# Differentiation of Strains in the Aster Yellows Mycoplasmalike Organism Strain Cluster by Serological Assay with Monoclonal Antibodies

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**ABSTRACT**

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Monoclonal antibodies (MAbs) raised against the tomato big bud (BB) mycoplasmalike organism (MLO), a member of the aster yellows (AY) MLO strain cluster, were employed in dot immunobinding assays. The MAbs reacted only with strains in the AY MLO cluster, and not with any of several other MLOs not affiliated with the AY MLO strain cluster. However, reactions with MLOs in the AY cluster varied by strain. All MAbs reacted with BB and several MLO strains previously termed "aster yellows," including NAY (eastern AY), OKAY1 (Oklahoma strain), NJAY (New Jersey strain), and AY27 (Alberta strain); but none of the MAbs reacted with certain other strains of AY MLO, including strains termed SAY3 (western AY), OKAY3 (Oklahoma strain), NYAY (New York strain), and MNAY (Minnesota strain). Thus, the BB MLO-MAbs distinguished a group of interrelated MLO strains within the AY MLO strain cluster. This serogroup corresponds to the type I subcluster in the AY MLO strain cluster, previously identified on the basis of nucleic acid dot hybridizations and restriction fragment length polymorphism analyses.

Plant pathogenic mycoplasmalike organisms (MLOs) are believed to be the causal agents of disease in several hundred plant species (19). Because of the inability to isolate MLOs in pure culture, the genetic relationships among them remain largely undetermined. For most MLOs, including aster yellows (AY) strains, relatedness among strains was postulated earlier based on the similarity of their biological properties, such as plant host range, symptoms produced in common hosts, and the specificity of transmission by insect vectors (2,3,6–8, 10). Based on these biological criteria, two strains of AY MLO, eastern and western AY, were identified and differentiated (7,8,10,11). However, some MLO strains share a common vector(s) and/or host plant(s) with these two strains, yet induce disease symptoms unlike those of either strain (2,7,8). Likewise, other MLO strains share common hosts with these two AY MLO strains and induce similar symptoms in the hosts, but are transmitted by different vectors (2,18). Thus, classification and identification of MLOs based solely on their biological properties may result in confusion. It is certainly questionable whether strain affiliations based on biological criteria alone consistently reflect phylogenetic relationships among MLOs.

The recent development of MLO-specific serological and DNA hybridization assays has provided rapid and accurate means for MLO detection and identification (1,13). Lin and Chen (17) reported a monoclonal antibody produced against a strain of AY MLO and the use of the antibody for detection of this MLO in plant hosts. MLO-specific DNA fragments cloned from infected plants or insect vectors have been used as probes in the detection of MLOs, in the diagnosis of MLO-induced diseases, and in investigations of genetic relationships among various MLOs (13). Dot hybridization analysis using cloned DNA probes from strains of Maryland AY, tomato big bud (BB), and periwinkle little leaf (CN1) MLOs has indicated close genetic relatedness, not only among known eastern and western strains of AY MLO, but also among these and other MLOs associated with yellows diseases designated by names other than AY (5,12–15). This has resulted in the proposed recognition of an AY MLO strain cluster comprised of AY and other MLO strains that share substantial sequence homology. Three subclusters (types) were identified within this strain cluster on the basis of restriction fragment length polymorphism (RFLP) patterns (14).

The objective of the present study was to employ a serological assay, using monoclonal antibodies (MAbs) produced against the BB MLO strain (a member of the AY MLO strain cluster), to differentiate strains in the AY MLO strain cluster. An abstract of this work has been published (16).

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## MATERIALS AND METHODS

**Source of MLOs.** BB MLO-infected periwinkle (*Catharanthus roseus* (L.) G. Don) (4) was kindly provided by James Dale, University of Arkansas, Fayetteville. Maryland strain of aster yellows (AY1) MLO-infected periwinkle was originally field collected at Beltsville, Maryland (12). Other MLO strains were provided by the researchers listed in Table 1, who provided each strain separately in periwinkle or other plant host tissue.

**Hybridomas and production of MAbs.** Partially purified MLO preparations from periwinkle plants infected with BB MLO were used in the production of MAbs against this MLO as previously described (9). Twenty hybridomas that secreted MAbs specific to BB MLO were obtained. Two hybridoma cell lines, 10B3G11 (IgG2a) and 4E5C2 (IgM), were used for the production of ascites fluid in mice. The fluid was induced by injecting each of the two hybridomas into mice as described (9). The diluted ascites fluid (1:10,000) containing high MAb titer was used for immunoassays to investigate the serological relationships between BB MLO and each of the other strains previously placed (14) in the AY MLO strain cluster.

**Preparations of healthy and MLO-infected plant samples.** Extracts from healthy plants and from plants infected with BB MLO or with other strains of MLO were prepared by a method similar to that described elsewhere (9). In brief, nitrocellulose membranes (0.2 $\mu$m pore size) were immersed in TBS buffer (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.5) for 10 min and then assembled onto a Minifold I apparatus (Schleicher & Schuell, Keene, NH). A 50 $\mu$l sample of antigen (plant extract) diluted in TBS was added to each well while a vacuum was applied. Each well was then rinsed twice under vacuum with 100 $\mu$l of TBS. The manifold was disassembled and the nitrocellulose membrane removed before the vacuum was discontinued. For the immunoassay, the membranes were rinsed in TBS containing 1% natural nonfat dry milk and 0.5% bovine serum albumin (BSA) at room temperature for 60 min to block unbound sites. The membranes were then incubated at 40 C in diluted (1:10,000) ascitic fluid in TBS-BSA (TBS containing 1% natural nonfat dry milk and 0.05% BSA). After overnight incubation, the membranes were washed three times for 5 min with TBS-BSA. For signal detection, the blots were incubated for 2 hr in a solution of goat anti-mouse immunoglobulin-alkaline phos-

phatase conjugate prepared in TBS-BSA. The blots were then washed five times in TBS-BSA for 5 min and incubated 10 min in a substrate solution containing 14 mg of nitro blue tetrazolium and 7 mg of 5-bromo-4-chloro-3-indolyl phosphate in 40 ml of substrate buffer containing 0.1 M Tris, 0.1 M NaCl, and 5 mM $MgCl_2$, pH 9.5. Reactions were stopped with TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5). To remove green pigment from the blots, the membranes were briefly rinsed in approximately 1% sodium hypochlorite and then in TE.

## RESULTS AND DISCUSSION

No false positive reactions were observed in tests with any of the four healthy plant samples tested (Fig. 1). Both of the BB MLO MAbs, 10B3G11 and 4E5C2, reacted strongly with tissue extracts from BB, CN1, CN13, CN13w, CN13G, OK1, NAY, AY27, and NJAY MLO-infected plants. They did not cross-react with tissue extracts from AY1, OK3, NYAY, MNAY, SAY3, TLAY2, DAY, CY3, MBS, ITABB, PWB, CP, ASHY, VR, CX, WX, EY, and CPH MLO-infected plants (Table 1, Fig. 1).

Unlike the MAbs previously prepared against a strain of AY MLO (NJAY) (17), which were monospecific to strain NJAY, the MAbs produced in this study reacted with the immunogen BB MLO and with eight other MLO strains in the AY MLO cluster. The cross-reactivity of the BB MLO MAbs indicated serological relatedness among these MLO strains. As shown previously, MLO strains in the AY MLO cluster can be differentiated into three distinct groups or subclusters, types I, II, and III, on the basis of Southern hybridizations and RFLP analyses with cloned MLO DNA probes (14). All nine MLO strains that reacted with BB MLO MAbs were previously designated type I. Thus, the MAbs differentiated the AY MLO strain cluster into at least two serogroups. This differentiation of a type I serogroup from other MLO strains in the AY MLO cluster is consistent with results from genomic DNA analysis (14). Strains of type I AY MLO, which include strains of "classic" eastern AY, are distinct from type II and type III strains. However, the differentiation does not coincide with previous groupings on the basis of biological properties. The serogroup identified in this work consists of MLOs that include strains previously designated as eastern AY (NAY), western AY (AY27, OKAY1), or New Jersey AY (NJAY), as well as MLOs associated with other yellows diseases, i.e., periwinkle little leaf (CN1, CN13, CN13w, and CN13G) and BB.

Differentiation and classification of the AY MLO strain complex traditionally relied on symptoms produced in common plant host species (6,8,10,11). Results from our study strongly suggest that reliance solely on symptomatology can be misleading. For instance, based on our study, although BB in North America (4) and Italian tomato big bud MLO (ITABB) both belong to the AY MLO strain cluster and elicit similar big bud symptoms in tomato, they are distinct from each other. On the contrary, BB and CN1 MLOs, two closely related strains on the basis of genetic (5,12,14) and serological characterizations (present study), induce very different symptoms in periwinkle plants. CN1 MLO induces "classic" AY disease symptoms (i.e., virescence or phyllody of floral part), whereas BB MLO induces formation of small flowers with normal color (15).

Highly specific serological reagents, such as the MAbs used in this study, provide sensitive and reliable means for detection and identification of uncultured MLOs in hosts. As more MAbs are developed against other MLOs in the AY MLO strain cluster, use of the various MLO-specific MAbs should provide further information on the antigenic relationships among these genetically related MLO strains.

**Table 1.** Results of dot-blot (alkaline phosphatase) immunoassays of various mycoplasmalike organism (MLO) samples with monoclonal antibodies (MAbs) produced against tomato big bud MLO

| MLO | Original disease name | Reaction with MAbs[a] | Source[b] | Reference |
|---|---|---|---|---|
| AY MLO strain cluster (type)[c] | | | | |
| BB (I) | Tomato big bud | + | Arkansas | J. L. Dale (4) |
| CN1 (I) | Periwinkle little leaf 0-1 | + | Connecticut | S. M. Douglas (5) |
| CN13 (I) | Periwinkle little leaf 0-13 | + | Connecticut | S. M. Douglas (5) |
| CN13G (I) | Periwinkle little leaf 0-13 | + | Connecticut | (15) |
| CN13w (I) | Periwinkle little leaf 0-13 | + | Connecticut | (15) |
| OK1 (I) | Oklahoma AY (western AY) | + | Oklahoma | J. Fletcher (6) |
| NAY (I) | Eastern AY | + | Canada | L. N. Chiykowski |
| AY27 (I) | Alberta AY (western AY) | + | Canada | C. Hiruki |
| NJAY (I) | New Jersey AY | + | New Jersey | T.-A. Chen (17) |
| AY1 (II) | Maryland AY | − | Maryland | (12) |
| OK3 (II) | Oklahoma AY (western AY) | − | Oklahoma | J. Fletcher (6) |
| NYAY (II) | New York AY | − | New York | C. Hiruki |
| MNAY (II) | Minnesota AY | − | Minnesota | E. E. Banttari |
| SAY3 (II) | Severe western AY | − | California | A. H. Purcell |
| TLAY2 (II) | Tulelake western AY | − | California | A. H. Purcell |
| DAY (II) | Dwarf western AY | − | California | A. H. Purcell |
| CY3 (II) | Chrysanthemum yellows | − | Italy | A. Bertaccini |
| CPH (III) | Clover phyllody | − | Canada | L. N. Chiykowski |
| MBS (UD) | Maize bushy stunt | − | Mexico | L. R. Nault |
| ITABB (UD) | Italian tomato big bud | − | Italy | A. Bertaccini |
| Other MLOs | | | | |
| PWB | Potato witches'-broom | − | Canada | C. Hiruki |
| ASHY | Ash yellows | − | New York | W. A. Sinclair |
| CP | Clover proliferation | − | Canada | C. Hiruki |
| VR | Beet leafhopper transmitted virescence | − | California | G. N. Oldfield |
| CX | Canada peach X-disease (eastern) | − | Canada | L. N. Chiykowski |
| WX | Western X-disease | − | California | B. C. Kirkpatrick |
| EY | Elm yellows | − | New York | W. A. Sinclair |

[a] 10B3G11 And 4E5C2. + = positive reaction with both MAbs, − = negative reaction with both MAbs.
[b] All MLO strains were maintained in periwinkle plants except NAY, MBS, and ITABB, which were maintained in China aster (*Callistephus chinensis* (L.) Nees), corn (*Zea mays* L.), and tomato (*Lycopersicon esculentum* Mill.), respectively.
[c] Number in parentheses is type (subcluster) designation (14). UD = undesignated.

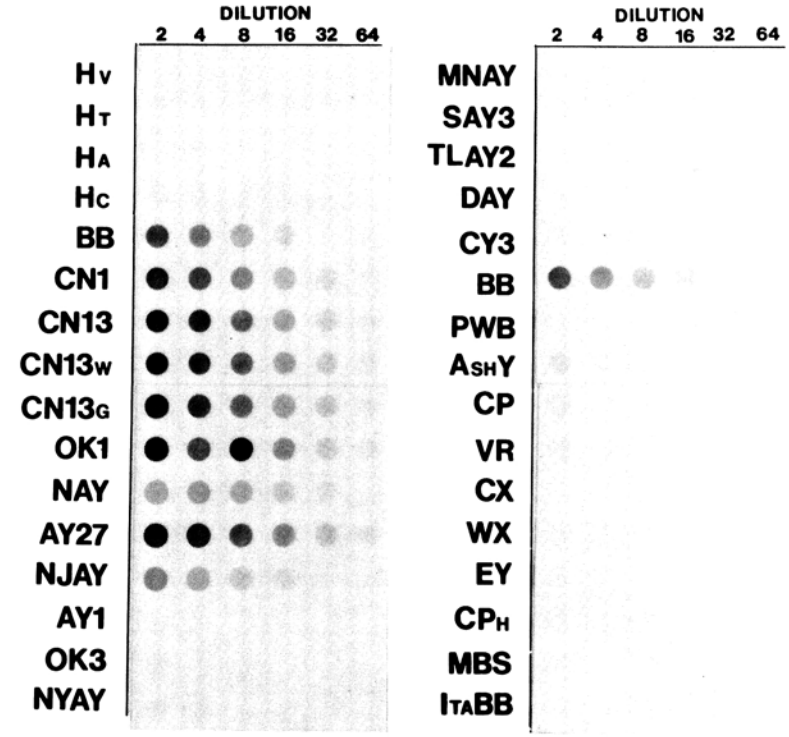


**Fig. 1.** Dot-blot (alkaline phosphatase) immunoassay of various mycoplasmalike organism (MLO) samples with a tomato big bud (BB) MLO-monoclonal antibody, 10B3G11. Antigen samples (plant extracts) diluted in TBS buffer were blotted onto the nitrocellulose membranes. The monoclonal antibody (diluted ascites fluid) was used for the immunoassay. Hv, Ht, Ha, and Hc are extracts from healthy periwinkle, tomato, aster, and corn plants. BB, CN1, CN13, CN13w, CN13G, OK1, AY27, NJAY, AY1, OK3, NYAY, SAY3, TLAY2, DAY, CY3, PWB, AshY, CP, VR, CX, WX, EY, and CPH are extracts from MLO-infected periwinkle; NAY and MNAY are extracts from MLO-infected China aster; ItaBB is an extract from an MLO-infected tomato; MBS is an extract from an MLO-infected corn plant. Abbreviations are the same as in Table 1.


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