Identification and Detection of *Erwinia amylovora* with Monoclonal Antibodies

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ABSTRACT

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Forty-eight hybridoma clones secreting monoclonal antibodies (MAs) specific to *Erwinia amylovora* were produced to identify and detect epiphytic and endophytic *E. amylovora* in pome fruit tissues by indirect enzyme-linked immunosorbent assay (ELISA) and immunofluorescent staining. These MAs reacted positively in indirect ELISA against pure cultures of 75 *E. amylovora* isolates from different countries. Serological specificity of 37 of the MAs was further tested against 24 strains of bacteria in six genera and against 56 unidentified epiphytes from pome fruit trees collected in different states. Ten clones of MAs reacted specifically with *E. amylovora* and did not cross-react with any epiphytes or known bacteria. Nine of the 10 MAs produced strong fluorescent reactions in vitro to *E. amylovora* in indirect immunofluorescent staining and were further used for in situ detection in diseased apple tissues with an epifluorescent microscope. The MAs were also used to detect *E. amylovora* in vitro by a modified immunofluorescent direct-counting technique. Bacterial suspensions collected from infected pome fruit blossoms and apple fruits were concentrated in a microcentrifuge tube during staining, then collected on polycarbonate membranes by filtration. This technique conveniently detected 5×10^3 cells per membrane and was useful in detecting and monitoring infection by *E. amylovora* in pome fruit trees and fruits.

Additional key words: fire blight, hybridoma technique, immunodiagnostics.

Fire blight, caused by *Erwinia amylovora* (Burrill) Winslow et al, is a devastating bacterial disease of considerable economic significance, affecting many members of the family Rosaceae (30). The disease is established in North America and Western Europe, and is the object of strict quarantine activity in central and southern Europe as well as in areas of the southern hemisphere and the Far East, where it has not yet extended. Virulent cultures of *E. amylovora* (endophytic) have been isolated from symptomless pear and apple shoots (14) as well as in the epiphytic form from fruit collected in a blighted orchard (32). Symptoms of fire blight have recently been discovered after budding of commercial rootstock with symptomless scionwood (29). Successful quarantine programs are dependent on effective methods for detecting the bacterium on or in plant tissues. The current methods of confirming the identity of *E. amylovora* are by pathogenicity tests and time-consuming biochemical and physiological tests. In pathogenicity tests, days may elapse for symptoms to appear in inoculated fruit or seedling tissues.

Serological tests have been of general value in phytobacteriology for taxonomy and detection (25). Among these tests, immunofluorescent staining (3,4,11,22,24) and enzyme-linked immunosorbent assay (ELISA) (16) are the most rapid and accurate serological tests for identifying bacteria, if suitable antibodies are available. Because various bacteria share common epitopes, conventional (heterogeneous) antibodies can produce nonspecific cross-reactions and that hamper the use of serology as a diagnostic tool (3,4,22,24).

Hybridoma techniques, introduced by Kohler and Milstein (15), have made possible the production of homogeneous antibodies specific to distinct epitopes. Antibodies specific to bacterial species, races, or strains can now be obtained (9,28).

In recent years, MAs have been developed for various plant pathogens including phytopathogenic prokaryotes. Monoclonal antibodies against *Corynebacterium sepedonicum* were superior in immunofluorescence tests to conventional polyclonal antisera for detecting symptomless infections (8). Alvarez et al (1) developed specific MAs against *Xanthomonas campestris* pv. *campestris* and used them for subgrouping of strains. Monoclonal antibodies have also been produced against fastidious prokaryotes including spiroplasmas (17,18), mycoplasmalike organisms (19,20), and xylem-limited fastidious bacteria (J. M. Wells, *unpublished*) and used in disease detection.

In this paper, we describe the production of monoclonal antibodies for *E. amylovora*, their use in ELISA and with immunofluorescence staining for the in vitro and in situ identification and detection of the bacterium, and the enhancement of test sensitivity by use of a modified immunofluorescent direct-count method. Preliminary results of part of this study have been published (21).

MATERIALS AND METHODS

Bacterial strains. *E. amylovora* strain WC542, isolated from apple in West Virginia, was used as antigen for producing and screening MAs. An additional 74 strains (Table 1) from various rosaceous host plants were collected from six states and seven foreign countries. These proved to be authentic and quite homogeneous in fatty acid profile tests (31). In specificity studies, 24 bacterial strains belonging to six genera were collected by USDA, ARS, Appalachian Fruit Research Station, WV, and USDA, ARS, Rutgers University, New Brunswick, NJ (Table 2): *E. ananas* X9, T1, and X6 (Eaa X9, T1, and X6); *E. herbicola* 43.27 and 43.28 (Eh 43.27 and 43.28); *E. carotovora* pv. *carotovora* SR319 (Ecc SR319); *E. carotovora* pv. *atroseptica* SR8(Eca SR8); *E. chrysanthemi* 120A (E.chr 120A); *E. mallotivora* (Em; ATCC 29573); *Pseudomonas syringae* pvs. *syringae* 18 (Pss 18), *tomato* (Pst), *glycinea* (Psg; NCPPB 2070), and *lachrymans* 43.28 (Ps 143.28); *P. viridiflava* (Psv); *P. fluorescens* bioII (Pf bioII); *Xanthomonas campestris* pvs. *campestris* 42 (Xcca 42), *cucurbitae* (Xccu), *vesicatoria* (Xcv), and *phaseoli* (Xcp); *X. maltophilia* 43.61 (Xm 43.61); xylem-limited fastidious bacteria (PCE-R); *Escherichia coli* C600 (Eco C600); and *Spiroplasma citri* Maroc (SC Maroc). Fifty-six unidentified epiphytes isolated during

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spring monitoring from pome fruit trees (32) were also used to test antibody specificity (Table 3). Except for SC Maroc and PCE-R, which were cultured in special media (17,27), bacteria were grown and maintained in nutrient yeast-dextrose broth (0.8% [w/v] nutrient broth powder, 0.5% [w/v] yeast extract, and 0.5% [w/v] dextrose).

Antigen preparation. Log-phase cultures of bacteria were harvested by centrifugation at 13,000 *g* for 5 min (30 min for SC Maroc). Pellets were washed twice, 0.01 M phosphate-buffered saline (PBS, pH 7.4) at 40 C, then resuspended in the same buffer for further preparation as required. Bacterial suspensions were sonicated with a Fisher Sonic Dismembrator, Model 300 (Fisher Scientific, Springfield, NJ) for 2 min before use as immunogens or as coating antigens in ELISA screening.

Monoclonal antibody production. Six-week-old female BALB/c mice were used for immunization. The injection scheme was similar to that described previously (17). Intraperitoneal injections were given on days 1, 28, and 35, and an intravenous booster on day 38, with 30 μg of protein per injection (Bio-Rad protein assay; Bio-Rad Lab., Richmond, CA). Mice were killed on day 41, and splenic cells collected for each hybridization by repeatedly injecting the spleen with serum-free RPMI-1640 medium (Gibco Lab., Grand Island, NY).

Murine myeloma cell line P3-NSI/1-Ag-4 (NS-1) was used in hybridoma production. Fusions were based on procedures previously described (17). In this study, 45% polyethylene glycol (PEG) 3600 (Sigma) was used as a fusion agent. RPMI-1640 medium supplemented with 15% fetal bovine serum (complete medium) (Gibco Lab., Grant Island, NY) was used for routine cultivation of myeloma and hybridoma cells; and HAT selective medium (complete medium supplemented with 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine) was used after fusions to allow for growth of only spleen-myeloma hybrids. Incubation was in a humid atmosphere of 7% CO_2 at 37 C. Hybridoma clones producing antibody were subcultured and cloned to single cells for further screening and for preservation in liquid nitrogen.

Screening by ELISA. Indirect ELISA with biotinylated antimouse immunoglobulin (Vector Lab., Burlingame, CA) was used for screening antibody-producing hybridomas specific for *E.*

TABLE 1. Strains of *Erwinia amylovora* tested for reactivity with monoclonal antibodies

Geographic origin[a]	Strains tested (no.)	Strain designation[b]
North America		
Illinois	5	126 (110), 127 (NM22), 128 (MR22), 129 (BB1), 130 (BB2)
Missouri	8	203 (E-8), 208 (E-9), 209 (E101b), 216 (MO23), 218 (MO69), 219 (MO15), 220 (MO47), 221 (MO95)
New York	4	225 (103), 256 (251), 258 (257), 260 (273)
Utah	4	426 (111), 427 (112), 428 (113), 429 (114)
Washington	6	477 (212), 478 (214), 479 (216), 480 (218), 481 (220), 482 (228)
West Virginia	6	526 (WV12), 527 (WV17), 528 (WV26), 541 (WV54), 542 (WV55), 548 (WV65)
Canada	5	1101 (E2017P), 1103 (E4003P), 1105 (E7002M), 1108 (E7004M), 1109 (E81004A), 1228 (CNEP20001)
Europe and Oceania		
Belgium	6	1126 (CNEP1376), 1232 (NCPPB2291), 1233 (NCPPB2293), 1238 (VT5), 1265 (VT147)
England	9	1427 (595), 1428 (686), 1429 (770), 1431 (781), 1432 (1657), 1434 (1661), 1439 (1667), 1442 (2329), 1444 (P)
France	6	1451 (1365), 1452 (1368), 1456 (1431), 1463 (1992), 1464 (1993), 1470 (1999)
Netherlands	4	1601 (108), 1602 (117), 1604 (210), 1607 (455)
New Zealand	6	1626 (550), 1627 (1440), 1629 (1494), 1630 (1498), 1631 (1500), 1632 (1504)
West Germany	6	1827 (Ea3/74), 1829 (Ea6/74), 1832 (Ea5/75), 1833 (Ea9/75), 1834 (Ea1/79), 1837 (Ea7/79)

[a] Host origins recorded at USDA, Appalachian Fruit Research Station, West Virginia.
[b] Culture number at AFRS *Erwinia amylovora* collection and original isolate number (in parentheses).

TABLE 2. ELISA reactions (at 490 nm) of monoclonal antibodies and polyclonal antibodies with representative strains of various bacteria

Bacterial strain[a]	Monoclonal antibodies[b]																																			Polyclonal antibodies[c]
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	
Ea wc268[d]	6[e]	6	5	8	8	7	6	8	9	6	7	6	5	6	9	9	4	5	6	6	7	6	5	5	6	7	5	7	5	5	5	3	4	5	4	4	6	8
Eaa x9	1	–	1	1	–	–	–	–	1	1	3	–	–	–	4	4	–	1	–	2	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	7
Eaa T1	1	–	1	1	–	–	–	–	3	1	3	–	–	–	4	4	–	1	–	2	–	–	–	–	–	1	–	3	–	–	–	–	–	–	–	–	–	7
Eaa x6	–	–	–	–	–	–	–	–	3	–	3	–	–	1	4	4	–	–	–	2	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	6
Eh 4327	1	–	1	1	–	–	–	–	3	2	3	–	–	1	4	3	–	–	–	1	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	5
Eh 4328	1	–	1	1	–	–	–	–	3	2	2	–	–	1	4	3	–	–	–	1	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	6
Ecc SR319	3	3	2	3	5	6	2	–	3	3	5	–	2	2	–	4	1	1	–	–	–	–	–	3	–	2	–	4	–	–	3	2	–	2	3	4	–	8
Eca SR8	3	3	2	3	5	2	2	–	–	4	3	–	2	2	–	3	1	1	–	–	–	–	–	2	5	5	–	5	1	–	2	2	–	2	2	2	–	6
Echr 120	3	5	1	3	7	2	3	–	–	3	4	–	–	2	–	4	1	1	–	–	–	–	–	4	5	5	–	4	1	–	4	3	–	4	4	4	–	8
Em	–	3	–	–	3	–	–	–	1	–	4	–	–	2	6	3	1	–	–	1	–	–	–	1	6	5	–	1	2	–	1	–	–	1	1	1	–	7
Pss 18	–	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5	–	–	–	–	–	–	–	–	–	–	–
Pst	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–
Psg 2070	–	2	–	–	2	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5	–	–	–	–	2	–	–	–	–	–	–
Psl 43.29	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–
Psv	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6	–	–	–	–	–	–	–	–	–	–	1
Pf bio II	–	4	–	–	5	2	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	–	3	–	–	–	–	2	–	–	–	1	–	–	1
Xcca 42	–	4	–	–	5	3	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	3	–	–	3
Xccu	–	–	1	1	–	–	–	–	–	1	3	–	–	1	4	3	–	1	–	3	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	4
Xcv	–	4	–	–	5	4	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Xcp	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Xm 4361	–	3	–	1	5	1	3	–	3	1	2	–	–	–	5	–	–	1	–	–	–	–	–	3	1	3	–	2	2	–	1	1	–	2	2	1	–	5
PCER	–	1	–	–	2	2	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Eco C600	2	5	1	2	8	2	3	–	–	1	3	–	1	–	–	4	–	1	–	–	–	–	–	4	2	3	–	2	2	–	2	2	–	3	2	3	–	6
Sc MAROC	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Protein content of antigen was 1–2 μg/well.
[b] Protein content of antibody was 5 μg/ml.
[c] Conventional polyclonal antibody purified from blood collected during splenectomy.
[d] Ea = *Erwinia amylovora*, Eaa = *E. ananas*, Eh = *E. herbicola*, Ecc = *E. carotovora* pv. *carotovora*, Eca = *E. carotovora* pv. *atroseptica*, Echr = *E. chrysanthemi*, Em = *E. mallotivora*, Pss = *P. syringae* pv. *syringae*, Pst = *P. syringae* pv. *tomato*, Psg = *P. syringae* pv. *glycinea*, Psl = *P. syringae* pv. *lachrymans*, Psv = *P. syringae* pv. *viridiflava*, Pf = *P. fluorescens*, Xcca = *X. campestris* pv. *campestris*, Xccu = *X. campestris* pv. *cucurbitae*, Scv = *X. campestris* pv. *vesicatoria*, Xcp = *X. campestris* pv. *phaseoli*, Xm = *X. maltophilia*; PCER = xylem-limited fastidious bacteria, Eco = *Escherichia coli*, and Sc = *Spiroplasma citri*.
[e] Absorbance at 490 nm: 0.0–0.1 (–), 0.1–0.2 (1), 0.2–0.4 (2), 0.4–0.6 (3), 0.6–0.8 (4), 0.8–1.0 (5), 1.0–1.2 (6), 1.2–1.4 (7), 1.4–1.6 (8), and 1.6–1.8 (9).

amylovora (17). Antigen for coating ELISA microtiter plates (Vangard International, Neptune, NJ), strain WC542, was prepared by diluting the sonicated bacterial suspension in 0.05 M carbonate buffer (pH 9.6) to 10 μg/ml protein (Bio-Rad protein assay). Biotinylated antimouse immunoglobulin, Avidin-biotinylated peroxidase, and *o*-phenylenediamine (enzyme substrate) were used in indirect ELISA. Reactions were generally considered positive if the absorbance value was greater than 0.1, because nonspecific background readings of normal serum (negative control) were fourfold less than 0.1.

Serological specificity of MAs. Specificity of MAs was determined by indirect ELISA with biotinylated antimouse immunoglobulin. Bacterial antigens were prepared and diluted to 10 μg/ml protein as described for the coating antigen. Monoclonal antibodies were harvested from culture supernatants after cultures reached $5–7 \times 10^6$ cells per milliliter. Polyclonal antisera were purified from blood collected during splenectomy. Antibodies were purified as described previously (17) with 50% saturated ammonium sulfate and used diluted to 5 μg/well in 0.01 M PBS (pH 7.4).

Immunofluorescent staining. Ten clones of MAs that reacted specifically to *E. amylovora* (Tables 2 and 3) in indirect ELISA were further tested by indirect immunofluorescent staining by a procedure similar to that of Roberts (24). Duplicate drops of each bacterial preparation (5×10^5 cells per milliliter in PBS) were placed on a microscope slide, then air-dried and heat-fixed. A drop of test MA, purified from culture supernatant (100 μg/ml protein), was applied to each preparation, and slides were incubated at 37 C for 60 min in a darkened moist chamber. After incubation, slides were flooded with PBS containing 0.05% (v/v) Tween 20 for 5 min, rinsed briefly with distilled water, and air-dried. A drop (45 μl) of fluorescein isothiocyante (FITC)-conjugated antimouse immunoglobulin (Sigma) diluted 1:70 with PBS was then applied to each preparation. The slides were again incubated as above, then rinsed and mounted in glycerol-PBS (9:1). Slides were observed at 495 nm with an oil immersion objective (100×) on a Nikon epifluorescent microscope with an HBO 50W high-pressure mercury lamp, an IF410-485 excitation filter, a DM 505 dichroic mirror, a 460 auxiliary filter, and a 515W absorption filter.

Nine clones of MAs that reacted specifically with *E. amylovora* in indirect immunofluorescent staining were further used for detection of *E. amylovora* in situ and in vitro.

Apple fruits, collected from trees with and without fire blight symptoms, were used for in situ detection of *E. amylovora*. Diseased and healthy tissues were sampled with a no. 1 cork borer. Freehand sections were fixed with acetone, and incubated with purified MA at 100 μg protein per milliliter, then with FITC-conjugated antimouse immunoglobulin diluted 100-fold in PBS (20). Mouse normal serum was used as an antibody control.

For in vitro detection, a method of direct counting on polycarbonate membranes was modified from previously described procedures (6,7,10,13). Bacterial suspensions were prepared from infected apple blossoms by soaking two blossoms in 1 ml PBS for 1 hr or from diseased apple fruit tissue ground in 100 volumes (w/v) PBS. Preparations were passed through three layers of cheesecloth and clarified by centrifugation at 3,000 rpm for 5 min. Supernatants were then subjected to centrifugation in a microcentrifuge tube at 13,000 *g* for 5 min in a Fisher model 235B microcentrifuge (Fisher Scientific, Springfield, NJ). Pellets containing bacteria were resuspended and incubated in MA for 1 hr, centrifuged, and resuspended in FITC-conjugated antimouse immunoglobulin for another hour. Bacteria were pelleted, then resuspended in 100 μl PBS and collected on the surface of a polycarbonate membrane filter (0.2-μm pore size, 13 mm diameter; Nucleopore Corp., Pleasanton, CA) by filtration with a syringe-type holder. During filtration, a nitrocellulose filter (0.4-μm pore size; Fisher Scientific, Springfield, NJ) was placed under the polycarbonate filter to prevent the aggregation of bacteria. Protein content of MAs and the concentration of FITC-conjugated antimouse immunoglobulin were the same as those used for immunofluorescent staining on slides. Stained membranes were mounted on microslides with glycerol-PBS (9:1) and no. 1 coverslips. Preparations from healthy blossoms and fruit tissues were used as controls. Mouse normal serum was used as an antibody control.

Photomicrographs were made with a 35-mm Nikon automatic photomicrographic system (Microfles AFX) with ASA 400 film (Kodak Tri-X Pan).

Sensitivity of direct-count detection method. To evaluate the sensitivity of the direct-count method, 10-fold serial dilutions of *E. amylovora*, from 5×10^6 to 5×10^2 cells per milliliter, were stained. One milliliter of each preparation was then processed as described before and observed by epifluorescent microscope with a 100× oil-immersion objective lens. The effective surface area of the polycarbonate membrane was calculated at 75.43 mm^2, or 5×10^3 microscopic fields. Average number of bacteria per microscopic field was based on observation of 20 fields.

TABLE 3. Numbers of epiphytic bacteria isolated from pome fruit trees reacting with monoclonal antibodies

Antibodies	Geographic origin of strains tested (no. tested)				
	Maryland (10)	Pennsylvania (12)	Virginia (15)	West Virginia (15)	Miscellaneous (4)
Polyclonal[a]	7[b]	8	13	13	1
Monoclonal					
1	1	0	3	1	1
2	4	1	4	2	1
3	0	0	0	1	0
4	2	4	2	1	1
5	4	2	3	2	1
6	2	0	2	1	0
7	2	0	2	1	1
8	0	0	0	0	0
9	2	4	4	1	1
10	1	2	3	1	1
11	3	4	3	2	1
12	0	0	0	0	0
13	0	0	0	0	0
14	2	4	3	1	1
15	2	4	3	1	1
16	2	4	3	2	1
17	0	0	3	0	0
18	0	0	3	0	0
19	0	0	0	0	0
20	0	2	3	0	1
21	0	0	0	0	0
22	0	0	0	0	0
23	0	0	0	0	0
24	2	0	1	1	0
25	0	1	0	0	0
26	1	3	3	1	0
27	0	0	0	0	0
28	2	4	3	1	1
29	0	0	0	2	0
30	0	0	0	0	0
31	0	1	0	0	0
32	1	0	0	0	0
33	0	0	0	0	0
34	2	0	0	0	0
35	2	0	0	0	0
36	2	0	0	0	0
37	0	0	0	0	0

[a] Conventional polyclonal antibody purified from blood collected during splenectomy.
[b] $A_{490nm} > 0.1$ considered positive reaction.

RESULTS

A total of 48 stable hybridoma clones secreting monoclonal antibodies against *E. amylovora* strain WC 542 were obtained from four independent fusions. All reacted positively in indirect ELISA to 75 *E. amylovora* strains (Table 1) from different countries. Of these clones, the 37 that gave highest optical density readings were further tested for specificity against 24 identified bacterial strains and 56 unidentified epiphytes. Ten of the 37 MAs (MA 8, 12, 19, 21, 22, 23, 27, 30, 33, and 37) did not cross-react with any of the 24 known bacterial strains (Table 2). Other MAs reacted to one or more *Erwinia* species besides *E. amylovora* and some to one or more xanthomonads, pseudomonads, or *E. coli* C600. Polyclonal antibodies strongly cross-reacted with all *Erwinia* species tested and weakly cross-reacted with strains of other genera. The 10 MAs specific to *E. amylovora* did not react to any of the 56 epiphytes

collected. Cross-reactions were common with other clones and with the polyclonal antibodies, which cross-reacted with 75% of epiphytes tested (Table 3).

In immunofluorescent staining on slides, all MAs specific to *E. amylovora* (based on ELISA), except MA 8, reacted positively to all the *E. amylovora* strains. These nine MAs (MA 12, 19, 21, 22, 23, 27, 30, 33, and 37) did not cross-react with any of the known bacteria or the epiphytes.

In studies on in situ detection, positive FITC-specific, bright green fluorescence was easily observed with the epifluorescent microscope in infected apple tissues treated with MA. Controls treated with normal mouse serum produced brownish and/or greenish autofluorescence of low intensity with both healthy and diseased tissues. No fluorescence could be seen in healthy tissue controls tested with MAs, except very slight greenish autofluorescence.

Fluorescing *E. amylovora* were also readily observed with an epifluorescent microscope when MA-treated bacterial preparations from both infected blossoms and diseased apple tissues were collected on polycarbonate membranes (Fig. 1). Plant debris deposits on filters showed a brownish and/or yellowish autofluorescence of low intensity that could be accurately distinguished from the brightly fluorescing *E. amylovora* cells. No FITC-specific fluorescence was observed in any diseased or healthy preparation treated with normal mouse serum or in any healthy preparation treated with MAs.

The sensitivity of the modified immunofluorescent direct-count method was determined by the ease in observing and pinpointing fluorescing cells on polycarbonate filters. In bacterial suspensions of more than 5×10^4 cells, an average of more than five fluorescing cells could be seen per microscopic field. When 5×10^3 *E. amylovora* cells were stained and concentrated on the membrane, an average of one fluorescing cell could be easily located in every two fields. When the preparations had fewer than 5×10^2 cells, an average of one fluorescing cell could be found in about every 10–15 microscope fields.

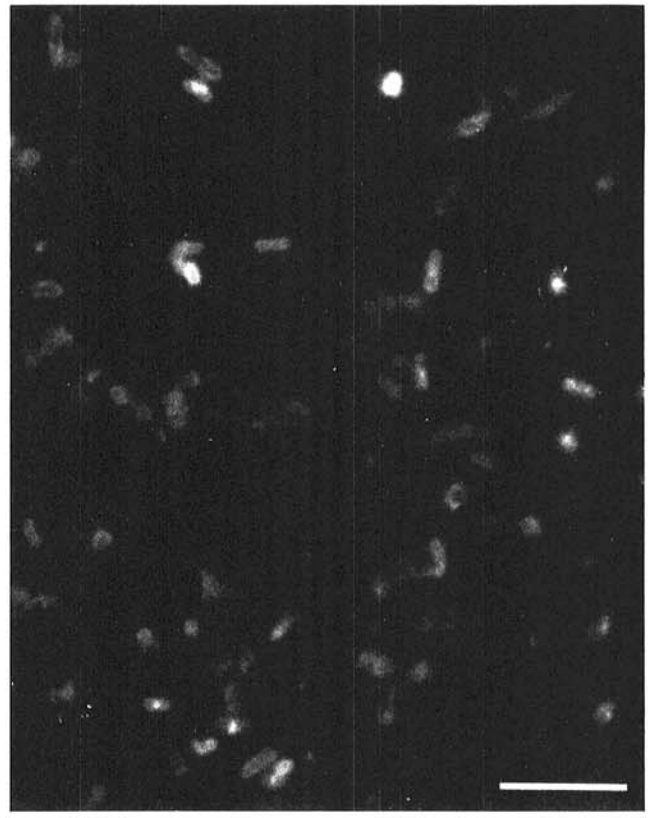

Fig. 1. Immunofluorescent staining with monoclonal antibodies for *Erwinia amylovora*. Fluorescing cells were collected on a polycarbonate membrane and observed under an epifluorescent microscope. Scale bar = 5 μm.

DISCUSSION

Nonspecific cross-reactions with other bacteria have been frequently reported with conventionally produced, polyclonal antibodies against *E. amylovora* (3,4,22,24). These problems have prevented accurate and unambiguous detection of *E. amylovora* in infected tissues, especially when visible symptoms are absent. In the present study, highly accurate detection was possible by ELISA with any of the MAs specific for *E. amylovora* and by immunofluorescent staining with any of nine MAs. In ELISA, all 10 MAs reacted with all isolates of *E. amylovora* collected from widely different geographic areas, indicating a high degree of conservation in antigenicity among isolates and the lack of recognition of strain or isolate-specific epitopes.

Monoclonal antibodies of various specificity levels were obtained. Some MAs reacted to one or more *Erwinia* species besides *E. amylovora*, and some MAs even cross-reacted with one or more xanthomonads or pseudomonads. The cross-reactions undoubtedly are due to the recognition by the MAs of common epitopes shared by *E. amylovora* and other bacterial genera or species. Various specificity levels of MAs also indicate the existence of a number of these shared epitopes. DeBoer and Wieczorek (8) had similar difficulty in developing species-specific monoclonal antibodies and attributed the problem to conservation of antigenic epitopes among different bacterial species.

The polyclonal and some monoclonal antibodies against *E. amylovora* could not be used for disease detection because of unwanted cross-reactions in ELISA with other species. The monoclonal MA8 also could not be used for disease detection by immunofluorescent staining because of negative reaction despite a positive and specific ELISA reaction. Because total antigens of broken cells were used in immunization, MA8 apparently reacted to an internal cytoplasmic antigen not exposed in stained intact cells but reactive in sonicated preparations as used in ELISA.

E. amylovora survives and overwinters in pear and apple trees as a result of infections occurring the previous year (30). The primary sources of inoculum in spring appear to be determinate and indeterminate cankers plus latent infections (26). Monitoring *E. amylovora* before and after symptom development in orchards has proven useful in forecasting fire blight (2,23). Selective media have been successfully used in epidemiology for monitoring epiphytic or endophytic *E. amylovora* (5,12,23). In our studies, we combined the specificity of MAs with the sensitivity of ELISA and immunofluorescent staining and for the identification and detection of *E. amylovora*.

The sensitivity of detection methods is also important. Using ELISA, Laroche and Verhoyen (16) were able to detect 10^4 cells per milliliter sample with conventional polyclonal antibodies. In our method, 5×10^3 cells, regardless of sample volume, were routinely detected using MAs without special efforts to increase the number of microscopic fields examined. Estimates of bacterial cell numbers determined by the membrane filter-immunofluorescent technique were similar to population estimates based on colony-forming units determined by standard plate count procedures (6,7). Ideally, we can elevate sensitivity with monoclonal antibodies to the order of 10^2 cells simply by increasing the numbers of microscope fields examined for pure-culture preparations or even for diseased samples if the autofluorescence from plant debris can be quenched and the fluorescing cells can be accurately recognized.

Sensitivity of detection is greatly influenced by the equipment used. An indirect biotinylated ELISA system was used to increase the sensitivity. We have also used an epifluorescent microscope to pinpoint fluorescing bacteria. A flow cytometer equipped with a fluorescent sensor could theoretically detect even one fluorescing cell per sample when no other fluorescing particles are present. For effective quarantine implementation, such an absolute level of detection sensitivity would be highly desirable. For epidemiological surveys and forecasting, the modified immunofluorescent direct-count method and indirect biotinylated ELISA with monoclonal

antibodies may provide suitable specificity and sensitivity (Lin et al, *unpublished*).

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