Techniques

Specific Recognition of a *Xanthomonas campestris* Florida Citrus Nursery Strain by a Monoclonal Antibody Probe in a Microfiltration Enzyme Immunoassay

T. A. Permar and T. R. Gottwald

U.S. Department of Agriculture, Agricultural Research Service, Orlando, FL 32803.

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ABSTRACT

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A monoclonal antibody (MCA), anti-X-4600 MCA, was developed to a Florida citrus nursery strain of *Xanthomonas campestris*. Application of this MCA in a microfiltration immunosorbent assay showed that at least two serologically distinct populations of the bacteria exist in Florida. A total of 30 strains representing 13 separate disease occurrences were assayed. Of these, 14 isolates from six locations tested positive. The anti-X-4600 MCA did not react with bacteria other than Florida citrus nursery strains. Comparisons were made of purified and unpurified anti-X-4600 MCA in ELISA and microfiltration enzyme immunosorbent assays with heat-treated and Formalin-fixed bacteria. Purity of the anti-X-4600 MCA had no effect on antibody specificity, and the ELISA and the microfiltration assay were not different in sensitivity. Heat treatment of the bacteria, however, did cause a substantial reduction in response to the MCA.

Additional keywords: citrus bacterial canker disease, immunoassay.

Since 1984, more than 22 million trees have been destroyed in an effort to eradicate a bacterial disease of citrus found in 18 commercial nurseries and two immature citrus groves in Florida (11). The progress of this effort has been the subject of numerous reviews (3,12,15). The causal organism has been referred to as the Florida nursery form of citrus bacterial canker, citrus bacterial canker disease strain "E," Xanthomonas leaf spot of citrus, and citrus bacterial spot. This bacterium, along with other citrus bacterial canker disease strains, is currently classified as *Xanthomonas campestris* pv. *citri* (Hasse) Dye. Considerable differences have been found, however, between Florida nursery strains and other citrus bacterial canker disease strains. Symptoms of the Florida citrus nursery strains include flat, water-soaked lesions, whereas those of citrus bacterial canker disease strains are erumpent and corky (10). Florida citrus nursery strains are also genetically distinct from other citrus bacterial canker disease strains (9). Because of these differences, the bacteria currently infecting Florida citrus nurseries will simply be referred to in this paper as Florida citrus nursery strains. In the absence of information on etiology and epidemiology of the Florida citrus nursery strains, regulatory guidelines have been based on data from other strains and other locations. An understanding of the Florida citrus nursery strains and their ability to move and survive in the field is paramount to more effective regulation of this disease. Research necessary to obtain this type of information has been hindered by the absence of a rapid and specific assay able to handle a large number of samples. Assays based on polyclonal (2,4,5) and monoclonal (1) antibodies have been developed to most citrus bacterial canker disease groups. The objective of this study was to develop such an assay and an immunological probe specific to the Florida citrus nursery strains of *X. c. citri*.

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MATERIALS AND METHODS

Preparation of antigen. Bacterial isolates grown in nutrient broth (Difco, Detroit, MI) at 25 C were washed three times by centrifugation for 15 min at 5,000 *g*, removal of the supernatant,

and resuspension of the cells in sterile 0.1 M phosphate-buffered saline (PBS), pH 7.4. After the final wash, the resuspended cells were divided into two samples. The first was heat-treated by boiling in water for 30 min. After it was cooled, sodium azide was added to 0.02%. The other sample was fixed in 0.5% Formalin.

Production of monoclonal antibody. Balb/c mice were immunized by intraperitoneal injection with 10^8 cells of X-4600, a Florida citrus nursery strain of *X. campestris*. The bacteria were a 1:1 mixture of Formalin-fixed and heat-treated cells. Six weeks later, the mice were hyperimmunized with 10^7 cells of the same mixture by intravenous injection. Spleen cells were harvested after 3 days and fused with Sp 2/0 Ag-14 myeloma cells by the technique of Van Deusen and Whetstone (14).

Two weeks after plating, primary hybridomas were screened by microfiltration immunoassay (see below). Hybridomas were selected for positive reaction to Florida citrus nursery strain X-4600 and negative reactions to *X. c. citri*, strain MF2PA, and control wells without bacteria. Positive hybridomas were cloned three times by limiting dilution. Antibody was produced by intraperitoneal injection of pristine primed mice with approximately 10^6 cloned cells. The resulting ascites fluid was centrifuged for 10 min at 1,000 *g* with Sure-Sep II serum-plasma separators (Gen. Diagnostics, Morris Plains, NJ) and filtered. Aliquots were either frozen or purified with an rProtein A antibody purification kit (Beckman, Fullerton, CA). Isotype class and subclass were determined by indirect enzyme-linked immunosorbent assay (ELISA) with reagents supplied in an isotyping kit (Zymed Lab., San Francisco, CA).

Immunoassays. Two types of indirect enzyme immunoassay (EIA) were employed in this investigation. The first was a standard ELISA with Immulon II plates (Dynatech Lab., Alexandria, VA) as a solid support, and the second was a microfiltration EIA with millititer GV 96-well filtration plates (Millipore Corp., Bedford, MA). Optimal dilutions of the immunoreagents were determined by checkerboard titration for both assay types. In the ELISA test, each well received 50 μl of bacterial protein (5 μg/ml; determined by Coomassie blue assay) in 0.1 M PBS. After overnight incubation at 37 C, plates were washed three times with a solution of 0.1 M PBS and 0.05% Tween 20, pH 7.4. Plates were incubated with 1% bovine serum albumin (BSA) in PBS for 1 hr at 37 C and washed as above, and 50 μl of monoclonal antibody (MCA) was added per well. After 1 hr at 37 C, plates were washed as above and blocked for 1 hr with BSA at 37 C. The plates were incubated with alkaline phosphatase-labeled goat antimurine IgG or IgM (Sigma, St. Louis, MO) for 1 hr at 37 C and then washed. Substrate solution (*p*-nitrophenyl phosphate, Sigma Chemical Co.) was added for 1 hr at 37 C, after which plates were read on an EL 309 microplate autoreader (Bio-Tek, Winooski, VT).

Microfiltration EIAs were performed in the same way as ELISA with the following exceptions. Due to the nature of the assay, cells did not have to be bound to the microfiltration plates. Washing between steps consisted of two washes with PBS-Tween followed by two washes with PBS only. Plates were blotted dry between steps. After incubation with substrate, contents of the microfiltration plates were transferred to a standard ELISA plate before being read. All assays were run three times (two replications per run) for each antigen treatment and three times (two replications per run) for each antibody treatment. The same protocol was used to establish a diluent control mean by substituting 0.1 M PBS, pH 7.4, for the unlabeled antibody.

RESULTS

Antigen preparation. Figure 1 shows the effects of various treatments on reactivity of Florida citrus nursery strain X-4600 with the anti-X-4600 MCA. Levels of the Formalin-fixed antigen between 10^5 and 10^6 cells per milliliter could be measured by the immunoassay, but a 10-fold higher concentration of heat-treated cells was needed before a positive result occurred. When Formalin-fixed samples of 31 Florida citrus nursery strains were tested by immunoassay (Table 1), 14 gave a positive reaction. Heat-treated samples of the same isolates tested positive in only the two most strongly reacting strains, F1 and X-4600 (data not shown). In addition, reaction levels of these two isolates were lower in the heat-treated cells.

Antibody purity. An isotype assay was performed before purification of the anti-X-4600 MCA, and the MCA was found to be IgG3 class with a lambda light chain.

Tests were made to determine whether purification of the antibody would alter its sensitivity or specificity. As shown in Figure 2, cell levels between 10^5 and 10^6 could be detected by both the purified antibody and the ascites fluid. Although absorbance values at a given cell level were less when tested with purified antibody, nonspecific background reactions were also considerably reduced (data not shown). When the two antibody preparations were tested against a variety of bacterial cells, the specificity of purified antibody was not much different from that of ascites fluid (Table 1 and data not shown). Likewise, little difference was found in antibody specificity regardless of antigen treatment.

Immunoassays. Two types of immunoassays were tested, one using a solid-phase plastic (ELISA) to bind the bacteria and the other a filter bottom plate (microfiltration EIA) to trap the cells. Both assays were able to detect bacterial concentrations between 10^5 and 10^6 cells per milliliter, i.e., 10^4–10^5 cells per well (Fig. 3). Figure 3 also shows that above 10^7 cells per milliliter, the binding capacity of the ELISA plate is saturated. In the ELISA test, sample size is determined by the size of the plate well. The microfiltration plate has the capability of filtering several samples through the same well, thereby effectively increasing the sensitivity of the assay.

MCA specificity. Data presented in Table 1 show that the anti-X-4600 MCA did not react with strain groups of *X. c. citri*, other pathovars of *X. campestris*, or the unrelated bacterial strains tested. Within the Florida citrus nursery strains, the anti-X-4600 MCA reacted with samples taken from locations A, B, D, E, L, and O. All samples within these locations were positive with one exception: two location A samples (F1 and F3) reacted positively, and one sample (F14) did not.

DISCUSSION

This investigation has shown that MCAs can serologically differentiate Florida citrus nursery strains of *X. campestris*. Although the usefulness of a single MCA for categorizing Florida citrus nursery strains is limited, two points can be made. The first is that some Florida citrus nursery strains contain an epitope recognizable by the anti-X-4600 MCA that is not present in any of the group strains of *X. c. citri*, and therefore Florida citrus

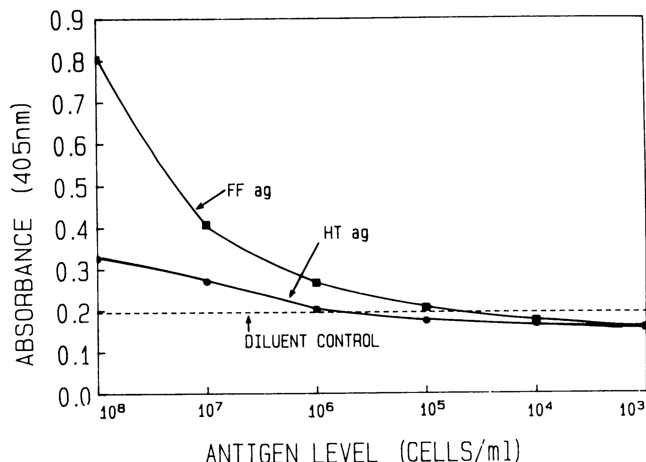

Fig. 1. Effect of treatment of Florida citrus nursery strain X-4600 on sensitivity of microfiltration enzyme immunoassay using purified antigen (ag) IgG3 from anti-X4600 monoclonal antibody. HT = heat treated, FF = Formalin fixed.

TABLE 1. Immunological reactivity of purified anti-X- 4600 monoclonal antibody to Formalin-fixed *Xanthomonas campestris* and nonrelated bacteria

Genus/species or pathovar[a]	Strain	Source of strain[b]	Geographical source	A_{405}
X. c. pv. *citri*				
Group A	XC62	5	Japan	0.05 ± 0.05
	XC63	5	Japan	0.02 ± 0.01
	XC118	5	New Zealand	0.02 ± 0.00
	MF2PA	1	Florida	0.03 ± 0.05
Group B	XC64	5	Argentina	0.02 ± 0.01
	XC69	5	Argentina	0.03 ± 0.01
	XC94	5	Argentina	0.02 ± 0.01
	PLG1	1	Argentina	0.02 ± 0.01
	PLG2	1	Argentina	0.02 ± 0.01
Group C	XC70	5	Brazil	0.01 ± 0.04
Group D	XC90	5	Mexico	0.02 ± 0.01
	T-20	5	Mexico	0.01 ± 0.01
	T-22	5	Mexico	0.01 ± 0.01
	T-23	5	Mexico	0.01 ± 0.01
	T-24	5	Mexico	0.02 ± 0.01
Florida citrus nursery strains	F1	5	Florida A[c]	0.53 ± 0.08
	F3	5	A	0.52 ± 0.11
	F14	5	A	0.02 ± 0.04
	F6	5	B	0.61 ± 0.07
	F29	5	B	0.46 ± 0.15
	F30	5	B	0.60 ± 0.12
	F49	5	B	0.46 ± 0.12
	F228	5	B	0.15 ± 0.08
	X-4600	2	B	0.88 ± 0.11
	F5	5	C	0.03 ± 0.05
	F7	5	D	0.46 ± 0.15
	F10	5	E	0.25 ± 0.08
	F18	5	F	0.03 ± 0.05
	F20	5	G	0.08 ± 0.04
	F33	5	H	0.03 ± 0.05
	F56	5	I	0.05 ± 0.05
	F59	5	I	0.05 ± 0.05
	F79	5	J	0.05 ± 0.05
	F83	5	J	0.06 ± 0.05
	F86	5	K	0.03 ± 0.05
	F88	5	K	0.03 ± 0.05
	F90	5	L	0.17 ± 0.12
	F92	5	L	0.28 ± 0.09
	F95	5	M	0.03 ± 0.05
	F97	5	M	0.05 ± 0.08
	F128	5	M	0.02 ± 0.04
	F130	5	M	0.03 ± 0.05
	F100	5	N	0.03 ± 0.05
	F101	5	N	0.02 ± 0.04
	F163	5	O	0.45 ± 0.16
	F167	5	O	0.21 ± 0.09
pv. *campestris*	X6	5	Oregon	0.03 ± 0.05
	···	3	Florida	0.00 ± 0.00
pv. *vesicatoria*	···	4	Florida	0.03 ± 0.05
pv. *manihotis*	XM6	5	Nigeria	0.03 ± 0.05
Pseudomonas sp.	PS1	1	Florida	0.02 ± 0.04
	PS2	1	Florida	0.00 ± 0.00
	PS3	1	Florida	0.02 ± 0.04
Uncharacterized yellow colony forming non-*Xanthomonads*	HP8-58	1	Florida	0.00 ± 0.00
	HP6-91	1	Florida	0.00 ± 0.00
	HPe3-30	1	Florida	0.00 ± 0.00

[a]Groups A–D have been previously described by Stall and Seymour (13). Florida citrus nursery strains provided by E. L. Civerolo, Beltsville, MD, were originally isolated and confirmed to be pathogenic by the Division of Plant Industry, Gainesville, FL.

[b]Source of strain: 1, this study; 2, FL Dept. Agric. Cons. Serv./DPI; 3, J. H. Graham, FL; 4, R. E. Stall, FL; E. L. Civerolo, Beltsville, MD.

[c]Florida sources identified with the same letter were taken from a single citrus nursery.

nursery strains are serologically distinct from group strains of *X. c. citri*. The second point is that the anti-X-4600 MCA did not react with all strains from the Florida citrus nursery group, which demonstrates a serological variability among strains within this group. Analytic evidence of the dissimilarity between Florida citrus nursery strains and other groups of *X. c. citri* and evidence of variation between strains within the Florida citrus nursery strain group have already been reported by Hartung and Civerolo (9), using genomic DNA fingerprints and by Gabriel et al (6), using restriction fragment length polymorphism analysis. Data presented here lend support to the argument that the recent outbreaks of the disease caused by Florida citrus nursery strains of *X. campestris* did not arise from a single event.

Because the primary objective of this study was to develop a rapid as well as specific assay for Florida citrus nursery strains, two types of EIA were examined. There were no differences in sensitivity between the microfiltration EIA and ELISA tests for the Florida citrus nursery strains. The anti-X-4600 MCA did not react with other pathovars of *X. campestris*, *Xanthomonas* spp., or unrelated bacteria in either test. The microfiltration EIA has some advantage, however, because multiple samples can be filtered through a single well, thereby effectively increasing the sensitivity of the test. Assays comparing anti-X-4600 ascites and the purified IgG3 fraction alone also showed no differences in reactivity with the Florida citrus nursery strains. But heat treatment of the bacteria caused a substantial reduction in response from the anti-X-4600 MCA relative to that from Formalin-fixed cells. This

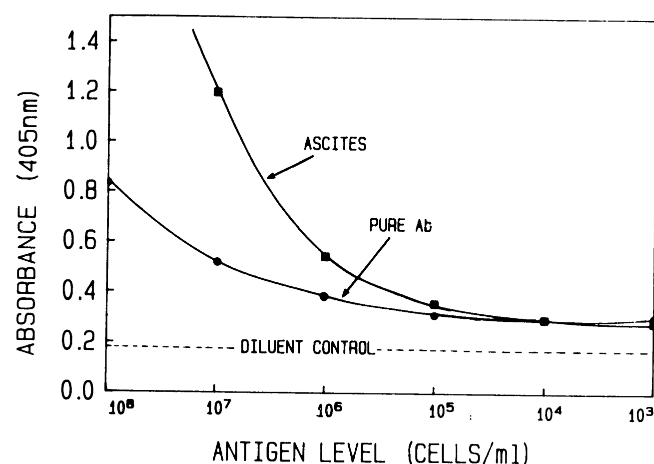

Fig. 2. Reaction of purified IgG3 versus ascites fluid from anti-X-4600 monoclonal antibody (ab) with Formalin-fixed Florida nursery strain X-4600 in a microfiltration enzyme immunoassay.

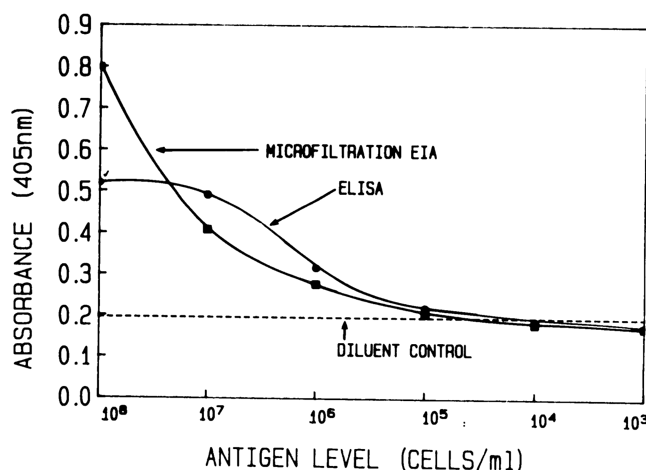

Fig. 3. Reaction of Florida citrus nursery strain X-4600 and purified anti-X-4600 monoclonal antibody IgG3 in microfiltration enzyme immunoassay (EIA) and ELISA tests.

would indicate that the epitope to which the anti-X-4600 MCA reacts is a proteinaceous flagellar "H" antigen (8). Ongoing research at this facility is aimed at developing additional MCAs to unique epitopes of the *X. campestris* Florida nursery strains in order to facilitate further serological characterization of these strains. During the time this paper was in press, a study by Gabriel et al (7) was published that proposed a reclassification of all strains of *X. c. citri*. Since it was not known whether this reclassification would be generally accepted, no changes were made in the present paper.

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