JOURNAL OF BACTERIOLOGY, Oct. 1984, p. 454–457
0021-9193/84/100454-04$02.00/0

Vol. 160, No. 1

Monoclonal Antibodies to *Rhizobium meliloti* and Surface Mutants Insensitive to Them

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Received 16 April 1984/Accepted 4 June 1984

Monoclonal antibodies were produced to the surface of the symbiotic nitrogen-fixing bacterium *Rhizobium meliloti*. Bacterial lysis in the presence of complement or cycles of agglutination and growth were used to select mutants no longer recognized by the antibodies. The mutants were used to produce new antibodies with different specificities. Several mutants had altered sensitivity to one or more bacteriophages. *R. meliloti* strains from different sources had distinct patterns of sensitivity to monoclonal antibodies and phages, which together can be used for discriminative typing.

Rhizobium meliloti forms nitrogen-fixing nodules on the roots of alfalfa. Rhizobia are, in general, plant species specific, and although direct evidence is still sparse, specificity is thought to involve bacterial surface components (2, 22, 29). We have been studying the *Rhizobium* cell surface (24, 26; E. Johansen, Ph.D. thesis, Massachusetts Institute of Technology, 1983), and we report here the production of monoclonal antibodies specific for the surface of *R. meliloti* SU47 and derivatives and the partial characterization of antibody specificity through the isolation of mutants no longer recognized by the antibodies. The approach should be equally applicable to other bacterial species.

Monoclonal antibodies are secreted by hybridomas, which are continuous lines made by fusion of myeloma cells with spleen cells from an immunized animal (14, 33). The exquisite specificity of monoclonal antibodies makes them particularly suitable for revealing subtle antigenic alterations. In bacteria they have been used to probe the structure and function of cell surfaces (4, 11, 28) and also to identify pathogenic organisms (20, 23, 25).

Monoclonal antibodies have been used for selection of antibody-insensitive surface mutants of several animal viruses (8, 12, 15, 34). To our knowledge, monoclonal antibody-insensitive mutants have not been reported previously for bacteria (although their isolation has been suggested; 9). Selection of antibody-insensitive mutants is not usually feasible with a polyclonal serum, which is a heterogenous population of antibodies directed against many different antigenic determinants, because a mutant missing one determinant should still react immunologically with other antibodies in the serum. However, an antibody-insensitive mutant of *Acinetobacter calcoaceticus* has been selected with a polyclonal serum, presumably because all determinants were on the same immunodominant surface component missing in the mutant (3).

In the present work, hybridomas were produced after immunization with *R. meliloti* (see below); the resulting antibodies were then used for isolation of antibody-insensitive mutants, which served to distinguish different antibodies, and the antibodies, together with a set of bacteriophages, were also used for identification of *R. meliloti* strains fom various sources.

R. meliloti EJ300 is a spontaneous mutant of the streptomycin-resistant SU47 derivative Rm2011 (18) that is also resistant to 50 μg of rifampin per ml. Phages φM2 and φM8 were isolated from soil by the method of Vincent (30); other φM phages have been previously described (10) (φM5 has also been given to other laboratories as F20); phage L5 was from M. Kowalski, and phage DF2br was from J. Olivares, both via K. Bergman. Growth medium was LB (19), with 2.5 mM $MgSO_4$.

Hybridoma lines producing antibodies 1 through 4 (below) were derived from SP2/0-Ag14 (27), and those producing antibodies 5 and 6 were derived from X63-Ag8.653 (13). Medium and methods for animal cell culture and for polyethylene glycol 1000-induced fusion of myeloma with spleen cells were as described previously (17), except a 1/15 ratio of myeloma to spleen cells was used. Mice were BALB/c, either born at the animal facility of the Massachusetts Institute of Technology or purchased from Jackson Laboratories, and were immunized intraperitoneally with 4×10^7 bacteria in Hanks balanced salt solution (GIBCO Laboratories) buffered with 0.01 M KH_2PO_4 (pH 7.2).

Hybridomas were tested for antibody production 2 weeks after fusion. About 3×10^8 fresh stationary-phase bacteria suspended in phosphate-buffered saline (0.85% NaCl, 0.01 M KH_2PO_4 buffer [pH 7.2]) were spread on LB agar containing 50 μg of rifampin per ml, 500 μg of streptomycin per ml, 0.5 mM $MgSO_4$, and 0.15 mM $CaCl_2$. Drops from microtiter wells were spotted on with a MIC-2000 inoculator (Dynatech Laboratories), and each was overlaid with a 2-μl drop of filter-sterilized guinea pig complement in Hanks solution. Plates were incubated for 2 days at 34°C; after this time, if antibody is present a hole is produced in the bacterial lawn due to complement-induced lysis. Positive wells were retested for complement-induced lysis; positive wells were also tested for agglutination by mixing 3×10^7 bacteria in phosphate-buffered saline with 5 μl of tissue culture supernatant, incubation on a microscope slide under a cover slip for 8 min at 34°C, and inspection under a phase-contrast microscope (×400). Hybridomas were cloned over fibroblasts (5) and used for production of ascites tumors (17).

Antibody-insensitive mutants were isolated by one of three methods. (i) Occasional rare colonies appearing in complement-induced lysis spots were purified; about half proved to be antibody insensitive. (ii) For agglutination cycles, 1.0 ml of saturated culture concentrated 1.5 times in phosphate-buffered saline, plus tissue culture supernatant or appropriately diluted ascites fluid, was incubated for 1 h at 34°C and centrifuged; the supernatant was added to 1.5 ml of

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TABLE 1. Representative monoclonal antibody-insensitive mutants

Strain[a]	Parent	Antibody selected with	Method[b]	Response[c] to antibody					
				1	2	3	4	5	6
EJ300	Wild type			+	+	+	+	+	+
EJ301	EJ300	Ab1	i	−	+	+	+	+	+
EJ312	EJ301	Ab2	ii	−	−	−	+	+	+
EJ318	EJ301	Ab3	ii	−	+	−	+	+	+
EJ322	EJ312	Ab4	ii	−	−	−	−	−	+
EJ329	EJ312	Ab4	ii	−	−	−	−	+	+
EJ332	EJ312	Ab5	ii	−	−	−	−	−	+
EJ337	EJ312	Ab5	ii	−	−	−	+	−	+
EJ355	EJ312	Ab6	ii	−	−	−	+	+	−
EJ363	EJ312	Ab6	iii	−	−	−	+	+	−
EJ358	EJ322	Ab6	ii	−	−	−	−	−	−

[a] In each case, at least three other independent mutants with the same antibody sensitivity were isolated.
[b] i, Colonies in complement-induced lysis spot, ii; agglutination cycles; iii, complement-induced lysis cycles.; see text.
[c] +, Agglutination; −, no agglutination.

fresh LB medium and grown to saturation again. After three to five cycles, cultures contained 95% or more antibody-insensitive bacteria. (iii) For complement-induced lysis cycles, 10^9 bacteria in phosphate-buffered saline containing 0.15 mM $CaCl_2$ and 0.5 mM $MgSO_4$, plus 50 μl of antibody and 100 μl of complement, were incubated for 1 h at 34°C; an additional 100 μl of complement was added, incubation was continued for 1 h more, and bacteria were subcultured in LB. After two cycles, cultures contained 80% or more antibody-insensitive bacteria. All mutants isolated by complement-induced lysis failed to agglutinate and vice versa; only one mutant from a given culture was saved.

Hybridomas were first produced from myeloma-spleen cell fusions carried out after immunization with *R. meliloti* EJ300, either 6 days after primary immunization (one mouse) or 4 days after the last of several secondary immunizations (three mice). Among ca. 475 microtiter wells with visible hybrids, 40 wells produced anti-EJ300 antibody. A mutant of EJ300 insensitive to antibody from one of those wells (Ab1) was isolated and called EJ301 (Table 1). Antibody from 34 of the wells failed to recognize EJ301 and thus could not be distinguished from one another or from Ab1 by means of that mutant. Antibody from the remaining six wells recognized both EJ300 and EJ301. Isolation and testing of antibody-insensitive mutants (Table 1) showed that these wells included two new monoclonal antibodies, Ab2 and Ab3.

To minimize the repeated isolation of hybridomas producing the same antibody, antibody-insensitive mutants were used in some immunizations. In one such experiment, two mice were initially immunized with another *R. meliloti* strain, L5-30 (chosen because it is antigenically distant from EJ300; see below); 2 days before myeloma-spleen cell fusion, these mice were then immunized again with strain EJ312 (= EJ300 Ab1 insensitive, Ab2,3 insensitive; Table 1). Among 700 wells with visible fusion hybrids, 6 wells produced antibody recognizing EJ312, although none of the 6 recognized L5-30. Isolation of antibody-insensitive mutants with antibody from these six wells showed that three new monoclonal antibodies had been produced, Ab4, Ab5, and Ab6 (Table 1). For Ab1, Ab2, and Ab6, antibodies with the same specificity have been obtained independently from different mice.

The response to antibodies of representative spontaneous independent antibody-insensitive mutants is summarized in Table 1. All mutants have the same drug sensitivities as the parental strain EJ300, and all are agglutinated by rabbit anti-Rm2011 serum. All mutants nodulate alfalfa, and nodules from all but one fix nitrogen as judged by acetylene reduction; the exception, EJ355, will be the subject of a separate report. Table 1 also shows how antibody-insensitive mutants were used for successive isolation of antibodies with new specificities.

The properties of the surface antigens recognized by these monoclonal antibodies and of the antibody-insensitive mutants span a wide range. The immunodominant Ab1 antigen is heat labile and therefore probably protein; it is not flagellar because Ab1 agglutinates NR1000, a mutant lacking flagella (1), and conversely, Ab1 insensitive mutants are motile (Johansen, Ph.D. thesis). In R-factor-mediated conjugation (18), four independent Ab1 insensitive mutants were tightly linked to *his-39* and loosely linked to *trp-33*. The Ab2 insensitivity of EJ312 is suppressed by growth in streptomycin (250 μg/ml), to which the strain is resistant; however, the Ab3 insensitivity of this strain, which probably results from the same mutation, is not suppressed by streptomycin. The properties of mutants isolated with either Ab2 or Ab3 (Table 1) suggest the surface antigen(s) recognized by these two antibodies may be on the same molecule. Both these antigens are heat stable and therefore probably polysaccharide; the Ab2 antigen can be extracted with hot phenol (Johansen, Ph.D. thesis), suggestive of lipopolysaccharide (32). Neither of two Ab2-insensitive mutations and neither of two Ab3 insensitive mutations showed any linkage in conjugation to *his-39*, *trp-33*, or *pyr-49*. Antibody insensitivity of mutants (Table 1) suggests Ab4 and Ab5 are related as well. The antigens of both Ab4 and Ab5 are heat stable, whereas the Ab6 antigen is heat labile. Although Ab4, Ab5, and Ab6 were isolated after immunization with EJ312, these antibodies recognize its parental strain EJ300 as well; therefore, the corresponding antigenic determinants of EJ300 were neither altered nor created anew by the Ab1 insensitive or Ab2,3 insensitive mutations of EJ312.

Bacterial surface alterations leading to antibody insensitivity affect sensitivity to bacteriophage as well. Mutants of EJ312 that are Ab4 insensitive invariably became resistant to phages ϕM2 and ϕM7 irrespective of their Ab5 insensitivity even though mutants selected with one of these two antibodies were often resistant to the other (Table 1). Therefore, the Ab4 antigen and the receptors for ϕM2 and ϕM7 are probably all on the same molecule, although results with *R. meliloti* from different sources (see below) suggest that they are not identical. Mutants of EJ312 that are Ab6 insensitive fall into a number of different classes with respect to phage sensitivity (Table 2); interestingly, the only antibody-insensi-

TABLE 2. Phage sensitivity of antibody-insensitive mutants of EJ312

Strain or class (no. in class)	Response[a] to phageϕM									
	1	2	5	6	7	9h1[b]	10	11	12	14
EJ312	+	+	+	+	+	+	+	+	+	+
Class I (29)	−	+	−	−	+	+	+	+	+	+
Class II (8[c])	+	+	+	+	+	+	+	+	+	+
Class III (2)	+	+	−	+	+	+	+	+	+	+
Class IV (1[d])	+	+	±[e]	+	+	−	−	+	+	−

[a] +, Lysis in spot test (10); −, no lysis; mutants are grouped in classes according to pattern of response to phages.
[b] Host range mutant of ϕM9 isolated for improved plating on EJ312.
[c] Includes EJ363 (Table 1).
[d] EJ355 (Table 1).
[e] Efficiency of plating 10^{-4} relative to EJ312.

tive mutant with altered symbiotic properties (EJ355; see above) also has a unique pattern (class IV). Evidently, sensitivity to a single monoclonal antibody, Ab6, can be abolished by any one of a number of different surface alterations. These results confirm that antibody insensitive mutants have altered surfaces, as expected. Together with the apparent relatedness of the Ab2-Ab3 and the Ab4-Ab5 antigens, they begin to indicate the complexity of surface architecture.

Many different rhizobial strains have been isolated from soil, and rapid serological identification is of value in the laboratory, in field studies, and in quality control of commercial inocula (7, 31). For *R. meliloti*, both polyclonal serology (21) and bacteriophage sensitivity (16) have been used. Among 36 *R. meliloti* strains from various sources, sensitivity to monoclonal antibodies is considerably more strain specific than phage sensitivity (Table 3). The only strains agglutinated by all six nmonoclonal antibodies are derivatives of SU47, the parent of the strains used for antibody isolation, and of the remaining 30 strains only 7 react with any of the antibodies. None of these monoclonal antibodies agglutinate *Rhizobium leguminosarum*, *Rhizobium phaseoli*, *Rhizobium japonicum*, *Agrobacterium tumefaciens*, or *Escherichia coli* (Johansen, Ph.D. thesis).

Since the bacterial surface is thought to be involved in nodulation specificity, all *R. meliloti* strains might be expected to share one or more common alfalfa-specific surface components. Indeed, by comparison, for the clover symbiont *Rhizobium trifolii*, polyclonal serology has identified an oligosaccharide present in wild-type strains but apparently missing from the surface of mutants defective in nodulation (6). To the contrary, however, Table 3 suggests that even though these different *R. meliloti* strains all nodulate alfalfa, their surfaces vary considerably. This could easily obscure any common component(s).

The results presented here indicate the resolving power of monoclonal antibodies and mutants insensitive to them with regard to the bacterial surface. Particularly in conjunction

TABLE 3. Response of *R. meliloti* strains to antibody and phage[a]

Strain[b]	Rabbit anti-RM1201	Monoclonal antibody						Phage						
		1	2	3	4	5	6	ϕM1	ϕM2	ϕM5	ϕM6	ϕM7	L5[c]	Df2br
SU47, NA219,[d] RCR2011,[d] RM1021,[d] and RM2011[d]	+	+	+	+	+	+	+	+	+	+	+	+	−	−
RCR2006, RCR2012, and SU8	+	−	+	+	−	−	+	+	−	+	+	−	−	−
NA39 and SU134	+	−	+	+	+	+	+	+	+	+	+	+	−	−
SU101/W1	+	−	+	+	+	−	+	+	−	+	+	+	−	−
NA218-6	+	+	+	+	−	−	+	+	−	+	+	+	−	−
RMJ2	+	−	−	−	−	−	−	+	+	+	+	+	−	+
102F51	+	−	−	−	−	−	−	+	−	+	+	+	−	+
NA355, NA2290, and R2	+	−	−	−	−	−	−	+	−	+	+	−	−	+
SU126 and R9	+	−	−	−	−	−	−	+	−	+	+	−	−	−
SU27	+	−	−	−	−	−	−	+	−	−	+	−	−	−
L5-30	−	−	−	−	−	−	−	+	+	+	+	+	+	+
RM11	−	−	−	−	−	−	−	+	+	+	+	+	−	+
NA216, RCR2001, and SU51/13L	−	−	−	−	−	−	−	+	+	+	+	+	−	−
SU 101/W2 -ellI	−	−	−	−	−	−	−	+	−	+	+	+	−	+
R3 and R10	−	−	−	−	−	−	−	+	−	+	+	−	−	+
NA298, NA299-2, NA359, R14, and SU66	−	−	−	−	−	−	−	+	−	+	+	−	−	−
102F34	−	−	−	−	−	−	−	−	+	+	−	+	−	−
RM41	−	−	−	−	−	−	−	−	+	−	−	+	−	−
NA315-2	−	−	−	−	−	−	−	+	−	+	−	−	−	−

[a] +, Agglutination or lysis; −, no agglutination or lysis.
[b] Strains were obtained as follows: NA, G. Bullard, New South Wales Horticultural Research Station; RCR, Rothamsted Experimental Station; R and SU, M. Wilson, University of New South Wales; RMJ2, *Melilotus alba* nodule, Cambridge, Mass.; others: reference 9, all strains form effective nodules on alfalfa.
[c] L5 is temperate; identical results are found with lytic ϕM8.
[d] Derived from SU47.

with bacteriophages, monoclonal antibodies should be useful for highly discriminative typing of rhizobial strains.

We thank Ann Marshak-Rothstein, Christine Norton, and Charles Maplethorpe for assistance and members of the Gefter and Signer laboratories for discussion.

E.J. was supported by a graduate fellowship from National Chemicals and Distillers Corp. Research support was from Public Health Service grants CA 28900-03 and AI 13357-07 to M.L.G. and AI 16873 to E.R.S. from the National Institutes of Health, from American Chemical Society grant NP6L to M.L.G., and from U.S. Department of Agriculture grants 59-2253-0-1-498-0 and 80-CRCR-1-0498 to E.R.S.

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