A Monoclonal Antibody Specific to *Agrobacterium tumefaciens* Biovar 3 and its Utilization for Indexing Grapevine Propagation Material

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ABSTRACT

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A monoclonal antibody, designated AbF21-1D3G7C8, reacts in enzyme-linked immunosorbent assay with biovar 3 strains of *Agrobacterium tumefaciens*, causal agent of grapevine crown gall, from North America, Europe, and Asia, regardless of tumorigenicity. AbF21-1D3G7C8 does not react with *A. tumefaciens* biovars 1 or 2, or with other plant pathogens, symbionts, or saprophytes. AbF21-1D3G7C8 is useful for laboratory diagnosis of *A. tumefaciens* biovar 3, and thus for detection and diagnosis of grapevine crown gall, indexing of propagation wood, and quarantine testing. *A. tumefaciens* biovar 3 was identified in grapevine cultivars and rootstocks from New York and Washington states by means of the monoclonal antibody.

Agrobacterium tumefaciens (E. F. Smith & Townsend) Conn. biovar 3, causal agent of crown gall of grapevine (*Vitis* spp.) (6,16,24,26), survives saprophytically in grapevine xylem and is transmitted by vegetative propagation (6,18). Tumor production follows wounding of systemically infected vines by freezing temperatures or mechanical means (12,18,19). Possible strategies for control of this disease include indexing and certification of propagation wood (4,27) and planting vines free of the pathogen (8). Proposed schemes for testing propagation stocks rely on colony appearance on a medium developed by Roy and Sasser (RS) (7) and subsequent pathogenicity testing (7,27) or on immunofluorescent assay (4) for diagnosis of biovar 3. The morphology of colonies of biovar 3 strains vary on RS and colonies of other endophytic bacteria from grapevine may appear similar (T. J. Burr and B. H. Katz, *unpublished*). These other bacteria may constitute more than 90% of the colonies in isolations from grapevine or soil samples (7,23). Basing diagnosis on colony morphology alone thus may lead to unnecessary rejection of plant material. The use of biological and physiological tests for biovar identification of numerous strains of *Agrobacterium* from isolation plates is time consuming and labor intensive (7,21,22). Serological assays may provide more rapid diagnosis, but the presence of common epitopes in nontarget species isolated from plant tissue can lead to false positive diagnoses (9,10). Previous attempts to develop biovar-specific antisera have not succeeded (1,3,4,15,21).

Hybridoma techniques of Kohler and Milstein (17) allow production of antibodies specific to single epitopes selected according to the investigators' design, thereby eliminating the problem of cross reaction. Application of monoclonal antibodies to diagnosis or detection of several bacterial plant pathogens has been reported (2,11,13,20). We report the production of a monoclonal antibody specific to *A. tumefaciens* biovar 3 and its application to indexing of grapevine propagation material.

MATERIALS AND METHODS

Bacterial strains. *A. tumefaciens* biovar 3 strain CG49 was used as immunogen for production of antibodies. Twenty-four other tumorigenic and nontumorigenic biovar 3 strains were screened against the antibody. These were isolated from five states in the United States, from Afghanistan, Crete, Italy, Spain, and West Germany. Strains were isolated from grapevine tumors, xylem extracts, callus, and sap and from vineyard soils. Nineteen biovar 1 strains and five biovar 2 strains (tumorigenic and nontumorigenic) also were tested. All strains of *Agrobacterium* were characterized to biovar by previously published methods (24,26). Strains of *Erwinia*, *Pseudomonas*, *Rhizobium*, and *Bradyrhizobium* and 43 unidentified saprophytic bacteria from grape sap and rhizospheres also were tested. Additional details of strain designation and origin are available from the authors on request.

Strains of *Agrobacterium, Pseudomonas,* and *Erwinia* were grown on potato-dextrose agar (PDA) or 523 medium (14). *Rhizobium* strains were grown on yeast mannitol agar (per liter: 1 g of yeast extract, 10 g of mannitol, 0.65 g of $K_2HPO_4 \cdot 3H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 15 g of agar, pH 7.4). Cultures were grown at 28 C.

Immunogen preparation. Cells from 3-day-old PDA cultures of CG49 were harvested by washing plates with phosphate-buffered saline containing 1.5 mM KH_2PO, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 150 mM NaCl, pH 7.4. Cells were then washed by three cycles of centrifugation (10,000 *g*, 10 min) and resuspended in the same buffer. Suspensions were adjusted to $A_{600nm} = 0.1$ with buffer and heated (10 min, 80 C) before immunization.

Monoclonal antibody production. BALB/c mice were immunized at 6 wk of age (300 μl intraperitoneal and 200 μl subcutaneous) with bacterial suspensions mixed 1:1 with Freund's complete adjuvant. Booster injections (500 μl intraperitoneal) 2 wk after immunization were prepared with Freund's incomplete adjuvant. Three days before fusion, 250-μl intraperitoneal booster injections were administered. Mouse spleens were removed 5 wk after initial immunization. A concentration of 10^8 splenocytes was fused with 10^7 SP2/O-AG14 myeloma cells (American Type Culture Collection, Rockville, MD) in 1 ml of 50% polyethylene glycol (mol wt 1,450) and diluted to 15 ml with amended Dulbecco's modified Eagle medium (Gibco Laboratories, Grand Island, NY)(DMEM). Cells were centrifuged, resuspended in 10 ml of DMEM with 20% fetal bovine serum. 1.02×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.65×10^{-7} M thymidine (DMEM/HAT/20% FBS), diluted to 70 ml with DMEM/HAT/20% FBS, and distributed in 100-μl aliquots to seven 96-well cell culture plates (Corning). Culture plates had

been seeded the previous day with mouse macrophages (10^3 per well in 100 μl of DMEM/HAT/20% FBS) collected by peritoneal lavage of pristane-primed mice. Cell cultures were fed by aspiration of spent medium and replacement with DMEM/HAT/20% FBS 4–6 days after fusion, DMEM/HAT/20% FBS (lacking aminopterin) 6–11 days after fusion, and screened for antibody production 11–13 days after fusion. Hybrids secreting antibodies that reacted with *A. tumefaciens* biovar 3, but not with other biovars, were cloned and subcloned by limiting dilution. Selected cell cultures were scaled up to 500 ml in DMEM/20% FBS, and supernatants were harvested by centrifugation after 7–10 days. Cell line F21-1D3G7C8 was used to produce ascites fluid by intraperitoneal injection of two 11-wk-old pristane-primed BALB/c mice with 2×10^6 hybridoma cells in 500 μl of phosphate-buffered saline. Cell culture supernatants and ascites fluids were stored frozen or at 4 C after adjusting to 0.05% NaN_3.

Screening of hybridomas. Hybridoma culture supernatants initially were tested for production of antibodies specific to *A. tumefaciens* biovar 3 in microtiter plate enzyme-linked immunosorbent assay (ELISA). Antigens were prepared from washed bacterial cells harvested from 3-day-old cultures growing on PDA or medium 523. Bacteria were suspended in phosphate-buffered saline (0.01 M sodium phosphate, 0.85% NaCl, pH 7.2) (PBS) with 0.05% sodium lauryl sarcosine added, centrifuged (10,000 *g*, 10 min), and then suspended and pelleted twice more in PBS. Before final centrifugation, suspensions were heated for 10 min at 80 C. The final pellet was suspended in coating buffer (40 mM sodium carbonate, pH 9.6) and adjusted to $A_{600nm} = 0.1$.

All incubations were in moist chambers at 37 C, unless otherwise noted. Microtiter plates (Immulon 2 "U" well, Dynatech) were incubated overnight with 100 μl of antigen per well. Antigen was shaken out and wells were washed three times, for 3 min each, with PBS 0.05% Tween 20 (PBST). Plates were incubated at room temperature for 1 hr with 200 μl of blocking buffer (5% nonfat-dry milk in 50 mM Tris-HCl, pH 7.2) per well. After washing (as above), hybridoma culture supernatant (100 μl per well) was added. After incubation for 2.5 hr in 5% CO_2, plates were washed and incubated 20 min at room temperature with blocking buffer (200 μl per well) which had been heated to 55 C. After washing, plates were incubated 1.5 hr with 100 μl of goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma) per well, diluted 1:1,000 or 1:400 in PBST. Plates were washed a final time and 200 μl of enzyme substrate (1 mg of *p*-nitrophenylphosphate per milliliter of 9.7% diethanolamine, pH 9.8), was added to each well and incubated at room temperature. A_{405nm} was measured periodically with a Dynatech MR580 MICROELISA auto reader. Polyclonal rabbit antiserum $FC_2$14 to *A. tumefaciens* biovar 3 (a gift from Dr. Carlo Bazzi) was used with goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) as a positive control.

Isotype determination. Isotypes were determined in Ouchterlony double diffusion tests with anti-mouse immunoglobulins (Sigma) in 0.8% noble agar buffered with 1.5 mM borate, pH 8.3, 5 mM KCl, 0.85% NaCl.

Purification of antibody. Antibody was purified from ascites fluid and culture supernatant by protein A affinity chromatography (MAPS II kit, Bio-Rad), according to the manufacturer's instructions, except that culture supernatants were not concentrated before chromatography. Concentration of antibody was estimated by UV spectrophotometry after purification. Binding buffer fractions were dialyzed against PBS and tested in ELISA to determine efficiency of binding of antibody to the protein A column.

Specificity of antibody. ELISA was modified to facilitate testing of many strains. Bacterial suspensions in coating buffer were adjusted to $A_{600nm} = 0.1$, about 10^7 colony-forming units (cfu)/ml, and 100 μl were added to each well. The suspensions then were dried by incubating microtiter plates overnight in a 37 C circulating air incubator. Immediately before use, dried plates were incubated with 200 μl of fixative (25% ethanol, 10% acetic acid) per well for 15 min at room temperature, and then rinsed with distilled water. Other procedures previously described in the ELISA protocol were followed, except that incubation with the monoclonal antibody was not in 5% CO_2. Purified antibody (1 μg/ml) was used in specificity tests.

Sensitivity of modified ELISA. Tenfold serial dilutions of a cell suspension of strain CG49 (about 10^7 cfu/ml) were prepared in sterile distilled water and coating buffer. Aliquots of sterile distilled water dilutions (100 μl) were spread on PDA and colonies were counted after 5 days. Bacterial suspensions diluted in coating buffer were used in the modified ELISA to determine the number of *A. tumefaciens* biovar 3 cells required to give a positive reaction.

Indirect fluorescent antibody stain. Drops of a suspension of CG49 in distilled water (about 10^7 cfu/ml) were dried and then heat fixed on glass microscope slides. A drop of antibody, diluted to various concentrations in PBST, was incubated on each slide for 1 hr in a moist chamber. Slides were washed in a stream of PBST and blotted dry, and a drop of diluted goat anti-mouse IgG-fluorescein isothiocyanate (FITC) conjugate (Sigma) was placed on each smear and incubated 1 hr in a moist dark chamber. Slides were washed again in PBST, air dried, and No. 1 cover slips were mounted in buffered glycerol (9:1, glycerol:0.1 M phosphate buffer, pH 7.6). Fluorescence was observed on a Zeiss Photomicroscope III with HBO 50W illuminator, band pass 450-490 nm exciter filter, FT 510 chromatic beam splitter and band pass 520-560 nm barrier filter. Antiserum $FC_2$14, in conjunction with FITC-labeled goat anti-rabbit IgG (Sigma), was used as positive control.

Immunoblot. An ELISA procedure with nitrocellulose membranes as the solid support phase (immunoblot) was developed to minimize sample preparation in testing colonies from isolation plates. Colonies grown for 48 hr on 523 medium were sampled by collecting a mass of cells on the flat end of a sterile toothpick and suspending the cells in 100 μl of sterile distilled water. These suspensions (4 μl each, applied as two superimposed 2-μl spots, 1 spot/cm^2) were air dried on nitrocellulose membranes. Membranes were blocked by slowly shaking in PBSTM (PBST + 5% nonfat dry milk) for 30 min. They then were incubated for 1 hr in 1 μg of AbF21-1D3G7C8 per milliliter of PBSTM. The membrane was washed three times (3 min each) in PBSTM (100 μl/cm^2 of membrane) and then incubated 1 hr in goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma) diluted 1:400 in PBSTM. Antibody incubations were carried out in heat-sealable plastic bags to minimize reagent volume. After washing in PBST three times, the membrane was incubated with enzyme substrate/dye solution of 330 μg of nitroblue tetrazolium and 167 μg 5-bromo-4-chloro-3-indolyl phosphate per milliliter of 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5. After adequate color development of positive controls (10–20 min), the reaction was stopped by washing the membrane in 20 mM Tris-HCl, 0.5 mM Na_2EDTA, pH 7.5. A negative control was included, consisting of the same procedure as above but omitting the monoclonal antibody incubation. Known biovar 3 strains were included in each experiment.

The sensitivity of the immunoblot procedure was tested with five strains of *A. tumefaciens* biovar 3 diluted in sterile distilled water to give 10^6 to 10^2 cfu per spot. The effect on sensitivity of the assay of fixing membranes for 15 min in 10% acetic acid-25% ethanol after drying also was examined. Fixed membranes were washed in distilled water after fixation and before the blocking step.

Indexing of grapevine cuttings. Dormant symptomless cuttings of five rootstocks and one cultivar were collected from a vineyard in New York and 25 samples (16 cultivars) were collected from two vineyards in Washington State. Riesling sample 1 was from a crown gall diseased vineyard. All other samples from Washington were taken from apparently healthy vines. Actively growing symptomless cuttings of three cultivars were obtained from a nursery field in western New York.

Attempts were made to isolate *A. tumefaciens* biovar 3 from New York nursery and Washington vineyard cuttings by two previously described procedures and a root wash method. The callus method involves planting cuttings in sterile perlite in individual containers in the greenhouse for 4–6 wk, triturating the callus tissue that develops at the base of the cuttings, and

plating this suspension on RS medium (6,23). The flush method involves forcing sterile distilled water through individual cuttings by reduced pressure, and plating the recovered fluid on RS medium (4,27). In the root wash procedure cuttings of rootstocks and cultivar Steuben collected in New York were first grown in a potting mixture in the greenhouse for 3 mo. About 5 g of roots per cutting was soaked 3.5 min in 10% commercial bleach and then rinsed in tap water. Roots (1 g, about 1.3 mm in diameter) were cut into small pieces (about 1 cm length) and shaken in 10 ml of sterile distilled water for 1 hr. Aliquots (10 μl) of the root wash were plated on RS medium. Colonies consistent with biovar 3 morphology after 5 days at 28 C on RS medium (opaque red center, domed, mucoid, white translucent margin) were spotted on 523 medium and then assayed by the immunoblot procedure.

RESULTS

One hybridoma line, F21-1D3G7C8, secreted IgG1 (AbF21-1D3G7C8), which reacted specifically with *A. tumefaciens* biovar 3. AbF21-1D3G7C8 reacted with all 25 strains of biovar 3 regardless of tumorigenicity but did not react with any other strains. Values of A_{405nm} for biovar 3 strains ranged from 0.572 to 1.500 in ELISA tests (1 μg AbF21-1D3G7C8 per milliliter, antigen dried and fixed), biovars 1 and 2, and other bacteria, gave readings from 0.001 to 0.070.

Large volume cultures of F21-1D3G7C8 yielded about 80 μg IgG1 per milliliter. After purification and concentration on the protein-A column, concentration of IgG1 was 295 μg/ml. Less than 1.0% of antibody activity passed through the column with the binding buffer. Ascites fluids from two mice injected with F21-1D3G7C8 yielded 1.7 mg/ml (3 ml total) and 750 μg/ml (3.5 ml total), respectively. Saturation with AbF21-1D3G7C8 was reached at about 1 μg/ml in ELISA tests with CG49 as antigen.

Drying and fixation of antigens on microtiter plates significantly improved ELISA tests. A_{405nm} readings of tests of dried, fixed plates coated with CG49 were 5–10 times higher than moist-coated plates under the same assay conditions, but readings of negative controls did not increase significantly. Also, dried plates could be stored desiccated at 4 C for at least 3 mo (the longest period tested) without detectable loss of antigenic activity.

AbF21-1D3G7C8 detected as few as 2.3×10^4 cells per well in the modified ELISA ($A_{405nm} = 0.332$ at 40 min, vs. $A_{405nm} = 0.011$ for control; antibody concentration was 1 μg/ml and conjugate dilution was 1:400, $P < 0.001$). Samples containing tenfold fewer cells gave A readings that were not significantly different from negative controls.

AbF21-1D3G7C8 was not suitable for the indirect fluorescent antibody stain. Even at high concentrations of antibody (40 μg/ml) and FITC conjugate (1:2 dilution of stock) fluorescence of stained CG49 smears was very weak. The conventional (polyclonal) rabbit serum $FC_2$14, used as a positive control, gave strong bright fluorescence, but lacked specificity for *A. tumefaciens* biovar 3 (3).

Positive responses with the immunoblot procedure were indicated by a dark purple spot on nitrocellulose membranes. Negative responses gave either no spot or a faint spot not noticeably darker than the negative control, but easily distinguished from positive controls. AbF21-1D3G7C8 had the same specificity for *A. tumefaciens* biovar 3 in the immunoblot procedure as in ELISA. Fixation of spotted nitrocellulose membranes resulted in about a tenfold increase in sensitivity of the assay. A clearly positive reaction was obtained in the sensitivity experiments for all five biovar 3 strains when 10^5 cfu/spot or greater were used.

A. tumefaciens biovar 3 strains were recovered from the three cultivars collected in the nursery and from two of six samples from the New York vineyard (Table 1). Three of the 16 cultivars from Washington State also yielded biovar 3. A relatively high number of biovar 3 cells were detected in the Riesling sample from Washington State collected from a vineyard with severe crown gall. Biovar 3 was recovered from two of 16 samples collected from a vineyard with no apparent galls. All of the isolation methods successfully recovered strains of biovar 3. As in previous studies (7,23) most of the colonies on RS medium were not biovar 3, indicating that it is not possible to identify biovar 3 accurately by colony morphology alone.

DISCUSSION

AbF21-1D3G7C8 is the first reliable serological reagent for identification of *A. tumefaciens* biovar 3 and provides a means for rapid diagnosis of strains in culture. Positive reactions of AbF21-1D3G7C8 with all 25 biovar 3 strains from North America, Europe, and Asia, and the absence of cross reactions with other biovars of *Agrobacterium*, other species of plant pathogens, and saprophytes associated with grapevines, represent a significant improvement over previous attempts to produce biovar-specific antisera for diagnosis of *Agrobacterium* (1,4,15,21). The monoclonal antibody does not differentiate between tumorigenic and nontumorigenic biovar 3, but this does not detract from its utility in indexing propagation stocks. It is important to index for the presence of all biovar 3 in cuttings, because both tumorigenic and nontumorigenic strains of biovar 3 decay grapevine roots (5).

The ELISA and immunoblot procedures described here are now routinely used for rapid identification of biovar 3 in our laboratory. They are simpler to perform and interpret and are more rapid than previously published methods for diagnosis of biovar 3 (7,21,22). These assays, together with previously published methods for isolating the bacterium from symptomless grapevine cuttings, provide a method for indexing grapevine propagation wood. The monoclonal antibody was used to identify biovar 3 from cuttings of various cultivars and rootstocks in New

TABLE 1. Identification of *Agrobacterium tumefaciens* biovar 3 from grapevine cuttings with monoclonal antibody AbF21-1D3G7C8

Site	Cultivar or rootstock	Indexing method[a]	No. biovar 3/ no. colonies assayed[b]
New York State			
Nursery	Seyval	Callus	1/5
	Catawba	Flush	2/9
	Vidal	Flush	2/4
Vineyard	44-53	Root	0/9
	5BB	Root	0/9
	101-14	Root	2/10
	18-815	Root	0/10
	3309	Root	8/9
	Steuben	Root	0/10
Washington State			
Vineyard	Riesling-1	Callus	7/20
	Riesling-2	Callus	0/28
	Cabernet Sauvignon	Callus	1/45
	Cabernet Franc	Callus	0/15
	Petite Sirah	Callus	0/8
	Meunier	Callus	0/21
	Gewurtztraminer	Callus	0/25
	Pinot Noir	Callus	0/13
	Merlot	Callus	0/3
	Grenache	Callus	0/17
	Chardonnay	Callus	0/21
	Chenin Blanc	Callus	2/37
	Limberger	Callus	0/13
	Semillon	Callus	0/14
	Sauvignon Blanc	Callus	0/11
	Muscat Canelli	Callus	0/21
	Muller Thurgau	Callus	0/10

[a]Callus = callus and/or roots were triturated in distilled water and plated on RS medium (6,23). Flush = water was flushed through cuttings by reduced pressure and the fluid recovered was plated on RS medium (4). Root = roots from cuttings were shaken in distilled water and 100-μl suspensions plated on RS as described in text. Colonies with morphology similar to biovar 3 were spotted on medium 523 and assayed with the immunoblot procedure as described in text.

[b]The number of colonies selected for immunoassay, based on colony morphology similar to biovar 3.

York and Washington states and has also been applied to identification of biovar 3 strains in Italy and South Australia (3,23).

Immunoassays for direct testing of plant material with AbF21-1D3G7C8 are under development. Preliminary results indicate that sufficient antigen is present in crown gall tissue for direct testing with AbF21-1D3G7C8 (data not shown). We do not anticipate similar success in direct ELISA testing of symptomless systemically infected grape cuttings because small numbers of biovar 3 cells (below the estimated sensitivity) are present in some cases (4,6). Weak staining in immunofluorescent assay with AbF21-1D3G7C8 does not support the application of this assay to indexing grapevine propagation wood. Weak fluorescence may result from low concentration of the epitope recognized by AbF21-1D3G7C8 on individual biovar 3 cells.

The antigen recognized by AbF21-1D3G7C8 has not been characterized. The presence of a common antigen in strains of *A. tumefaciens* biovar 3 from around the world leads us to speculate that this antigen plays a role in the ecological and physiological specialization of this biovar to grapevines (5,25). Such homogeneity might not be expected in widely distributed soil microorganisms such as *A. tumefaciens* biovars 1 or 2, but is consistent with the notion that biovar 3 is disseminated primarily through the vegetative propagation of grapevines.

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