Agglutinin from Alfalfa Necessary for Binding and Nodulation by *Rhizobium meliloti*

Abstract. *A protein that specifically agglutinates* Rhizobium meliloti, *the alfalfa root nodule endosymbiont, has been purified from alfalfa seed. Material cross-reactive to antiserum prepared against the purified agglutinin is present in all alfalfa varieties that were tested but is absent in corn and other legumes not nodulated by* Rhizobium meliloti. *Studies with nonnodulating mutants of this microorganism incapable of binding to alfalfa roots suggest that the agglutinin is responsible for specific recognition between* Rhizobium meliloti *and alfalfa and that this recognition is an essential step in nodule formation.*

Infection of legume roots by *Rhizobium* that results in the formation of nitrogen-fixing root nodules is generally species-specific (*1*). The recognition process between infective rhizobia and their susceptible hosts is believed to be mediated by plant proteins that specifically recognize and bind to the surface of the bacteria (*2*). Some of these proteins are lectins and recognize specific cell-surface carbohydrates (*3*). Clover and soybean lectins have been purified and were found to bind specifically to the corresponding infective species of *Rhizobium* (*2*); in contrast, lectins from several legumes, such as jack bean, pea, and kidney bean, fail to bind infective rhizobia or have been shown to bind nonspecifically to species that do not infect these host plants (*4*). In these latter studies, however, the lectins used were isolated originally as hemagglutinins and not as specific agglutinins for rhizobia. We now describe an alfalfa agglutinin that specifically agglutinates cells of *Rhizobium meliloti*. This protein may play a role in the specific binding of the endosymbiont to the legume root, resulting in nodulation.

Table 1. Agglutination of *Rhizobium* and erythrocytes by purified alfalfa agglutinin. None of the strains except *R. meliloti* have been documented to nodulate alfalfa. Hemagglutination was assayed with glutaraldehyde-fixed cells at 2 percent final concentration. Symbols: +, positive agglutination; −, no agglutination.

Strains	Agglutination	Strains	Agglutination
R. meliloti		*R.* "lotus"†	
102F28	+	95C11*	−
102F51	+	95E8*	−
102F65	+	95C6*	−
104A12	+	95C8*	−
104A14	+	95E6*	−
2011	+		
		R. "cowpea"†	
R. phaseoli		176A17	−
127K14	−	176A27	−
127K26*	−	176A40	−
127K44*	−		
127K55*	−	*R. japonicum*	
		61A76	−
R. trifolii		61A84*	−
162E8	−	61A88	−
162T2	−	61A92*	−
0403*	−	61A103	−
		61A119*	−
		505 (Wisconsin)	−
R. leguminosarum		*R.* "sesbania"†	
175P1*	−	145A1	+
175G9	−	145A4*	−
128C82	−	145A10	−
175F3*	−	145B1	−
		145E1	−
R. lupini			
96A6	−	Erythrocytes	
86B8	−	Human A	−
96E8	−	Human B	−
		Human O	−
		Sheep	−

*These strains were filtered through glass wool to eliminate flocs caused by autoagglutination before the assay was initiated. †These *Rhizobium* strains have not been identified as to species; the name in quotes indicates the source.

A seed extract (*5*) of alfalfa (*Medicago sativa* L. cultivar Vernal), when assayed under the proper conditions (*6*), agglutinated cells of *R. meliloti* 102F51, a strain that produces nitrogen-fixing alfalfa nodules. Preliminary studies showed that this crude seed extract agglutinated other infective *R. meliloti* strains as well. The agglutination assay was used to purify to homogeneity (*7*), from alfalfa seed extract, an agglutinin that migrated as a single protein band (molecular weight, ~ 14,000) after electrophoresis in a sodium dodecyl sulfate polyacrylamide gel and that aggultinated six infective *R. meliloti* strains tested (Table 1) but had no hemagglutination activity. Thirty-four other strains, representative of all the major *Rhizobium* species that do not nodulate alfalfa, were tested and only one was agglutinated (Table 1). The sole "false positive" strain, 145A1, was originally isolated from nodules of *Sesbania macrocarpus*, and its taxonomic identity and geographical source are still unclear (*8*). This strain appears to be closely related to *R. meliloti* according to two-dimensional polyacrylamide gel analysis of total cellular proteins (*9*), and it also binds to the root hairs of alfalfa but does not form nodules. Therefore, a positive correlation exists between agglutination of infective *Rhizobium* by alfalfa agglutinin and the ability of these strains to bind to alfalfa root hairs.

Three independently isolated nonnodulating mutants of *R. meliloti* 102F51 were obtained by direct screening of alfalfa plants (*10*) after mutagenesis (*11*). Mutants WL113 and WL131 failed to bind to alfalfa root hairs (*12*), but mutant WL188 as well as the wild type did bind (Fig. 1). In addition, assays with purified agglutinin showed that WL188 and the wild type were agglutinated, whereas WL113 and WL131 were not. The concomitant loss, in the two mutants, of root hair binding ability and agglutination strongly suggests that alfalfa agglutinin is responsible for recognition and binding of *R. meliloti* to alfalfa root hairs prior to nodulation. Since the nonnodulating mutants were agglutinated by an antiserum prepared against the wild-type parent, were sensitive to phage specific for *R. meliloti* (*13*), and had a total cellular protein pattern identical to the wild type on two-dimensional polyacrylamide gels (*9*), it appears certain that the nonnodulating mutants were not contaminants but were derived from the wild-type parent.

Three other nonnodulating mutants derived from another wild type, *R. meliloti* 2011, were isolated in the laboratory of F. Ausubel after transposon (Tn5) mutagenesis. One of these mutants, strain 1028, did not react strongly with alfalfa agglutinin, whereas strains 1027 and 1126, as well as the wild type, were agglutinated. Therefore, strain 1028 is

Table 2. Quantitation of alfalfa agglutinin on the root surface with plant age, and its correlation with the ability of the roots to be nodulated. Agglutinin was measured (*15*) at various times after germination. Seedlings of the same ages were also inoculated with 10^6 cells of *Rhizobium meliloti* per plant to test their receptivity to infection and nodulation at these times. The number of nodules was determined 3 weeks after inoculation. Plants were grown as described (*10*) in serum vials, and the nodule number reported per plant is the average from five seedlings. Results for the change in absorbance at 405 nm ($\Delta A_{405\ nm}$) (experimental minus control) are the averages of duplicate samples.

Number of days after germination	Alkaline phosphatase activity ($\Delta A_{405\ nm}$)	Nodules per plant (± standard deviation)
2	0.114	5.8 ± 3.9
4	0.241	7.4 ± 3.4
6	0.310	10.4 ± 2.1
8	0.199	10.8 ± 1.5
10	0.182	7.2 ± 0.8

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phenotypically similar to WL113 and WL131, and strains 1027 and 1126 are similar to WL188. More than 30 other *R. meliloti* mutants that formed nodules on alfalfa reacted strongly with the purified agglutinin.

Rabbit antiserum prepared against purified alfalfa agglutinin was used to demonstrate the ubiquitous and unique presence of agglutinin in different alfalfa varieties nodulated by *R. meliloti*. Cross-reactive material to the antiserum was detected by Ouchterlony double immunodiffusion (*14*) in seeds of eight varieties of *Medicago sativa* tested (Buffalo, Ranger High Saponin, Ranger Low Saponin, Hairy Peruvian, Sorona, Saranac, NC8376 High Saponin, and NC8376 Low Saponin) and one variety of *M. falcata*. The agglutinin in the extract of one of these varieties (Buffalo) was further purified, and it specifically agglutinated infective cells of *R. meliloti*. Cross-reactive material to the antiserum was also detected in concentrated extracts from Vernal alfalfa cotyledons and young seedling roots but was not detected in seed extracts of corn or other legumes (*Trifolium repens, Glycine max* Corsoy, *Pisum sativum, Vigna radiata, Vigna unguiculata* cultivar Speckled Purple Hull, and *Vicia faba*). Indirect immunoassay with alkaline phosphatase-conjugated goat antiserum against rabbit immunoglobulin G (AP-GAR) (*15*) was used to detect agglutinin on the surface of young Vernal alfalfa seedling roots (Table 2). A low level of AP-GAR was bound nonspecifically to seedling roots previously incubated with rabbit serum obtained before immunization. A significantly larger amount of AP-GAR was bound to roots previously incubated with antiserum against alfalfa agglutinin, indicating the presence of agglutinin on the root surface. The appearance of agglutinin on the seedling roots was transient and correlated with the time at which the seedlings were most competent for nodulation by *R. meliloti*. These results indicate that the agglutinin purified from alfalfa seeds, like trifoliin in clover (*16*) and the soybean lectin (*17*), is present on the surface of the root where it may play a role in the recognition and binding of the endosymbiont.

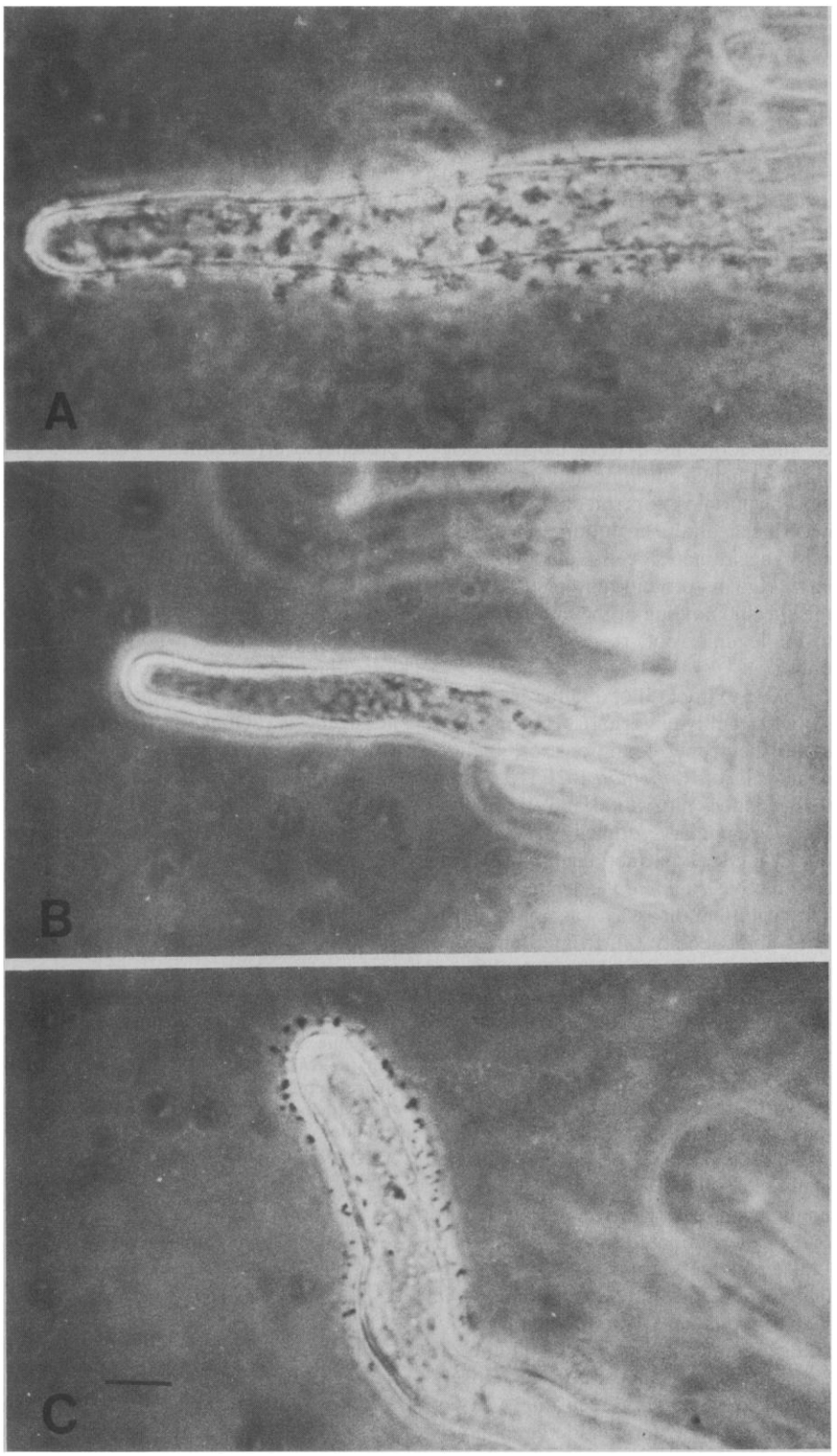

Fig. 1. Binding of *Rhizobium meliloti* 102F51 and the nonnodulating mutants to root hairs of alfalfa seedlings. (A) Strain 102F51, (B) mutant strain WL113, and (C) mutant strain WL188. Scale bar, 5 μm.

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5. Alfalfa seeds were ground to 20 mesh and defatted with two volumes of hexane in a dry ice–ethanol bath for 1 hour. The defatted seed powder was vacuum dried. Eight grams of seed powder was stirred in 60 ml of 0.2*M* phosphate-buffered saline (PBS) (*p*H 6.8) containing 0.6 g of sodium ascorbate, 70 mg of $MgSO_4 \cdot 7H_2O$, and 1.2 g of acid-washed polyvinyl-polypyrrolidone. The slurry was centrifuged at 27,000*g* for 60 minutes, and the supernatant solution was dialyzed overnight against 0.1*M* sodium acetate (*p*H 4.0) in the cold. Insoluble material was removed by centrifugation and discarded.
6. *Rhizobium* strains were grown on an agar–yeast

extract–mannitol medium (*10*) for 4 to 5 days and then harvested in 10 ml of PBS. The cells were washed once in the same buffer and then in 0.1*M* sodium acetate (*p*H 4.0) before finally being suspended in 10 ml of the acetate buffer. The final cell suspension was adjusted to approximately 12.0 units at an absorbance of 660 nm. The cell suspension (80 μl) was mixed with 80 μl of the seed extract and incubated at 30°C for 15 to 60 minutes.

7. Alfalfa agglutinin was purified by the following method. The crude seed extract was heated at 80°C for 30 minutes. After centrifugation, the supernatant was treated with 60 percent ammonium sulfate; the precipitate was dissolved in 0.1*M* sodium acetate buffer (*p*H 4.0) and loaded onto a Sephadex G-50 column. Active fractions were dialyzed against distilled water. Further purification was achieved by preparative flat-bed isoelectric focusing in Sephadex G-75 (*p*H 3.5 to 10.0). Fractions containing purified agglutinin were pooled and concentrated by ultrafiltration (Amicon, UM2). The ampholytes were removed by gel filtration on a Sephadex G-75 or G-100 column. Biochemical characterization and evidence that the agglutinin is a protein will be presented elsewhere.
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11. Exponentially growing cells of *R. meliloti* 102F51 in yeast extract–mannitol medium were harvested by centrifugation and washed once with 50 m*M* PBS. The cells were suspended in 50 m*M* PBS containing 160 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per milliliter for 30 minutes. They were then pelleted and washed three times with 50 m*M* PBS before being suspended in yeast extract–mannitol medium and incubated overnight at 30°C to allow phenotypic expression. The cells were cultured in minimal mannitol medium for over 30 generations before clones were isolated for testing on alfalfa plants. The mutants grew as well as the wild type on a variety of different media.
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13. The nonnodulating mutants were tested for susceptibility to 20 phage isolates that lyse *R. meliloti* 102F51. Plaque formation on lawns of the mutants in 0.7 percent yeast extract–mannitol agar was noted after incubation at 30°C overnight.
14. Ouchterlony plates contained 0.7 percent agarose (Sigma, type II), 0.05*M* sodium barbital (*p*H 8.4), 0.2 percent NaCl, and 0.05 percent sodium azide. The wells were filled with 20 μl of extract or antiserum, and precipitin bands were allowed to develop at 30°C for 4 to 24 hours.
15. Cotyledons were removed from seedlings, and the cut ends were sealed by dipping them in melted paraffin. The seedling roots were then washed successively in 0.1*M* PBS, 5 mg of bovine serum albumin per milliliter, and 5 mg of bovine gamma globulin per milliliter, followed by two more washings in PBS. Control seedlings were incubated at room temperature with rabbit serum obtained before immunization. Experimental seedlings of matched size were incubated with rabbit antiserum prepared against purified agglutinin. The seedlings were again washed as described above. They were then incubated at 30°C for 1 hour with alkaline phosphatase–conjugated goat antiserum against rabbit IgG (Miles) before being washed as before and placed in 1 ml of 10 percent ethanolamine (*p*H 9.9), containing 1 mg of 2-nitrophenylphosphate per milliliter, at room tempeature for 10 minutes. The reaction was stopped by the addition of 0.17 ml of 3*M* NaOH, and the activity of the alkaline phosphatase was determined at an absorbance of 405 nm.
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Mutator Activity in Maize: Timing of Its Activation in Ontogeny

Abstract. *Mutator activity in maize seems not to be active throughout the development of the plant, but to arise late in ontogeny, and to be developmentally rather than chronologically triggered.*

The mutator system (*Mu*) (*1*) increases the frequency of mutations in outcross progeny 30 to 50 times (*1*, *2*). This *Mu* activity is not expressed throughout the development of the plant but seems to be preferentially expressed late in the development of the germ-line tissue of the sporophyte (*2*). Whether the timing of *Mu* activity is related to total elapsed time from germination (chronologically determined) or is related to a particular developmental stage of the germ line (developmentally determined) that occurs late in ontogeny was investigated in this study.

Several different small sectors (or clusters) of seeds bearing allelic mutants were found in ear maps of plants showing *Mu* activity. The largest sector extended over a minimum of five seeds (*2*). More sectors have now been found in other ear maps, the largest sector containing a minimum of 11 seeds and the smallest, a minimum of 3 seeds. The occurrence of clusters of mutants establishes that some *Mu*-directed mutational events occur in mitotic divisions late in the development of the ear. Solitary mutants on some of the ears may be the result of very late mitotic events giving rise to a sector in which only one of the cells produced a seed with a *Mu*-induced mutant. Alternatively, some or all of these single mutants may be meiotically

Table 1. Frequency of mutants in five serial outcrosses of plants sampled on 5 to 7 consecutive days of pollen shedding, and the distribution of putative clusters in these outcrosses. Heterogeneity χ^2 for totals of each plant outcrossed is 34.0471; d.f. = 4; $P < .01$.

Day	Total plants self-pollinated	Total mutants per outcross	Percent mutants per outcross	Heterogeneity χ^2	Degrees of freedom	*P*	Number of putative clusters	Average putative cluster per plant
				Cross 5062-3				
1	77	8	10.4				1	
2	63	16	25.4				1	
3	52	11	21.2				3	
4	47	10	21.3				1	
5	60	12	20.0				4	
6	71	16	22.5				3	
7	57	7	12.3				2	
Total	427	80	18.7	8.0540	6	.20 to .30		2.5
				Cross 5061-9				
1	49	5	10.2				0	
2	51	7	13.7				1	
3	40	5	12.5				1	
4	44	6	13.6				1	
5	55	7	12.7				2	
6	52	6	11.5				1	
Total	291	36	12.4	0.4038	5	>.99		1.0
				Cross 5062-4				
1	27	2	7.4				0	
2	43	5	11.6				2	
3	32	2	6.2				0	
4	41	2	4.9				0	
5	35	2	5.7				0	
6	30	5	16.7				1	
Total	208	18	8.7	4.3268	5	.50 to .70		0.5
				Cross 5061-8				
1	51	4	7.8				1	
2	53	3	5.7				1	
3	46	5	10.9				1	
4	64	7	10.9				3	
5	52	3	5.8				1	
Total	266	22	8.3	1.9267	4	.70 to .80		1.4
				Cross 5061-6				
1	12	3	25.0				0	
2	10	1	10.0				0	
3	30	8	26.7				2	
4	30	9	30.0				2	
5	19	6	31.6				1	
Total	101	27	26.7	1.8393	4	.70 to .80		1.0

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