NRG282, NRG289, and NRG312
19	102F28, NRG90, NRG131, NRG149, NRG170, Balsac, NRG230, and NRG284
20	NRG61, NRG84, NRG147, and NRG238
21	NRG60, NRG118, and URB165
22	NRG53 and NRG286
23	NRG50, NRG185, NRG277, NRG278, NRG296, NRG304, NRG308, NRG315, NRG352, URB164, and URB166
24	NRG43
25	NRG38, NRG47, NRG55, NRG57, NRG71, NRG79, NRG85, NRG86, NRG124, NRG126, NRG139, NRG232, NRG299, NRG302, 104A13, 102F66, and 102F77
26	NRG34
27	NRG24 and NRG70
28	NRG23

[a] On the basis of steamed- and unsteamed-cell reactivities considered together.

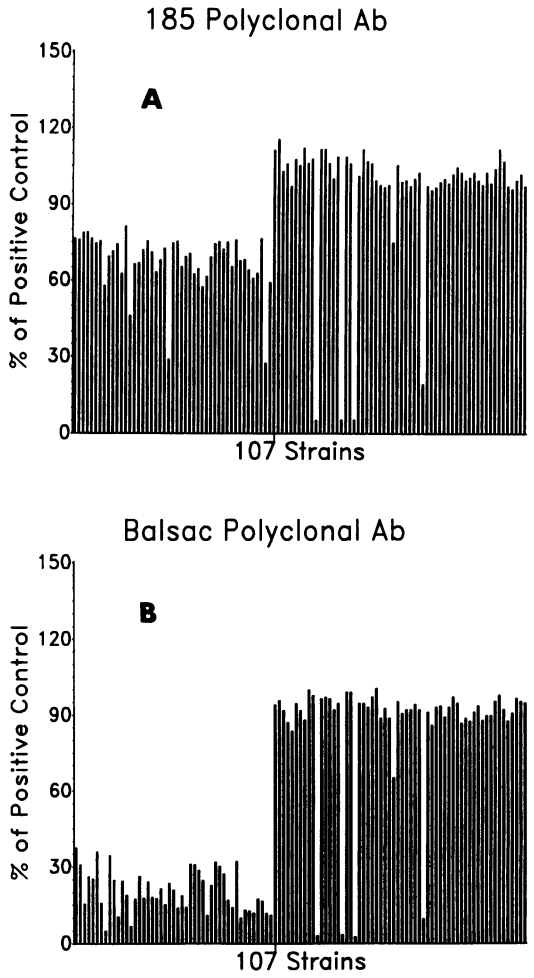

FIG. 2. ELISA reactivities of the 107 strains with rabbit polyclonal antisera (Ab) raised separately against *R. meliloti* NRG185 and Balsac, showing cross-reactivity between most strains within each species and also between strains of the two *Rhizobium* species. The ELISA reactivity of each antiserum with its immunogen strain (see Table 1) was defined as 100%. Strains to the left of the *x* axis tick mark are *R. leguminosarum* bv. trifolii; strains to the right of the mark are *R. meliloti*.

Nine of the 11 MAbs detected antigen secreted into the culture broth by the growing rhizobia (Fig. 1C). The pattern of reactivity between MAbs and culture broth antigens was such that if a MAb reacted with a strain's culture broth, that MAb would also react with cells of that strain. However, the reverse situation was not true in many cases.

Eight of the 11 MAbs reacted with nodule squash material. Nodule squash reactivity was generally much less than vegetative-cell reactivity (Fig. 1A, B, and D).

R. leguminosarum bv. trifolii USDA2165 and 162Y13 have been considered to be the same strain (8). Results between an isolate obtained from the Nitragin Company (162Y13) and one obtained from the U.S. Department of Agriculture collection (USDA2165) were compared. The results of this study indicate that these strains are not identical. Strain USDA2165 did not react with any of the MAbs, while 162Y13 reacted with MAb 3. By contrast, *R. leguminosarum* bv. trifolii 162P30 was entered into the test twice from different sources, and the pattern analysis placed 162P30a and 162P30b into the same group (Table 4, group 7). This group was co-occupied by none of the other 105 strains tested. Strain NRG40 had been listed as a *R. meliloti* strain in the NRG culture collection (Agriculture Canada, Beaverlodge) and was entered into the test as such. However, strong reactivity with anti-*R. leguminosarum* bv. trifolii MAb 3 and lack of reactivity with any of the anti-*R. meliloti* MAbs indicated that NRG40 was in fact an *R. leguminosarum* bv. trifolii strain, and this was confirmed by plant nodulation testing. Pattern analysis placed NRG40 in group 12 (Table 4) of *R. leguminosarum* bv. trifolii strains.

DISCUSSION

On the basis of the presence or absence of reactivity with the 107 strains, none of the 11 MAbs were identical in patterns of reactivity; therefore, they may be presumed to have different specificities. When they were reactive with a strain, the MAbs tended to also react with each of the strain's antigen forms (steamed or unsteamed cells, cell-free culture broth, and nodule squash). In some cases, however, a MAb did not react with secreted antigen in culture broth (anti-*R. leguminosarum*

bv. trifolii MAb 1), or nodule squash material (anti-*R. leguminosarum* bv. trifolii MAb 3 and anti-*R. meliloti* MAb 8), or both (anti-*R. leguminosarum* bv. trifolii MAb 2) (Fig. 1C and D). Nodule squash reactivity was generally much less than that for the other antigen forms; however, nodule squash antigen is a relatively impure form of the antigen compared with washed vegetative cells. In the indirect ELISA format (in which antigen is added to microplates as the first step), the impurity of nodule squash antigen would be expected to affect the intensity of the ELISA result. In addition, nodule squash (and culture broth) results are reported in this study as a percentage of the reactivity of the respective positive controls which were relatively pure washed and steamed cells. Low nodule squash reactivity in these ELISAs, therefore, does not suggest that the ELISA is generally poor for nodule squash strain identity work. Nevertheless, it has been previously reported (15) that some of the anti-*R. meliloti* MAbs used in the present work (MAbs 8 and 9) do not react well with nodule squash antigen because the reactive antigen has been lost or is diminished on bacteroid cells. Similarly, the stability of some rhizobial MAb-specific antigenic epitopes under conditions of stress has been questioned elsewhere (9, 26). It is therefore essential for investigators to be circumspect in the interpretation of rhizobial strain identification results and to demonstrate the validity of the assay used in relation to the form of antigen being tested.

Several reports exist suggesting that the practice of steaming *Rhizobium* or *Bradyrhizobium* antigen prior to testing by ELISA may increase reactivity or specificity (11, 24–26). The results of the present study indicated that the steaming of washed rhizobial whole cells for use as antigen in the ELISA caused few overall qualitative differences in reactivity compared with the reactivity of unsteamed cells. Some exceptions to this generalization did occur, and most of these exceptions were of the type in which steaming caused a strain's cells to react with the MAb sufficiently to be classified as a positive reaction (more than 15% of the positive control) in the ELISA, whereas the unsteamed cells of that strain were classified as negative in reaction. Nevertheless, the most notable overall effect of cell steaming was a smoothing out of the variability of reactivity of unsteamed cells to the anti-*R. leguminosarum* bv. trifolii cross-reacting MAb 3 and to the anti-*R. meliloti* cross-reacting MAbs 6 and 11. This effect may have been a result of a loosening and shedding of extracellular material to a relatively uniform level during the steaming process and subsequent washes.

The MAb-strain reactivity data found little cross-species reactivity. The only cross-species reactivity found in this study was associated with anti-*R. meliloti* MAb 9, which reacted positively with two *R. leguminosarum* bv. trifolii strains. In contrast, polyclonal antisera prepared against *R. meliloti* NRG185 and Balsac (which had been used as immunogen during creation of MAbs 6, 8, 9, and 11) and tested by ELISA against the 107 strains showed extensive cross-reactivity between both strains and species, indicating the presence of common antigens. Despite the fact that the MAbs used in this study demonstrated little cross-species reactivity, it is likely that a MAb could be selected which would show a high degree of cross-species reactivity.

The interpretation of the ELISA reactivity data to establish a system for analysis of antigenic differences between the strains used in this study is based on the simple premise that if two strains, under standardized conditions, give different patterns of positive and negative reactions with a panel of MAbs, then it is reasonable to assume that they possess antigenic differences. The basic principle of selection of an ELISA cutoff point involves attempting to minimize both false-positive and false-negative results (13, 20). Inevitably, however, minimizing false-positive results is associated with increasing the number of false-negative results and vice versa. The reproducibility, background noise, and precision of the data in this study were generally such that a meaningful cutoff absorbance value of 10%, or even 5%, of the level of the positive control could have been used instead of the 15% which was selected. The use of a 15% cutoff value minimized false-positive classifications so that the number of antigenic groupings and strains deduced and reported tends to be a minimum rather than a maximum number.

The finding that many *R. leguminosarum* bv. trifolii and *R. meliloti* strains secrete significant amounts of antibody-reactive antigen into the culture broth has significant implications. Since these secreted antigens are soluble, they could, presumably, be isolated and attached to beads, where they could serve as the basis for affinity purification systems as alternatives to adsorption of polyclonal antisera with heterologous cells for purposes of improvement of specificity of cross-reactive sera. At least some of the secreted antigens contain very specific epitopes (anti-*R. meliloti* MAb 7; Fig. 1C). Purified secreted antigen could possibly be used to immunize mice, resulting in an increased production of useful hybridomas. In a negative sense, it is possible that the occurrence of secreted antibody-reactive antigen may make it difficult to use immunoaffinity beads or immunomagnetic bead systems to isolate cells of *Rhizobium* species or strains from situations in which the bacteria have grown and secreted or shed antigen from the cells.

The abilities of anti-*R. leguminosarum* bv. trifolii MAb 3 and anti-*R. meliloti* MAbs 6, 10, and 11 to identify, at the species level, *R. leguminosarum* bv. trifolii and *R. meliloti* have already found application in regulatory legume inoculant analysis in Canada, where these MAbs have been used to presumptively confirm package labelling of clover and alfalfa inoculants through immunoblot analysis of plate counts. The immunological distinction between *Rhizobium* species may also prove useful in studies of microbial rhizosphere succession or other ecological studies involving rhizobial persistence. The specificities of MAb 2 for *R. leguminosarum* bv. trifolii WV22 and MAb 7 for *R. meliloti* NRG34 also have practical utility. MAb 2 will be used to trace strain WV22 in the soil during successive crop rotations, and MAb 7 is already used to monitor strain NRG34, which will be introduced commercially in Canada as a cold-tolerant strain in 1994.

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