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Monoclonal Antibodies against *Agrobacterium tumefaciens* Strain C58

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Abstract. Hybridoma cell lines were derived from the fusion of mouse myeloma cells with spleen cells from a mouse that had been immunized with *Agrobacterium tumefaciens* strain A759, a flagella-less mutant of strain C58 containing the Ti plasmid of strain B6. All of the 20 antibodies produced by the cloned hybridomas reacted with strain C58 and with other strains derived from C58. The antibodies did not react with 34 other strains of *A. tumefaciens*, representing the three biovars, or with strains of *A. radiobacter*, *A. rubi*, *Rhizobium leguminosarum*, *R. meliloti*, or other plant-associated bacteria such as *Erwinia herbicola* and *Pseudomonas syringae*. In addition to reacting with whole cells of strain A759, the antibodies reacted with phenol–water extracts of A759, indicating that they may recognize the lipopolysaccharide. These antibodies may be useful for ecological and epidemiological studies of *A. tumefaciens* strain C58 in the agroecosystem.

Tools for the detection of plant pathogenic bacteria are important for accurate disease diagnosis and for studying the ecology and epidemiology of plant pathogens in the agroecosystem. Isolation of bacteria on selective media, isolation on nonselective media coupled with taxonomic identification, the satisfaction of Koch's postulates, and reaction with polyclonal antisera have been the major methods for diagnosis of bacterial diseases of plants [11, 29, 32]. In general, these methods do not differentiate among strains of the same species or pathovar and thus lack the specificity required to monitor survival of a particular strain in soil and air, or on plant surfaces. There is a need for studies designed to monitor particular strains in order to improve our understanding of the behavior of pathogens and biocontrol agents in the environment. Such an understanding will enable us to predict the behavior of pathogens and manipulate the agroecosystem to accomplish more successful biocontrol of plant disease.

Strain specificity is critical to the success of methods designed to determine the fate of a particular unaltered or genetically engineered organism introduced into the agroecosystem. Recent advances in the development of DNA probes [14, 16, 33] and monoclonal antibodies [1, 3, 4] suggest that these technologies may provide the requisite specificity to track particular bacterial strains.

Monoclonal antibody technology has provided a new set of immunological tools that have exquisite antigen specificity. The high degree of specificity has been useful in many aspects of bacteriology, including the study of the structure of the bacterial cell surface [12, 15, 25, 26, 30], the detection of bacterial toxins [2, 31] and the detection and identification of pathogens of animals [7, 22, 30] and plants [1, 3, 4, 8, 19, 20, 23, 34]. Here we report the production of monoclonal antibodies that are specific to *Agrobacterium tumefaciens* strain C58, which causes crown gall disease of dicotyledonous plants.

Materials and Methods

Bacterial strains and culture conditions. *Agrobacterium tumefaciens* strains A759, A1045, and A2505 were obtained from E. W. Nester (University of Washington). The other *A. tumefaciens* strains were obtained from L. Moore (Oregon State University). The remaining bacterial strains are part of the culture collection in the Department of Plant Pathology at the University of Wisconsin.

Bacteria were grown in AT minimal broth [27]. Broth medium was inoculated with a loopful of bacteria from an AT plate (containing AT broth plus 1.5% agar) and incubated at 28°C for 2 days with vigorous shaking. For mouse immunization or for the

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immunoassay, the broth culture was centrifuged at 10,000 *g* for 10 min, the pellet was resuspended in sterile, phosphate-buffered saline (PBS), and the washing process was repeated once. PBS contained, per liter: 8.76 g NaCl, 5.22 g K_2HPO_4, and 1.36 g KH_2PO_4 and was adjusted to pH 7.3. The washed bacterial cells were resuspended in PBS to a final concentration of approximately 4.0×10^8/ml; the cell concentration was determined by dilution plating on AT agar.

Mouse immunization. An 8-week-old BALB/c mouse was immunized with *A. tumefaciens* strain A759. The first immunization consisted of an intraperitoneal injection with an emulsion of 4.0×10^7 bacteria suspended in 0.1 ml PBS and 0.1 ml Freund's complete adjuvant (Sigma Chemical Co., St. Louis, Missouri). Subsequently, the mouse was injected intraperitoneally biweekly with an emulsion of 4.0×10^7 bacteria in 0.1 ml PBS and 0.1 ml Freund's incomplete adjuvant (Sigma Chemical Co.). Emulsions were obtained by forcing the mixture through an 18-gauge needle with a syringe 10 to 20 times. After two biweekly injections the serum titer was greater than 1/1000 as determined in the enzyme-linked-immunoadsorbent-assay (ELISA) described below.

For serum titer determinations, the mouse was bled through the tail vein. The blood was collected in a microfuge tube, centrifuged at 10,000 *g* to remove the red blood cells, and serial tenfold dilutions of the serum were made in PBS. When the serum titer reached 1/1000, the mouse received a series of two combination injections 48 h apart. The combination injections consisted of 8.0×10^6 bacteria in 0.05 mL of PBS administered intravenously in the tail vein, and 3.2×10^7 bacteria in 0.2 ml PBS administered intraperitoneally. Two days after the final injection, the mouse was killed and the spleen was removed aseptically.

Production of hybridomas. Hybridomas were produced by fusing mouse spleen cells with mouse myeloma NS-1 cells by a modification of the Kohler and Milstein procedure [21]. The spleen cells (approximately 10^8) and the NS-1 cells (approximately 10^7) were washed separately with 10.0 ml DMEM medium (Sigma Chemical Co.) each, and suspended in separate tubes in 11.0 ml of DMEM. Then, 10.0 ml of each suspension was transferred into a single 50-ml plastic centrifuge tube, and the two cell types were centrifuged together at 1,000 *g* for 5 min. The remaining 1.0 mL each of the NS-1 and spleen cell suspensions were used as controls. After centrifugation, the supernatant was decanted and the pellet was gently loosened by flicking the tube manually. One milliliter of a 40% solution of 1,450 mw polyethylene glycol at 37°C was added to the cell mixture over a period of 45 s. The mixture was then drawn up into a plastic pipette twice in 15 s, maintained at room temperature for an additional 45 s, and then diluted in 30 mL DMEM with 20% fetal calf serum (Gibco Co., Grand Island, New York) and HT medium supplement (Sigma Chemical Co). The diluted suspension was incubated at 37°C in an atmosphere of 5% CO_2 for 1 h and then centrifuged for 5 min at 1,000 *g*. The cells were resuspended in 230 ml DMEM containing 20% fetal calf serum, 1% rabbit red blood cell suspension, and HAT medium supplement (Sigma Chemical Co.). The 1% rabbit red blood cell suspension was prepared by bleeding a New Zealand white rabbit from the central ear vein. Immediately upon being drawn, 10 ml of blood was placed into 100-ml sterile PBS to prevent clotting, centrifuged at 2,000 *g* for 15 min, resuspended up to 10.0 ml with sterile PBS, and stored at 4°C.

The fused-cell suspension was dispensed into 96-well culture dishes and incubated at 37°C in an atmosphere of 6% CO_2. Expansion and cloning of cell lines was performed according to Mierendorf and Dimond [24], except that ascites tumors were not used for antibody production, and antibody solutions were taken from culture supernatants.

Screening hybridomas. Ten days after fusion, supernatants from the hybridoma cultures were screened by ELISA. Bacteria were prepared for the immunization procedure as described, resuspended in PBS to a final concentration of 1.0×10^8 cells/ml, and 0.05 ml was placed in each well of a 96-well microtiter plate. The plates were centrifuged at 1,000 *g* for 5 min in a centrifuge with microtiter plate adapters. The ELISA was conducted with 4-methylumbelliferyl phosphate as substrate according to the method of Mierendorf and Dimond [24], except that the BSA blocking buffer was replaced with 0.5% nonfat dry milk buffer [17], and 0.25 mg/ml levamisole (Sigma Chemical Co.) was added to the substrate solution to inhibit the mouse alkaline phosphatase activity in the culture supernatants. In addition, for the blocking step before the addition of goat-anti-mouse antibody, the blocking buffer was heated to 55°C before it was added to the wells. Heating the blocking buffer at this step substantially reduced the background in the assay.

Results and Discussion

When mouse myeloma cells were fused with the spleen cells from a mouse that was immunized with cells of *Agrobacterium tumefaciens* A759, 3,000 of the resulting hybridomas produced colonies, and 20 independent colonies produced antibodies that reacted positively with strain A759 in two ELISA screenings.

The 20 positive colonies were cloned to produce pure cell lines. All 20 of these lines produced monoclonal antibodies (mAb's) that were highly specific for strain A759 and other strains derived from the C58 parent. The data in Table 1 are for mAb-007, but are representative of all 20 mAb's. All of the antibodies reacted with strain C58 when they were diluted at least tenfold. The undiluted hybridoma culture supernatants containing the mAb's were tested by ELISA against 34 strains of *Agrobacterium* spp., two *Rhizobium leguminosarum*, one of *R. meliloti*, and one strain each of *Erwinia herbicola*, *Pseudomonas syringae*, and *Escherichia coli*. All 20 mAb's reacted positively with strain C58, A759, and two avirulent mutants, A1045 and A2505, that map in the *chvB* locus, which affects attachment to plant cells and production of a cyclic β-1,2-D-glucan [10, 28]. A759 and the mutants all contain a C58 chromosomal background and differ from C58 only in the nature of the Ti plasmid present [13], lack of flagella [6], and the *chvB* mutations [10].

None of the antibodies reacted positively with any other *Agrobacterium* strains, including strains of biovars 1, 2, and 3, or with any of the other genera of bacteria tested. Thus, the mAb's described here

Table 1. Reactions of bacterial strains with monoclonal antibodies raised against *Agrobacterium tumefaciens* strain A759

Bacterial species and strain	Biovar[a]	Geographical origin	Host origin	Reaction with antibody mAb-007[b]
A. tumefaciens				
A759 (C58-derived)	1	Mutant[c]	—	+
A1045 (C58-derived)	1	Mutant[c]	—	+
A2505 (C58-derived)	1	Mutant[c]	—	+
C58	1	New York	Cherry	+
B6	1	Iowa	Apple	—
G2/79	1	Oklahoma	Cottonwood	—
G18/79	1	Oklahoma	Poplar	—
GA001	1	Georgia	Pecan	—
GA002	1	Georgia	Pecan	—
GA003	1	Georgia	Pecan	—
GA012	1	Georgia	Pecan	—
GA015	1	Georgia	Pecan	—
GA105	1	Georgia	Pecan	—
H27/79	1	Colombia	Rose	—
K24	1	Australia	NA[d]	—
K30	1	Australia	Peach	—
M63/79	1	Oklahoma	Cottonwood	—
S1/73	1	Arizona	Lippia	—
AB2/73	2	Arizona	Lippia	—
B234	2	California	NA	—
U11	2	Oregon	Willow	—
Ag63	3	Greece	Almond	—
CG48	3	New York	Grapevine	—
CG54	NA	NA	NA	—
CG56	3	New York	Grapevine	—
CG64	3	New York	Grapevine	—
6/6	3	Hungary	Grapevine	—
16/5	3	Hungary	Grapevine	—
A. rhizogenes				
A4	2	California	NA	—
K47	2	Australia	NA	—
A. rubi				
RR5	1	Oregon	Raspberry	—
TR2	2	Washington	Raspberry	—
A. radiobacter				
T20/73	1	Oregon	Rose	—
K84	2	Australia	Soil	—
Rhizobium leguminosarum				
CE3	phaseoli	Mexico	Bean	—
KIM5	phaseoli	Idaho	Bean	—
Rhizobium meliloti				
2011	—	NA	Alfalfa	—
Escherichia coli J53 pSa				—
Erwinia herbicola Eh01				—
Pseudomonas syringae Ps01				—

[a] Information regarding biovar, geographical origin, and host was obtained from Bouzar et al. [5].
[b] Reactions with mAb-007 are representative of all 20 mAb's.
[c] Indicates strains that contain the C58 chromosomal background [6, 10, 13].
[d] NA indicates information not available.

may be useful for monitoring populations of C58 in soil, on plant surfaces, or in crown gall tissue, since they can differentiate between *A. tumefaciens* strain C58 and other bacteria.

It is intriguing that we identified only strain-specific mAb's with this technique, whereas other investigators have produced genus-, pathovar-, and subgroup-specific mAb's against *Xanthomonas* spp. [1], species-specific antibodies against *Corynebacterium sepedonicum* [9], and biovar-specific antibodies against *A. tumefaciens* biovar 3 [4]. Our results suggest that there is a strong, immunodominant antigen on strain C58 that is not present or exposed on the cell surfaces of other strains of *Agrobacterium* and of other genera of bacteria. Bouzar et al. [5] recently showed that polyclonal antiserum raised against lipopolysaccharide (LPS) from *A. tumefaciens* strain B6 reacted with whole cells of B6 and not with cells of other strains of *Agrobacterium* spp. or other bacteria. Preliminary results suggest that our strain-specific mAb's may be directed against LPS. In an ELISA, the mAb's reacted strongly with crude phenol–water extracts [18] of strain A759 and not with extracts of strain B6. Although LPS is a major component in phenol–water extracts, we cannot rule out the possibility that the antibodies detected another antigen in the preparations.

It may be significant that we injected live bacteria into the mice, whereas other workers have injected heat-killed cells. Heat killing is often used to denature flagella antigens, but since strain A759 is a flagella-less mutant, heating did not seem necessary. It is fortuitous that the cells used as the antigen were not heat killed, since it has been demonstrated recently that antiserum directed against heat-killed cells does not react with unheated cells (L. Moore, personal communication). Since the major use of our antibodies is likely to be the detection of live bacteria in plant and soil samples, it is important that the antibodies react with bacteria that have not been heat killed.

Further work is required to determine whether the mAb's are sufficiently specific and sensitive to detect bacteria in plant and soil samples. If they are, these mAb's could provide a useful tool for understanding the behavior of *A. tumefaciens* strain C58 in the agroecosystem.

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