Monoclonal Antibodies Against *Erwinia amylovora*: Characterization and Evaluation of a Mixture for Detection by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

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Eight monoclonal antibodies that specifically react against antigens of *Erwinia amylovora* were evaluated. These antibodies were characterized to determine the type of antigens that they recognize and whether they are directed against different epitopes. Six reacted with protein antigens, as determined by loss of reactivity in indirect enzyme-linked immunosorbent assay (ELISA) after treatment of sonicated cells with proteinase K. Two of the antibodies (MA-8 and MA-23) reacted with purified lipopolysaccharide from *E. amylovora*; reactivity of these antibodies with sonicated antigen was not lost after proteinase K treatment. Four antibodies belonged to immunoglobulin class IgG1, three to subclass IgG2b, and one to subclass IgG2a. In an ELISA designed to determine epitope specificity, antibodies MA-12, MA-21, and MA-37 clearly bound to different epitopes. Antibodies MA-27 and MA-33 were complemented by MA-8, indicating similarity of binding sites, and two (MA-23 and MA-30) were too low in reactivity to determine epitope specificity. Antibodies MA-8, MA-12, and MA-21 were chosen, based on epitope binding specificity and reactivity in ELISA, to determine whether a mixture of antibodies would improve detection of *E. amylovora* by ELISA. Increased sensitivity of detection was observed with the mixture at the detection limits of ELISA (4.0 log cfu) and at cell concentrations up to 5.5 log cfu.

Additional keywords: fire blight, serology.

Fire blight, caused by *Erwinia amylovora* (Burrill) Winslow et al, is a disease that affects members of the family Rosaceae and causes considerable economic loss in pear- and apple-producing areas where it is endemic: North America, Western Europe, and more recently, the Middle East (3,21). Serological tests with polyclonal antisera have demonstrated a great degree of uniformity among strains of *E. amylovora* (21). Serology is thus regarded as a practical method for facilitating rapid and specific detection of the bacterium. Polyclonal antisera have been used in immunofluorescence staining (4,5,9,16,18) and enzyme-linked immunosorbent assay (ELISA) (11) but have shown inadequate specificity, particularly for differentiation of the fire blight bacterium from epiphytes on or in susceptible plant tissue (4,5,9, 11,16,18).

In recent years, monoclonal antibodies have become widely used because they are more specific than conventional polyclonal antisera and are useful for the detection and identification of various plant pathogenic prokaryotes (1,6,7,12). Lin et al (12) described 10 monoclonal antibodies that react specifically against antigens of *E. amylovora*. These antibodies are useful for immunofluorescent detection of *E. amylovora* in vitro and in situ as they do not react with other plant pathogenic bacterial species or with epiphytic bacteria isolated from pome fruit trees (12).

In preliminary studies we have observed improved detection of *E. amylovora* in immunofluorescence and ELISA assays with a mixture of these monoclonal antibodies. However, since these antibodies are not well characterized, we did not know the specific components in the mixture that significantly contribute to improved detection. In this report, this panel of antibodies was evaluated in terms of relative titer, isotype, and epitope specificity to determine the characteristics of each monoclonal antibody. These data were used to formulate a cocktail of monoclonal antibodies to assess improvement of detection by ELISA. Brief accounts of this research have been published (13,15).

MATERIALS AND METHODS

Bacterial strains. Two virulent strains of *E. amylovora* were generously provided by Tom van der Zwet (USDA Appalachian Fruit Research Station, Kearneysville, WV). Strain 477, a nonmucoidal isolate from Wenatchee, WA, was used in antigen preparations in all tests. Strain 542, a mucoidal isolate from Kearneysville, WV, and strain 477 were used in the ELISA with monoclonal antibody mixtures.

Antigen preparation. Bacterial cells were harvested from streak plate cultures grown on nutrient-yeast extract-dextrose agar (0.8% nutrient broth, 0.5% yeast extract, 0.5% dextrose, and 1.5% agar) at 27 C for 24 hr. Bacteria from each plate were suspended in 10 ml of phosphate-buffered saline (PBS, 0.02 M phosphate, 0.14 M NaCl, 0.2 M KCl, pH 7.4), adjusted to 10^9 cfu/ml ($A_{600nm} = 0.45$), pelleted by centrifugation at 13,000 *g* for 5 min, washed twice in PBS, and then resuspended in the same buffer. Cell suspensions were then sonicated for 2 min in an ice bath as previously described (12).

Monoclonal antibodies. Eight monoclonal antibodies, selected for their specificity against *E. amylovora* by Lin et al (12), were used in the ELISA assays. The antibodies were typed by indirect ELISA to determine immunoglobulin class and subclass. Goat anti-mouse antibodies of different class and subclass-specificities (Sigma Chemical Co., St. Louis, MO) were suspended in 50 mM of sodium carbonate buffer, pH 9.6, and added to microtiter plates (Nunc, Denmark) for a final concentration of 5 μg/well. Plates were incubated for 4 hr at 37 C. Blocking of wells was done with crystalline grade bovine serum albumin (1%, w/v in coating buffer) for 1 hr at 37 C. Hybridoma supernatants were then added at a dilution of 1:10 in PBS and incubated for 1 hr at 37 C. Goat anti-mouse immunoglobulin, conjugated with horseradish peroxidase (0.5 μg/ml, Kirkegaard and Perry Laboratories, Gaithersburg, MD), was added as a final step. The enzyme

substrate was *o*-phenylenediamine (Sigma Chemical Co.). Incubation, washing, and reaction conditions were as described for ELISA methods.

Protein concentrations of the monoclonal antibodies were standardized for the epitope specificity assay, after purification by column chromatography on Protein-A Sepharose CL-4B (Pharmacia, Inc., Piscataway, NJ). Chromatography was done according to manufacturer instructions.

ELISA methods. Antigen was prepared by diluting the sonicated bacterial suspension to 4.0 μg of protein per milliliter in carbonate buffer, and 50 μl was added to each microtiter plate well. Plates were incubated for 4 hr at 37 C and washed three times with PBS-Tween (PBS plus 0.05% Tween 20). Blocking of wells was done as mentioned previously. Wells were washed six times, and 50 μl of hybridoma supernatant (diluted 1:10 in PBS) was added per well. After 1 hr of incubation at 37 C, plate wells were washed six times and 50 μl of biotinylated anti-mouse immunoglobulin (0.5 μ/ml in PBS, Kirkegaard and Perry Laboratories) was added per well. Plate wells were washed 12 times after 1 hr of incubation and streptavidin-peroxidase conjugate (0.5 μg/ml in PBS) was added for an additional 45 min of incubation. Wells were washed 15 times, and *o*-phenylenediamine (4 mg/ml in 50 mM citrate buffer, pH 4.5, plus 0.015% H_2O_2) was used as an enzyme substrate. After 5 min of incubation at room temperature, the reaction was terminated by addition of 50 μl of 3 M H_2SO_4 to each well. Absorbance was read at 492 nm on a Titertek Multiskan microtiter plate reader (Flow Laboratories, Finland).

In the ELISA assay for determining epitope specificity of the monoclonal antibodies, each microtiter plate well was coated with 20 ng of antigen (total protein). The methods were essentially as described for additivity ELISA (8). After antigen coating, monoclonal antibodies were added to each well (0.25 μg of protein per well) individually or to the same well in pairs. Antibodies were previously purified by column chromatography on Protein A-Sepharose CL-4B as described above. All other ELISA steps were as described above.

In the ELISA assay with the mixture of monoclonal antibodies, cell sonicates were treated at 100 C before coating for 10 min, followed by an additional 1 min of sonication at 0 C. Microtiter plate wells were then coated with dilute antigen preparations of 4.5–7.5 log cfu/ml. Hybridoma supernatants (diluted 1:10 in PBS) were added after blocking of plate wells. All other ELISA steps were as described above.

Antigen characterization. Sonicated antigen (40 μg/ml) from strain 477 of *E. amylovora* and partially purified lipopolysaccharide (LPS, 100 μg/ml) were treated with proteinase K (100 μg/ml in 200 mM Tris HCl, 5 mM $CaCl_2$, pH 7.8) for 4 hr at 37 C. Subsequently, proteinase K was denatured by treatment at 100 C for 15 min. ELISA titer was then determined for diluted proteinase- and non-proteinase-treated solutions that were incubated at 100 C for 15 min. ELISA titer of each monoclonal antibody against purified lipopolysaccharide (LPS) from strain 477 of *E. amylovora* was determined also. Isolation and purification of LPS was done according to Westphal and Jann (22). After two cycles of ultracentrifugation at 100,000 *g* for 2.5 hr, there was 140 μg of protein per milligram of LPS. For ELISA with the LPS, each well was coated for 4 hr at 37 C with 50 μl of a 2 μg/ml suspension in coating buffer.

TABLE 1. Isotypes and enzyme-linked immunosorbent assay (ELISA) reactions of hybridoma supernatants against a sonicated cell suspension of strain 477 of *Erwinia amylovora*[a]

Hybridoma cell line	Monoclonal antibody[b]	Antibody isotype	Reactivity of supernatant[c]
8D10	MA-12	IgG1	0.92
37G11	MA-23	IgG1	0.19
33D4	MA-21	IgG1	0.22
37H5	MA-30	IgG1	0.13
42B6	MA-37	IgG2b	0.16
38H3	MA-27	IgG2b	0.31
37E4	MA-33	IgG2b	0.50
37A12	MA-8	IgG2a	1.57

[a]Protein concentration of the antigen was 0.4 μg/well.
[b]Designation of Lin et al (12).
[c]Absorbance at 492 nm, average of three replicates. Hybridoma supernatants were diluted 1:10 in phosphate-buffered saline. A_{492nm} values in control wells minus antigen or antibody were less than 0.025. Standard errors of the mean ranged from ±0.01 to ±0.03.

RESULTS

Isotyping. The eight monoclonal antibodies isolated by Lin et al (13) belong to one of three immunoglobulin isotypes (Table 1). Four belonged to class IgG1, three to subclass IgG2b, and one to subclass IgG2a. Reactivity of monoclonal antibodies MA-12, MA-8, and MA-33 was strong ($A_{492nm} > 0.500$). The remaining antibodies showed weak to moderate reactivity ($A_{492nm} < 0.40$).

Antigen characterization. Treatment of cell sonicates of *E. amylovora* with proteinase K caused loss of ELISA reactivity ($A_{492nm} < 0.030$) of antigen with all monoclonal antibodies except MA-23 and MA-8. Weak reaction ($A_{492nm} = 0.180$) of MA-23 was evident for proteinase-treated antigen and in wells coated with LPS. Strong reactivity ($A_{492nm} = 1.6$) of MA-8 was apparent with proteinase-treated antigen and LPS.

Epitope specificity of monoclonal antibodies. In this assay, paired monoclonal antibodies showed enhanced ELISA reactivity over that of single antibodies if they were directed against different epitopes. Several of the monoclonal antibodies (MA-12, MA-21, and MA-37) clearly reacted against unique epitopes (Table 2). Complementation of MA-8 by antibodies MA-27 and MA-33 (A_{492nm} = 0.594 and 0.448, respectively) indicate that either a similar epitope may be recognized or the binding of MA-8 to its epitope on LPS effects occlusion of the targeted epitopes. Antibodies MA-23 and MA-30 reacted weakly or not at all and could not be evaluated in this assay, presumably because of the

TABLE 2. Epitope specificity of the monoclonal antibodies as determined by the effect of mixtures on enzyme-linked immunosorbent assay (ELISA) reactivity[a]

Monoclonal antibody	Base reactivity[c]	Relative increase in ELISA reaction after addition of second monoclonal antibody[b]							
		MA-27	MA-23	MA-12	MA-33	MA-21	MA-37	MA-30	MA-8
MA-27	0.357	−	−	++++	+	++	+	−	++
MA-23	0.000	++++	−	++++	++	+++	++	−	+++
MA-12	0.573	+++	−	−	+	++	+	−	+++
MA-33	0.140	+++	−	++++	−	++	+	−	++
MA-21	0.199	+++	+	++++	++	−	++	+	++++
MA-37	0.116	+++	−	++++	+	++	−	+	++++
MA-30	0.044	++	−	++++	+	++	+	−	++++
MA-8	0.442	+	−	++++	−	++	+	+	−

[a]Strain 477 of *Erwinia amylovora* used as antigen. Protein concentrations of the antigen and antibody were 0.02 and 0.25 μg/well, respectively.
[b]Relative increase in reactivity (A_{492nm}): (−) = 0.050 or less, (+) = 0.051 to 0.150, (++) = 0.151 to 0.250, (+++) = 0.251 to 0.350, and (++++) = 0.351 to 0.451. Average of two replicates.
[c]Base reactivity = A_{492nm} of single monoclonal antibodies in the left-hand column.

TABLE 3. Enzyme-linked immunosorbent assay (ELISA) reactions of three monoclonal antibodies and a mixture against dilution series of cell sonicates from two strains of *Erwinia amylovora*

Bacterial strain[b]	Monoclonal antibody[c]	ELISA value at cell dilutions (log cells/ml)[a]						
		4.5	5.0	5.5	6.0	6.5	7.0	7.5
477	MA-12	0.000	0.004	0.006	0.123	0.452	1.607	>2.000
477	MA-21	0.007	0.000	0.000	0.046	0.230	1.306	>2.000
477	MA-8	0.004	0.014	0.065	0.287	0.820	>2.000	>2.000
477	Cocktail	0.004	0.027	0.100	0.497	1.362	>2.000	>2.000
542	MA-12	0.000	0.012	0.042	0.305	0.601	1.934	>2.000
542	MA-21	0.000	0.009	0.000	0.051	0.106	0.954	1.579
542	MA-8	0.000	0.013	0.052	0.602	1.224	>2.000	>2.000
542	Cocktail	0.006	0.038	0.161	1.029	1.754	>2.000	>2.000

[a] A_{492nm} after 10 min. Each value is the average of three replicates. Average values less than 0.000 are listed as 0.000.
[b] 50 μl of cell sonicate was added per well.
[c] Monoclonal antibody supernatant was diluted 1:10 in phosphate-buffered saline and 50 μl was added to each well. Each monoclonal antibody was diluted to a final concentration of 1:10 in the mixture.

dilute nature of the coating antigen that was used to facilitate comparison of antibodies having stronger reactivities.

Monoclonal antibody mixture for improved detection of *E. amylovora* by ELISA. The isotyping, enzyme digestion, and ELISA additivity assays demonstrate that several unique monoclonal antibodies are present in this panel, indicating that a mixture of antibodies, having unique epitope binding specificity, could be used to improve detection of *E. amylovora* by ELISA. The reactivity of several of the antibodies is low, however. Those monoclonal antibodies with the highest reactivities at low cell dilutions were MA-12, MA-21, and MA-8. The additivity ELISA also demonstrated that each of these were directed against different epitopes. Therefore, a cocktail of these antibodies was evaluated.

The strength of reaction of the cocktail and individual monoclonal antibodies was assessed against a dilution series of cell sonicates, from 4.5 to 7.5 log cfu/ml, of strains 477 and 542 of *E. amylovora* (Table 3). In this dilution series, MA-8 had the strongest reactivity at lower cell concentrations. Detection limits, arbitrarily defined as $A_{492nm} > 0.100$, with antibodies MA-12 and MA-21 were 6.0 and 6.5 log cfu/ml, respectively. The detection limit for the mixture was 5.5 log cfu/ml with both strains. Strong enhancement of the ELISA reaction was observed with the monoclonal antibody mixture against cell dilutions up to 7.0 log cfu/ml. Reaction of MA-12 and MA-8 was strongest with strain 542 at most cell dilutions.

DISCUSSION

The results of the characterization assays clearly show that several different monoclonal antibodies were present in this collection. Six of the antibodies were directed against protein antigens as indicated by loss of reactivity after proteinase K treatment. These protein antigens are thermostable. Monoclonal antibodies MA-8 and MA-23 apparently react with antigenic determinants on the LPS of *E. amylovora*.

The assay for epitope specificity verified that MA-12, MA-21, and MA-37 each bound to unique epitopes. This assay also shows that antibodies MA-12 and MA-27 appear to be directed against different epitopes; however, recent evidence indicates that these two antibodies are directed against a single antigen (14). This antigen is expressed from a 5.0-kb DNA region cloned in *Escherichia coli*. Monoclonal antibody MA-8 showed partial identity with MA-27 and MA-33. In certain cases, interpretation of epitope specificity with this assay is complex as it is reliable only if the affinities of both antibodies are similar or above the threshold where effects of washing are negligible (8). In addition, it was not possible to determine the epitope binding specificity of MA-23 and MA-30 with this assay. Weak reactivity of these antibodies at the cell dilution of 20 ng/well indicates that there may be relatively few antigenic sites present in cells of *E. amylovora*. The stronger ELISA reactivity of the remaining antibodies required a reduced antigen concentration to facilitate comparison of the epitope binding specificity.

Data from the epitope specificity and the isotyping assays were useful criteria for selecting monoclonal antibodies for improving detection of *E. amylovora* by ELISA. Each of the monoclonal antibodies in this mixture had unique epitope specificity and demonstrated strong reactivity in ELISA at low antigen concentrations. Detection of *E. amylovora* was enhanced by use of the mixture, especially at cell dilutions where detection was otherwise unapparent or ambiguous with single antibodies. The mixture could be simplified by elimination of MA-21, without sacrifice of sensitivity, since it appears that its contribution to increased titer is relatively low as compared to the other two antibodies in the mixture. Detection limits of 5.5 and 6.5 log cfu/ml are approximately equivalent to 4.0 and 5.0 log cfu, since 50 μl was added to each well of the microtiter plate. Further improvement of detection sensitivity, to 5.0 log cfu/ml, should be easily achieved with a slightly longer substrate incubation time. In addition, improvement of detection by an order of magnitude or more may be possible if other signal amplification methods are used (10).

An ELISA assay with this monoclonal antibody mixture allows enhanced specificity of detection as compared to polyclonal antisera. This method may be quite useful for epidemiological studies and quarantine needs, especially in situations where several samples need to be processed in a timely manner. A minimal detection level of 4 log cfu should be adequate for detecting epiphytic populations of *E. amylovora* from plant cankers and blossoms. Preinfection epiphytic populations of the bacterium of 4 to 5 log cfu per blossom have been commonly observed by using semiselective media (2,17,19,20). We have also found that a monoclonal antibody mixture, consisting of all of the antibodies, can enhance detection in immunofluorescence assays (data not shown). Immunofluorescent detection methods have been reported to have greater sensitivity, to 5×10^3 cells (12), but the processing of individual samples is time-consuming and can limit the scope of the study. This method is ideal in situations where only a few samples need processing. In addition, immunofluorescent detection of *E. amylovora* with the monoclonal antibody mixture needs further investigation, as there appear to be only a few components of that mixture that result in strong fluorescence.

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