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Serological Relatedness of *Rhizobium fredii* to Other Rhizobia and to the Bradyrhizobia

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Several isolates of *Rhizobium fredii* were examined for their serological relatedness to each other, to *Bradyrhizobium japonicum*, and to other fast- and slow-growing rhizobia. Immunofluorescence, agglutination, and immunodiffusion analyses indicated that *R. fredii* contains at least three separate somatic serogroups, USDA 192, USDA 194, and USDA 205. There was no cross-reaction between any of the *R. fredii* isolates and 13 of the 14 *B. japonicum* somatic serogroups tested. Cross-reactions were obtained with antisera from *R. fredii* and serogroup 122 of *B. japonicum*, *Rhizobium meliloti*, and several fast-growing *Rhizobium* spp. for *Leucaena*, *Sesbania*, and *Lablab* species. The serological relationship between *R. fredii* and *R. meliloti* was examined in more detail, and of 23 *R. meliloti* strains examined, 8 shared somatic antigens with the type strains from all three *R. fredii* serogroups. The serological relatedness of *R. fredii* to *B. japonicum* and *R. meliloti* appears to be unique since the strains are known to be biochemically and genetically diverse.

Immunodiffusion (ID), agglutination (AG), and fluorescent-antibody (FA) techniques have been widely used to identify and characterize strains of rhizobia and bradyrhizobia in culture (3, 7, 8, 11, 24), in soil (2), and in nodules (2, 4, 8, 9, 17). Based on the serological examination of a large number of rhizobia and bradyrhizobia isolates, three broad serological groups have been defined, namely: 1, *Rhizobium leguminosarum* bvs. *viceae*, *trifolii*, and *phaseoli*; 2, *Rhizobium meliloti*; and 3, *Bradyrhizobium japonicum* and *Bradyrhizobium* sp. (12, 29). Each of these groups appears serologically distinct. Results of these studies also indicate that the surface somatic antigens of the rhizobia and bradyrhizobia are more strain specific than are flagellar or internal antigens.

Keyser et al. (16) isolated 11 fast-growing strains of soybean-nodulating rhizobia from the People's Republic of China. In addition to these 11 strains, Dowdle and Bohlool (6) have subsequently isolated effective soybean-nodulating, fast-growing rhizobia from the Hubei province in central China. These fast-growing soybean rhizobia have since been described as a new species, *Rhizobium fredii* (23). Although *Rhizobium fredii* and *B. japonicum* have a similar host range, these organisms are considered to be physiologically and biochemically distinct (21, 23, 25, 31).

Given the usefulness of rhizobial antigens in strain identification and taxonomy, we studied the serological nature of the original 11 isolates of *R. fredii*. Our objective was to determine their serological relatedness to each other, to the typical slow-growing *B. japonicum*, and to other fast- and slow-growing species of the root nodule bacteria.

MATERIALS AND METHODS

Bacterial strains, sources, and maintenance of cultures. The strains used in this study and their sources are listed in Table 1. All strains were maintained on yeast extract-mannitol (YEM) agar slopes (27) or as lyophilized cultures. Agar slants used for the maintenance of fast-growing rhizobia contained 0.05% $CaCO_3$. All cultures were incubated at 28°C.

Antigen preparation. For ID, antigens were prepared from cells grown on the surface of B5 (10) agar medium. *Rhizobium* cells were harvested from 30-ml agar flats after 3 days of growth, whereas the bradyrhizobia were harvested after 7 days. Cells were suspended in 2 to 3 ml of 0.85% saline (containing thimerosal at a final concentration of 1:10,000) and stored at 4°C until use. For immunofluorescence (IF) and AG studies, antigens were prepared from cells grown in YEM liquid medium for 2 to 3 days. Somatic cell antigens for injections and for IF and AG studies were prepared by heating cell suspensions in a boiling water bath for 30 min.

Preparation of antisera. Boiled cell suspensions were used to produce somatic cell antisera by the method of Schmidt et al. (22). For whole-cell antisera, the same rabbits used for the production of somatic antisera were injected intramuscularly with 1 ml of a 1:1 mixture of unboiled cell suspensions and Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). After 3 weeks, 2 ml of the same culture was injected intravenously without adjuvant. All rabbits were bled 1 week after the last injection, provided that titers were greater or equal to 1,280.

IF analyses. FA were prepared against the somatic cell components of *R. fredii* USDA 192, USDA 193, USDA 194, USDA 201, USDA 205, and USDA 214 according to procedures described previously (22).

Smears from pure cultures and nodules were stained by the method of Schmidt et al. (22). For *R. fredii* cultures and nodules, suspensions were boiled for 30 min before FA staining. Gelatin-rhodamine isothiocyanate (1) was used to suppress nonspecific binding of FA. Stained smears were examined with a Zeiss Standard 16 microscope equipped for epifluorescence and phase-contrast microscopy. Incident illumination was provided by an HBO-50 (OSRAM) mercury-vapor light source with a fluorescein isothiocyanate filter pack.

The cross-reaction of FA-stained strains was assessed by subjective evaluation of the relative intensity of fluorescence, from 0 to 4+, with 4+ being the brightest.

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TABLE 1. Bacteria used and their sources[a]

Culture	Source
B. japonicum	
USDA 6, USDA 31, USDA 38, USDA 46, USDA 62, USDA 76, USDA 94, USDA 110, USDA 115, USDA 122, USDA 123, USDA 124, USDA 125, USDA 130, USDA 135, USDA 136	1
Webster 48	13
PRC005, PRC113-2, PRC121-6, PRCB15	3
R. fredii	
USDA 191, USDA 192, USDA 193, USDA 194, USDA 201, USDA 205, USDA 206, USDA 208, USDA 214, USDA 217, USDA 257	4
R. leguminosarum bv. *phaseoli*	
Bel7.1 (Viking-1)	2
CC511	5
NUM446	15
Tal1376	9
R. leguminosarum bv. *trifolii*	
TA1	5
NZP560 #2	6
R. leguminosarum bv. *viceae*	
Nitragin 92A3	5
PF2	6
R. loti NZP2037	5
R. meliloti	
L5-30	7
NZP4013	5
USDA 1002, USDA 1005, USDA 1021a, USDA 1024a, USDA 1027, USDA 1031, USDA 1035, USDA 1045, USDA 1093, USDA 1098, USDA 1107, USDA 1146, USDA 1148, USDA 1149, USDA 1150, USDA 1163, USDA 1170, USDA 1171, USDA 1174, USDA 1179, USDA 1180	1
Bradyrhizobium sp. (*Crotalaria*) 32H1	12
Bradyrhizobium sp. (*Macrotyloma*) CB756	5
Bradyrhizobium sp. (*Lupinus*) CC1814s	5
Rhizobium sp. (*Leucaena*)	
RL18	8
RL33	
RL38	
RL78	
RCR3817	1
Tal582	9
Tal1145	
UMKL19	6
Rhizobium sp. (*Sesbania*)	
3F4a3	1
3F4a4	
3F4c1	
Tal1113	9
Tal1114	
Tal1115	
Tal1116	
Tal1117	
Tal1119	
KH	10
PL146	11
Rhizobium sp. (*Lablab*)	
NGR234	14

[a] Sources: 1, U.S. Department of Agriculture, Beltsville, Md.; 2, E. L. Schmidt, University of Minnesota, St. Paul; 3, T. S. Hu, Institute of Soil and Fertilizers, Beijing, China; 4, H. H. Keyser, U.S. Department of Agriculture, Beltsville, Md.; 5, R. M. Greenwood, DSIRO, Palmerston North, New Zealand; 6, W. J. Broughton, Max-Planck Institute, Cologne, Federal Republic of Germany; 7, B. G. Rolfe, ANU, Canberra City, Australia; 8, H. Moawad, University of Hawaii, Honolulu; 9, NifTAL Project, Paia, Hawaii; 10, D. Evans, University of Hawaii, Honolulu; 11, origin unknown; 12, Nitragin Co., Milwaukee, Wis.; 13, B. B. Bohlool, University of Hawaii, Honolulu; 14, M. J. Trinick, Australia; 15, S. O. Keya, University Nairobi, Nairobi, Kenya.

ID analysis. ID of whole-cell and somatic cell antigens was done by a procedure modified from that of Vincent (27). Cell suspensions containing 10^{10} to 10^{11} cells per ml were added to the outer wells (6-mm diameter) of petri dishes containing 20 ml of ID agar (0.75% [wt/vol] Noble agar [Difco], 0.85% [wt/vol] NaCl, 0.01% [vol/vol] thimerosal, 8×10^{-5} M disodium EDTA, pH 6.5) and allowed to prediffuse for 4 h before the addition of antiserum to the center well. Gels were incubated in a moist chamber at room temperature for 3 to 4 days and stained with amido black solution (0.1% amido black, 4.25×10^{-1} M acetic acid, 4.25×10^{-2} M sodium acetate, 15% [vol/vol] glycerol). Gels were destained with a 2.0% (vol/vol) solution of acetic acid until the background was clear, and the precipitin bands were recorded photographically.

AG reactions. Tube AG reactions were performed essentially as described by Vincent (27). Antisera were diluted to a final concentration of 1:100 unless titers were to be determined, in which case antiserum concentrations were adjusted by using serial twofold dilutions in saline (0.85% [wt/vol] NaCl). Tubes were incubated at 37°C for 12 to 18 h followed by a 1-h incubation at 4°C. The cross-reactions of strains were assessed by subjective evaluation of the relative degree of AG, from 0 to 3+, with 3+ representing complete AG.

RESULTS

IF analysis. The results of the IF reactions of 11 strains of *R. fredii* with FA prepared from six *R. fredii* somatic antigens are shown in Table 2. Based on their reaction with FA to 6 of the *R. fredii* strains, the 11 *R. fredii* strains can be divided into at least three somatic serogroups: USDA 194 and USDA 201 (with a partial cross-reaction with USDA 257); USDA 192, USDA 217, and USDA 257 (with partial cross-reaction with USDA 206); and USDA 193, USDA 205, USDA 206, USDA 208, and USDA 214. Strain USDA 191 reacted weakly with antisera against USDA 193, USDA 205, and USDA 214. *R. fredii* USDA 193, USDA 205, and USDA 214 were virtually indistinguishable based on the reactions of their somatic antigens with FA.

Three of the *R. fredii* FA (192, 193, and 194) were also tested with other rhizobia and bradyrhizobia (Table 3). Of the 25 *B. japonicum* strains examined (comprising members of 18 different serotypes), only USDA 122 and USDA 136,

TABLE 2. IF analysis of somatic antigens of 11 isolates of *R. fredii*

Somatic serogroup	FA against	Isolates reacting 2+ or greater with FA[a]
1	192	192, 217, **206**, **257**
2	194	194, 201, **257**
	201	194, 201
3	193	
	205	193, 205, 214, 191, **206**, 208
	214	

[a] IF reactions were assessed based on fluorescence intensity from − = no fluorescence to 4+ = bright fluorescence. In all cases, homologous reactions scored 4+. The isolates designated in boldface type shared antigens with more than one reference strain.

TABLE 3. Cross-reaction of somatic antigens of *Rhizobium* and *Bradyrhizobium* species with antisera from *R. fredii*

Somatic antigens	FA against somatic antigens of *R. fredii* isolates[a]		
	USDA 192	USDA 193	USDA 194
B. japonicum			
23 strains	–	–	–
USDA 122 and USDA 136	–	–	2+
Bradyrhizobium sp. (2 strains)	–	–	–
R. leguminosarum bvs. *viceae* and *phaseoli* (5 strains)	–	–	–
Rhizobium spp.			
For *Leucaena*			
6 strains	–	–	–
Tal 82	3+	–	–
For *Sesbania*			
5 strains	–	–	–
Allen 770, Tal 1117, and PL 146	2+	NT	–
For *Lablab* (NGR 234)	3+	2+	2+

[a] Values represent relative degree of fluorescence: –, no fluorescence; 1+, weakly fluorescent; 4+, very fluorescent. NT, Not tested.

both from the USDA 122 serogroup, reacted with FA 194. Of the 21 fast-growing rhizobia examined, *Rhizobium* sp. (*Leucaena*) Tal 82 and *Rhizobium* spp. (*Sesbania*) Tal 1117, Allen 770, and PL 146 reacted with USDA 192 FA. In addition, the broad-host-range *Rhizobium* sp. (*Lablab*) NGR 234 cross-reacted with all three FA. However, the IF cross-reactivity of NGR 234 was greatest with FA prepared against USDA 192 and USDA 205. *Bradyrhizobium* sp. strains failed to react with any of the three FA.

R. fredii IF chracteristics differed from all other rhizobial systems that we have worked with in three fundamental ways. (i) Culture-grown cells, nodule suspensions, and rhizosphere populations had to be heat treated to obtain even and consistent IF staining; unheated cells either failed to react altogether or reacted weakly and unevenly. (ii) Cells grown in complex media devoid of added carbohydrates, i.e., TY (14) or PPM (20), did not react with FA produced against TY- or YEM-grown cells even after heat treatment; but both types of FA reacted strongly with YEM-grown cells; (iii) The intensity of IF reactions of YEM-grown cells was greatest with cultures in the logarithmic phase of growth; older cultures were less intense in fluorescence, and a smaller percentage of the cells reacted.

ID analysis. The ID cross-reactions of 33 strains of *Rhizobium* and *Bradyrhizobium* with somatic cell and whole-cell antisera produced against USDA 192, USDA 194, and USDA 205 are summarized in Table 4. The *R. fredii* strains could not be separated into serological groups based on their ID reactions with whole-cell antisera. All the strains examined shared at least one heat-labile antigen. However, they could be separated into three distinct serogroups based on ID reactions with the three somatic antisera (Fig. 1). These were the same groups that were found with FA. The ID patterns obtained with whole-cell antisera were quite complex and involved at least four precipitin bands, whereas reactions with somatic antisera produced one to two ID bands.

While none of the 11 strains of *Bradyrhizobium* examined cross-reacted with USDA 192, USDA 194, or USDA 205 antisera in ID testing, cross-reactivity was detected with six

TABLE 4. ID analysis of *Rhizobium* and *Bradyrhizobium* antigens

Antigen	Antiserum prepared against[a]:					
	Whole-cell antigen of[b]:			Somatic antigen of[c]:		
	192	194	205	192	194	205
R. fredii						
USDA 191	3	3	1	0	0	1
USDA 192	4	3	1	1	0	0
USDA 193	1	2	2	0	0	1
USDA 194	3	4	4	0	2	0
USDA 205	2	3	4	0	0	1
USDA 214	1	1	2	0	0	1
R. meliloti						
L5-30	1	2	2	1	0	0
NZP 4013	1	2	1	1	0	0
Rhizobium spp.						
For *Leucaena*						
1 strain	0	0	0			
RL 38	1	0	0			
For *Sesbania*						
2 strains	0	0	0			
PL 146	1	0	0	0	0	0
Allen 770	1	0	0	1	0	0
For *Lablab* (NGR 234)	2	2	3	0	0	1
Others[d]	0	0	0			

[a] Values represent the number of precipitin bands.
[b] Unboiled cells were used in antigen wells.
[c] Cell suspensions were placed in a boiling water bath before use.
[d] Other rhizobia: *B. japonicum* (strains from eight distinct serogroups); *Bradyrhizobium* sp. (three strains); *R. leguminosarum* bvs. *phaseoli* (two strains), *trifolii* (two strains), and *viceae* (two strains); and *R. loti* (one strain).

Rhizobium strains. Two *R. meliloti* strains, L5-30 and NZP 4013, produced one to two precipitin bands with whole-cell antisera prepared against USDA 192, USDA 194, and USDA 205 and one band of partial identity with antiserum from USDA 192 somatic antigens. *Rhizobium* spp. (*Sesbania*) PL 146 and Allen 770 formed one precipitin band with whole-cell antiserum from USDA 192; however, only Allen 770 reacted with USDA 192 somatic antiserum. The *Rhizobium* sp. (*Lablab*) strain NGR 234 formed precipitin bands with all

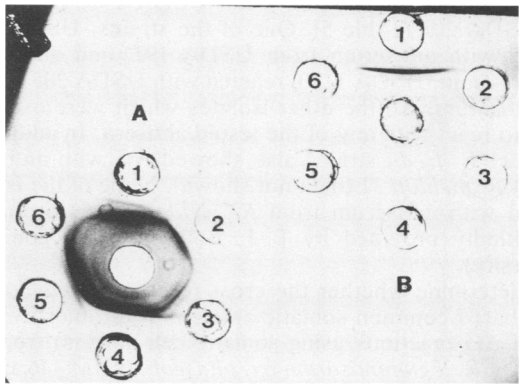

FIG. 1. ID analysis of whole-cell (A) and somatic cell (B) antigens from several *R. fredii* strains. Center wells are antiserum against whole cells (A) and somatic cells (B) of *R. fredii* USDA 192. Outer wells contain cell antigens of strains as follows: 1; USDA 192; 2, USDA 193; 3, USDA 191; 4, USDA 194; 5, USDA 205; and 6, USDA 214.

TABLE 5. Immunological cross-reactiveness of somatic antigens from several *R. meliloti* strains with antisera against *R. fredii*

Cross-reaction group	Isolates of *R. meliloti*	AG with *R. fredii* antisera[a]
		192
1	1002, 1005, 1027, 1031	194
	1045, 1093, 1146, 1174	205
2	1098	192
3	1170	205
4	1179, 1107, 1171, 1180, 1035, 1149, 1021a, 1024a, 1150, 1148, 1163	None[b]

[a] Reactions were performed at antiserum dilutions of 1:100. The cross-reactions of strains were assessed by subjective evaluation of the relative degree of AG, from 0 to 3+, with 3+ representing complete AG.
[b] None of the strains in group 4 reacted with any tested antisera.

three whole-cell antisera, but produced only one precipitin band when somatic cell antiserum from strain USDA 205 was used.

AG reactions. When 11 *R. fredii* strains were tested for AG reaction with somatic antisera produced against 15 strains of *B. japonicum*, the only case of cross-reactivity was with antiserum from USDA 122 (data not shown). This is the same as was found in the IF tests. However, in addition to the cross-reactions of *R. fredii* strains USDA 194 and USDA 201, strains USDA 217 and USDA 257 also agglutinated with antiserum 122. In the latter case, the AG titer was lower than that used with strains USDA 194 and USDA 201. The reciprocal AG of *B. japonicum* USDA 122 with antisera of USDA 194 and USDA 201 was also demonstrated. There was little or no AG cross-reaction with the other strains examined, with the exception of USDA 38 and 115. When the 11 *R. fredii* strains were examined for AG reaction with somatic cell antisera produced against *R. fredii* USDA 192, USDA 193, and USDA 194, the patterns of cross-reaction were the same as were found by IF.

Serological relatedness of *R. meliloti* and *R. fredii*. Of 21 *R. meliloti* strains examined, 8 (USDA 1002, USDA 1005, USDA 1027, USDA 1031, USDA 1045, USDA 1093, USDA 1146, and USDA 1174) demonstrated complete AG with somatic antisera against *R. fredii* USDA 192, USDA 194, and USDA 205 (Table 5). One of the strains, USDA 1098, reacted with antiserum from USDA 192, and another *R. meliloti* strain, USDA 1170, reacted with USDA 205 somatic cell antiserum. All the other isolates which were examined failed to react with any of the tested antisera. In addition, 8 of the 11 *R. fredii* strains also showed AG with antiserum against *R. meliloti* 31 (data not shown). None of the isolates reacted with antiserum from *R. meliloti* 17 (both antisera were kindly provided by P. J. Bottomley, Oregon State University).

To determine whether the cross-reactive *R. meliloti* isolates shared common somatic antigens with other rhizobia, we did AG reactions using somatic cell antisera from two strains of *R. leguminosarum* bv. *phaseoli*, NUM 446 and Tal 1376. Results of these studies (data not shown) indicated that of the six *R. meliloti* isolates examined, the four which cross-reacted with antisera from all three *R. fredii* serogroups (USDA 1005, USDA 1031, USDA 1045, and USDA 1093) also agglutinated with the two *R. leguminosarum* bv. *phaseoli* antisera. The other two *R. meliloti* isolates examined, USDA 1098 (*R. fredii* serogroup 192) and USDA 1170 (*R. fredii* serogroup 205) failed to react with these two antisera. These results indicate that while some of the *R. meliloti* isolates shared common somatic antigens with other fast-growing rhizobia, some have somatic antigens which appear species specific.

DISCUSSION

Although the 11 *R. fredii* strains were isolated from geographically diverse regions of China, all the isolates shared at least one heat-labile, agar-diffusible antigen. The heat-stable (somatic) antigens analyzed by IF, ID, or AG reactions were useful in separating these strains into at least three serological groups. Dowdle and Bohlool (6) have reported that only 9 of 15 *R. fredii* isolates they examined cross-reacted with these three somatic antisera, suggesting the existence of other serological groups in this species. Others (11, 19, 24, 29) have found somatic antigens of fast- and slow-growing rhizobia more specific than flagellar or internal antigens and suitable for separating strains within a species into distinct serological groups.

IF reactivity of *R. fredii* was found to be unique among rhizobia in that test antigens had to be grown in carbohydrate-containing media and heat activated before staining. Furthermore, the intensity of the IF reaction of cells diminished as they entered the stationary phase of growth (data not shown).

While *R. fredii* and the slow-growing *B. japonicum* both nodulate soybeans, they had little serological relatedness. However, four of the *R. fredii* strains (USDA 194, USDA 201, USDA 217, and USDA 257) serologically cross-reacted with *B. japonicum* somatic serogroup USDA 122. It should be noted that this cross-reaction with USDA 194 somatic cell antiserum was only evidenced by AG and IF reactions. No precipitin bands were detected in ID reactions between USDA 136 and USDA 194 somatic cell antiserum.

The serological cross-reaction between *R. fredii* and *B. japonicum* appears unique in the family *Rhizobiaceae*. In an analogous study, Pankhurst (19) found no sharing of somatic antigens between 62 fast-growing and 76 slow-growing *Lotus* rhizobia compared by ID reactions.

Unlike *R. fredii* and the other fast-growing rhizobia (11, 12), *B. japonicum* strains appear to be relatively serologically distinct. Of the 15 slow-growing strains tested by AG, only one cross-reaction was noted (USDA 115 and USDA 38). With minor exceptions, our results are similar to those found by others (5, 18) and show how the immunological distinctiveness of *B. japonicum* has been proved invaluable for ecological studies.

The *R. fredii* strains also showed some serological relatedness to other fast-growing rhizobia, with *R. fredii* antisera cross-reacting with *R. meliloti* and isolates from *Leucaena*, *Sesbania*, and *Lablab* species. Trinick (26) also noted AG of *R. meliloti* by antisera prepared against a fast-growing isolate from *Leucaena* species. Since somatic antigens are considered to be relatively specific among the root nodule bacteria, further taxonomic research seems warranted to determine whether *R. fredii* is closely related based on other important criteria. That is, *R. fredii* may be part of a taxonomic group that includes strains from diverse legume hosts.

The serological relationship between *R. fredii* and *R. meliloti*, as evidenced by ID and AG reactions, is significant since all taxonomic evidence (13, 15, 28), including serology, indicates that *R. meliloti* is unique among rhizobia and

deserves separate species status. In addition, a relationship between *R. fredii* and *R. meliloti* has also been noted by Wedlock and Jarvis (30) based on studies with rRNA-DNA hybridizations and DNA-DNA homologies.

In summary, despite the similarity with *B. japonicum* for legume host specificity for nodulation and nitrogen fixation, *R. fredii* is clearly more related to members of the genus *Rhizobium*, based on microbiological criteria (21, 23, 25). In addition, some of the strains of *R. fredii* have somatic antigens in common with the genus *Bradyrhizobium* (not necessarily the same antigens) and thus might represent an evolutionary link between the two divergent groups of organisms.

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