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Analysis of *Xanthomonas campestris* pv. *citri* and *X. c. citrumelo* with Monoclonal Antibodies

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ABSTRACT

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A monoclonal antibody (MAb), designated A1, reacted with lipopolysaccharide (LPS) epitopes of all tested strains of *Xanthomonas campestris* pv. *citri* isolated from the Asiatic form of citrus bacterial canker (CBC-A), with *X. campestris* strains pathogenic on ti (*Cordyline terminalis*), and with some Florida citrus nursery strains associated with citrus bacterial spot (CBS) disease. The A1 MAb did not react with strains associated with other forms of citrus canker (B, C, or D). Except for weak reactions with *X. c. manihotis,* MAb A1 did not react with 130 other *Xanthomonas* pathovars and species or with 89 strains of other genera. In contrast, the titers of a rabbit-anti-CBC-A antiserum with several other *X. campestris* pathovars were as high as titers with some CBC-A strains. A second MAb, A2, reacted only with a flagellar epitope associated with CP1 bacteriophage-sensitive CBC-A strains. The CBC-B strains appeared to be antigenically heterogenous, because no MAb was produced that reacted with all CBC-B strains; however, the CBC-B strains were grouped by reactions to three MAbs specific for LPS epitopes. One CBC-B MAb, B2, indicated a close antigenic relationship between strains in groups B, C, and D. Another MAb, C1, specific for CBC-C strain XC70 reacted with a heat-sensitive epitope associated with a molecule partially sensitive to proteolytic enzymes. MAbs (T1 and T2) specific for weakly virulent strains isolated in Mexico from *Citrus aurantifolia* (Mexican lime) did not react with any other strains from citrus. CBS strains from Florida were serologically heterogeneous but distinct from strains associated with CBC. Most of the strongly aggressive CBS strains reacted with a MAb (CBS1) generated to a strongly aggressive strain, whereas most moderately and weakly aggressive strains reacted with MAb Xct generated to a *X. campestris* pathogen of ti. Moderately to weakly aggressive CBS strains reacted with MAb A1, but those strains also reacted with MAb Xct, whereas CBC-A strains did not. The LPS banding patterns of CBC-A strains were similar to each other, with major bands at an average M_r of 80,000, and were distinguished from the LPS patterns of A1-positive CBS, ti, and *X. c. manihotis* strains (major bands at an average M_r of 60,000).

Citrus bacterial canker (CBC) disease, caused by *Xanthomonas campestris* pv. *citri* (10), is widespread and poses a serious threat to citrus in many areas of the world. Until recently, three different forms of CBC were recognized. The most severe form, Asiatic canker (CBC-A), occurs worldwide on the broadest range of plants. Cancrosis B (CBC-B) primarily affects lemon in Argentina and Uruguay, and cancrosis C (CBC-C) affects Mexican lime (*Citrus aurantifolia*) in Brazil (19). A bacterial disease of limes in Mexico is considered to be a fourth form (CBC-D) caused by *X. c. citri* (14,24). Bacteria associated with various forms of CBC were considered "pathotypes" of *X. c. citri*, but after adoption of the pathovar system (10,30), pathotypes became "groups" A, B, C, and D of *X. c. citri* (14,15,26).

Citrus bacterial spot (CBS) strains associated with a foliar disease in Florida citrus nurseries were first designated group E of *X. c. citri* (14), but later were proposed to have a separate pathovar status, *X. c. citrumelo* (12). The Asiatic form of citrus canker CBC-A also appeared at limited sites in Florida (15,26). In view of the differences among these xanthomonads, in preliminary studies (2,6) we generated monoclonal antibodies (MAbs) to *X. c. citri* and CBS strains in search of stable antigenic phenotypes that would reflect meaningful relationships within and among the bacteria associated with various forms of CBC and CBS. The feasibility of this approach has since been confirmed with the production of MAbs that revealed pathovar-specific epitopes for the following *X. campestris* pathovars: *campestris* (1), *oryzae* (5), *oryzicola* (5), *pelargonii* (7), *begoniae* (8), and *phaseoli* (*unpublished data*). In the present study, the MAbs and their respective epitopes that distinguish xanthomonads associated with various forms of CBC and CBS were characterized.

MATERIALS AND METHODS

Bacterial strains. Listed in Table 1 are 282 bacterial strains isolated from citrus species in widely dispersed geographical regions. The 130 strains of other *Xanthomonas* sp. and pathovars and 89 strains of other genera of phytopathogenic bacteria used to screen and characterize MAbs were described previously (1,5). Five additional strains of *X. c. manihotis* (XM1, XM4, XM6, and HMB253, HMB23; received from H. Maraite) and 10 strains (ti strains) of an undescribed pathovar of *X. campestris* isolated in Hawaii from leaves of ti (*Cordyline terminalis*) showing bacterial leaf streak symptoms also were used.

Production of MAbs. The procedures used were described previously (1). For immunization, mice were injected with formalin-killed, whole-cell suspensions of CBC-A strains XC59, XC62, XC64, XC69, XC70 (either individually or as mixtures), and individually with Mexican strain T22, ti strain A910-2, strains A1024-3 and A990-5 of *X. c. dieffenbachiae*, and CBS strains showing varying levels of aggressiveness by the detached-leaf assay (13). Immunizations also were done with a mixture of strains F1 (DPI 084-3048, strongly aggressive), DPI X169-1 (moderately aggressive), and DPI 084-3162 (moderately aggressive) isolated during the 1984 outbreak, or individually with weakly aggressive strain FP3 and moderately aggressive strain FP19, isolated in 1987, and strongly aggressive strain VE16, isolated in 1988. Supernatant fluids from culture wells with hybridomas were screened for antibody reactions with group A, B, C, and D strains of *X. c. citri*, CBS strains, and control strains (*Erwinia herbicola* Eh1 and *X. c. campestris* A249), by either radioimmunoassay (RIA) (1) or enzyme-linked immunosorbent assay (ELISA) (5). Selected hybridomas were tested for specificity by screening with all strains of *X. c. citri*, selected CBS strains, and with numerous other xanthomonads and nonxanthomonads described previously (1,5). The isotypes of MAbs were determined as described (5). All known or suspected strains of *X. c. citri* and CBS also were tested with *Xanthomonas*-specific MAbs, X1 and X11 (1).

LPS and heat-extraction methods. For preparation of LPS, either living or formalin-killed bacteria were extracted by the hot phenol method of Westphal and Jann (29). The water phases of the extracts were dialyzed against distilled water, concentrated under reduced pressure, and treated with RNase (Sigma Chemical Co., St. Louis, MO) and proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN). The LPS was pelleted by centrifugation of the extracts for 6 h at 80,000 *g*. The pellets were dissolved in distilled water.

Heat extracts were prepared by heating bacterial suspensions containing approximately 2×10^8 cells per milliliter in phosphate-buffered saline (PBS) for 1 h in a boiling water bath and were cleared by centrifugation.

Western blotting. For SDS-PAGE, the buffer system of Laemmli (17) was used. Samples consisting of either LPS, heat extracts, or of living or formalin-killed bacteria (1.5×10^9 cells per milliliter) were mixed (1:1) with 2× sample buffer (17) with or without 5% 2-mercaptoethanol, boiled for 3 min, and electrophoresed with 5% stacking gels and 7.5% separating gels. After electrophoresis, some gels were silver-stained by the method of Tsai and Frasch (28) to detect LPS. For western blotting, components were electrotransferred from gels to nitrocellulose (27) with 11- × 13-cm stainless steel plates in place of platinum wire electrodes. The nitrocellulose was blocked overnight with Blotto

TABLE 1. Strains of *Xanthomonas campestris* pv. *citri* and *X. c. citrumelo* (citrus bacterial spot [CBS] strains)

Number of strains	Group[a]	Strain designations	Host	Origin	Received from[b]
2	A	XC62, XC63	*Citrus* sp.[c]	Japan	1
1	A	XC59	Lime	Brazil	1
2	A	Xcc7, Xcc18	Grapefruit	Argentina	5
2	A	Xcc36, Xcc43	Orange	Brazil	5
3	A	Xcc45, Xcc49, Xcc52	Orange	Japan	5
2	A	Xcc70, Xcc84A	Lemon	Argentina	5
1	A	Xcc63	Lime	Argentina	5
1	A	Xcc62	Mandarin	Argentina	5
3	A	Xc74, Xc75, Xc77	Mandarin	Reunion Island	1
1	A	XC83	Lemon	Uruguay	1
1	A	XC87	Mandarin	Japan	1
2	A	XC89, XC91	Lemon	Argentina	1
1	A	XC98	Lime	Yemen	1
1	A	XC100	Lime	Pakistan	1
3	A	XC103, XC104, XC105	Orange	Thursday Island	1
2	A	XC106, XC107	Lime	Christmas Island	1
2	A	F132, F142	Orange	Florida	1
1	A	F133	Navel orange	Florida	1
1	A	F134	Mexican lime	Florida	1
1	A	F139	Sweet orange	Florida	1
1	A	F212	Grapefruit	Florida	1
10	B	Xcc83B, Xcc84B, Xcc85B, Xcc87B, Xcc88B, Xcc89, Xcc90, Xcc91B, Xcc93B, Xcc94B	Lemon	Argentina	5
2	B	XC64, XC69	Lemon	Argentina	1
1	C	XC70	Lime	Brazil	1
1	D	XC90	Lime	Mexico	1
5	...[d]	T20, T21, T22, T23, T24	Lime	Mexico	4
4	...	T25, T26, T27, T28	Lime	Mexico	2
16	CBS[e]	X73-084-3294[f]	*Citrus* sp.	Florida	6
15	CBS	F1–F100[f]	*Citrus* sp.	Florida	1
194	CBS	FP, LW, OC, VE[f]	*Citrus* sp.	Florida	3

[a] Based on citrus bacterial canker symptoms. "Group" is synonymous with a previously used term, "pathotype," for strains associated with different forms of citrus bacterial canker.

[b] 1. E. L. Civerolo, Maryland; 2. G. Garza, Mexico; 3. T. R. Gottwald, Florida; 4. S. Rodriguez, Mexico; 5. D. Zagory, Argentina; 6. J. Miller, Florida.

[c] Various *Citrus* sp.

[d] Strains have no group designation, but because of weak virulence, they are distinguished from the group D strain isolated from the same area.

[e] Strains originally referred to as group E of *X. c. citri*, and now designated CBS strains.

[f] The complete listing of strains is available upon request. FP = Frostproof; LW = Lake Wales; OC = Ocoee; VE = Venice nurseries.

(16), rinsed in borate buffer, and then reacted for 1 h with 1:1,000 dilutions of MAbs (ascitic fluids) in 1:3 dilution of Blotto. The blots were washed five to six times with borate buffer, reacted for 1 h with 1:1,000 rabbit-anti-mouse globulin diluted in the 1:3 Blotto. After washing five to six times with borate buffer, ^{125}I-protein A at 2.5×10^5 cpm/ml was added for 1 h. After extensive washing, the blots were autoradiographed on Kodak X-Omat AR5 film with intensifying screens at −70 C. Relative molecular weights (M_r) were determined with a ^{14}C-labeled methylated protein mixture (Amersham Corp., Arlington Heights, IL) consisting of phosphorylase (doublet at 92.5 and 100 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa).

Other immunological procedures. A rabbit-anti-CBC-A serum produced by immunizations with XC62 was described previously (8,9). The procedures for immunofluorescence, immunoelectron microscopy (22,23), and immunodiffusion have been described (7).

RESULTS

Group A MAbs. Numerous fusions made with spleen cells after immunization of mice with either individual strains or mixtures of group A, B, or C strains failed to produce a pathovar-specific MAb that reacted exclusively with all strains of *X. c. citri*. However, fusions made with cells from mice immunized with CBC-A strains (XC59, XC62, XC63) generated hundreds of hybridomas that produced similar MAbs; all reacted in ELISA or RIA with all group A strains, but not with any of the strains from group B, C, or D. These MAbs also reacted with all *Xanthomonas* strains pathogenic on ti, some CBS strains, and weakly with some strains of *X. c. manihotis*, but not with any other xanthomonads or nonxanthomonads tested. One MAb was selected and designated A1 (Table 2). Binding of MAb A1 to *X. c. manihotis* was considerably weaker than with *X. c. citri*, as shown by the binding curves with clone 147-7 in Figure 1. In titrations, MAb A1 bound ti strains as well as or better than CBC-A strains. Binding curves for the A1-positive CBS strains were essentially the same as for strain XC62 (Fig. 1). Although MAb A1 did not distinguish CBC-A strains from A1-positive CBS and ti strains, other MAbs generated to a ti strain to be described below did distinguish CBS from CBC-A strains.

A rare MAb, designated A2 (Table 2), reacted only with three CBC-A strains from Japan (XC62, Xcc45, Xcc52), XC103 from Thursday Island, and XC83 from Uruguay. This MAb did not react with any other xanthomonad or nonxanthomonad.

MAbs of groups B and C. Unlike the production of numerous "A1-type" MAbs reacting with all CBC-A strains, numerous fusions made with individual or mixtures of B strains failed to yield a specific MAb reacting with all B strains. However, three MAbs (B1, B2, B3) were produced that essentially grouped the B strains (Table 2). Furthermore, MAb B2 linked the Argentine B, Brazilian C, and Mexican D (XC90) strains (Table 2).

The sharing of epitopes between CBC-B and CBC-C strains was frequent. For example, in a fusion made after immunization with XC70 (CBC-C), 85 of 114 (60%) antibody-producing hybridomas yielded MAbs that detected determinants of XC70 in common with at least one group B strain. In addition to MAbs that reacted to B, C, and D strains, a MAb (designated C1) reacted only to a C strain, XC70 (Table 2).

Mexican strains T20–T28 MAbs. Nine Mexican strains (T20–T28) were serologically identical. Numerous MAbs generated to these strains reacted to all strains in the group, but not to Mexican strain XC90 or any other xanthomonads except some strains of *X. c. dieffenbachiae* and a strain from *Strelitzia*, A2169. Two MAbs designated T1 and T2 (Table 2) differed only in that T2 reacted to one Florida strain (F870) that did not react with T1.

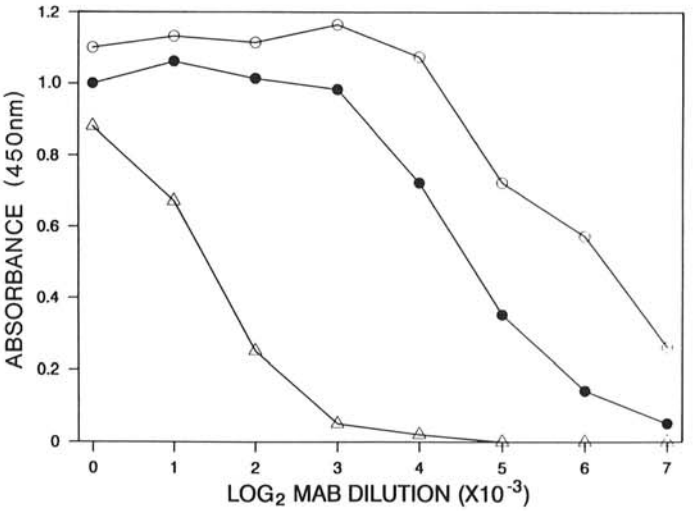

Fig. 1. Binding curves of monoclonal antibody A1 (ascitic fluid, clone 147-7) reacted with citrus bacterial canker (CBC)-A strain XC62 (●), ti strain A910-2 (○), and *Xanthomonas campestris* pv. *manihotis* strain HMB9 (△). Each point is an average of duplicate ELISA measurements.

TABLE 2. Monoclonal antibodies used to characterize strains of *Xanthomonas campestris* pv. *citri* and *X. c. citrumelo* (citrus bacterial spot [CBS] strains)

MAb	Clone no.	Isotype	Specificity	Other pathovars of *X. campestris*[a]
A1	147-7	IgG_3k	All CBC[b]-A strains	*manihotis*, "ti"[c] some Florida CBS strains
A2	80-10	$IgG_{2b}k$	CP1 bacteriophage positive CBC-A strains	None
B1	80-237	IgG_3k	CBC-B strain XC64	None
B2	83-14	IgM*k*	All CBC-B strains (Argentine strains) except XC64 and XC69; CBC-C strain XC70; and CBC-D strain XC90	*phaseoli*[d]
B3	150-42	IgM	CBC-B strains XC64 and XC69	*dieffenbachiae*[d]
C1	96-120	IgG1	CBC-C strain XC70	None
T1	92-16	IgM*k*	Mexican strains T20–T28	*dieffenbachiae*[d]
T2	92-59-1-1	IgG_{2a}	Mexican strains T20–T28	*dieffenbachiae*[d]
CBS1	93-19	IgM	98 CBS strains	*alfalfae*
CBS1a	159-135	IgM	98 CBS strains	*alfalfae*
CBS2	154-10	IgM	Eight CBS strains	*vitians*
Xct	163-118	IgG_{2a}	123 CBS strains	"ti", *alfalfae*[d]
Xcd1	72E-E3-B9-C5	$IgG_{2a}k$	Low reactions with many CBS strains	*dieffenbachiae*[d]
Xcd3	72B-F9-B6-E9	IgM	High reactions with 10 CBS strains	*dieffenbachiae*[d]

[a] One or more strains of the listed pathovars also reacted.
[b] Citrus bacterial canker.
[c] Unnamed pathogen on ti (*Cordyline terminalis*).
[d] One or more strains of one or more other pathovars also reacted.

TABLE 3. Serological reaction patterns (serogroups) of 225 strains of *Xanthomonas campestris* pv. *citrumelo* from Florida nurseries

MAb[a]	Serological reaction patterns (serogroups)							
	I	II	III	IV	V	VI	VII	VIII
X1	+[b]	+	+	+	+	+	+	+
X11	+	+	−	+	+	+	+	+
CBS1[c]	+	+	−	−	−	−	−	−
Xct	+	−	−	+	+	+	+	−
A1	−	−	−	+	−	−	−	−
CBS2[d]	−	−	−	−	+	−	−	−
Xcd3	−	−	−	−	−	−	+	+
Others[e]	−	−	−	−	−	−	−	−
Number of strains per group	10	93	4	98	8	2	5	5

[a] Monoclonal antibody.
[b] A positive value indicates an ELISA absorbance of 0.15 above that of the control, *Erwinia herbicola*.
[c] CBS1 is a MAb made to a mixture of citrus bacterial spot (CBS) strains recovered from the 1984 outbreak of CBS. A second MAb, CBS1a, had an identical reaction pattern.
[d] CBS2 was made to a weakly aggressive citrus bacterial spot strain, FP3, isolated during the 1987 outbreak.
[e] Reactions with MAbs A2, B1, B2, B3, C1, T1, and T2 made to other xanthomonads from citrus. Many CBS strains showed weak reactions (ELISA absorbance values = 0.05–0.15 above control) with MAb Xcd1 made to *X. c. dieffenbachiae*.

TABLE 4. Distribution of 46 serologically distinct citrus bacterial spot strains among 24 Florida nurseries[a]

Serogroup	Number of strains	Number of locations	Detached-leaf assay reactions[b]
I	10	4	Moderate (8); weak (2)
II	12	9	Strongly aggressive (6); moderate (6)
III	2	1	Strongly aggressive (2)
IV	8	2	Moderate (5); weak (3)
V	2	1	Weak (2)
VI	2	2	Moderate (1); weak (1)
VII	5	5	Moderate (4); weak (1)
VIII	5	4	Moderate (4); weak (1)

[a] Includes 31 strains from 20 locations in 1984–1985 and 15 strains from four locations in 1987–1988.
[b] The number of strains with each reaction is shown in parentheses.

MAbs used to characterize CBS strains. The specificities and characteristics of these MAbs are shown in Table 2. Antibody CBS1 produced to a mixture of strains (DPI 084-3048, DPI X169-1, and DPI 084-3162) reacted with strongly and moderately aggressive strains isolated from several nurseries in 1984. An antibody CBS1a with identical specificity was generated to a strongly aggressive strain (VE16) recovered from the 1988 outbreak. An antibody designated CBS2 made to a weakly aggressive strain (FP3) recovered from the 1987 outbreak reacted with only eight other CBS strains from the same location. MAbs designated Xcd1 and Xcd3 generated to strains of *X. c. dieffenbachiae* reacted with most strains of *X. c. dieffenbachiae* (*unpublished data*) and a few strains of several other pathovars including 10 CBS strains (Table 2).

A fusion performed after immunization with ti strain A910-2 resulted in MAbs of several specificities, two of which are pertinent to this study. One MAb (clone 163-111) apparently was specific for the A1 epitope, because it reacted with all the A1-positive strains (CBC-A strains, ti strains, *X. c. manihotis*) and A1-positive but not A1-negative CBS strains. The second MAb, designated Xct (Table 2) did not react with any CBC-A or *X. c. manihotis* strains but detected an epitope shared between the ti strains, both A1-positive and A1-negative CBS strains, *X. c. alfalfae*, and xanthomonads isolated from *Syngonium* and *Cynodon*.

Reaction patterns of CBS strains. Except for reactions with MAb A1, the CBS strains did not react by ELISA or RIA with MAbs (A2, B1, B2, B3, C1, T1, or T2) made to various groups of *X. c. citri*. Six MAbs showed differential reactions with the 225 CBS strains and formed eight reaction patterns or serogroups (Table 3). All the CBS strains reacted with the *Xanthomonas*-specific MAb X1; however, four of the 225 CBS strains did not react to another *Xanthomonas*-specific MAb, X11, and thus formed pattern III.

The relationship between reaction patterns and results of detached-leaf assays performed on all of the strains isolated in 1984–1985 and 15 strains isolated in 1987–1988 are shown in Table 4. Reactions with MAbs CBS1, Xct, and Xcd3 partially delineated moderately and weakly aggressive strains from strongly aggressive strains; that is, strongly aggressive strains were found only in serogroups II and III, and all but two strongly aggressive strains reacted with MAbs CBS1 and CBS1a. Although some moderately aggressive strains reacted with MAb CBS1, most of these also reacted with MAb Xct, whereas no strongly aggressive strains reacted with MAb Xct or Xcd3.

The serological relationships between and among strains of *X. c. citri* and *X. c. citrumelo* are diagramatically summarized in Figure 2. The CBC-A strains are clearly separated from CBC-B, CBC-C, CBC-D strains and from all CBS strains except those in serogroup IV, which react strongly with MAb A1. Except for weak reactions with the ti and CBS serogroup IV strains, MAb A1 is specific for CBC-A. The ti strains were a serological link between the CBC-A strains and the heterogeneous CBS strains (Fig. 2). Notably, the aggressive strains (serogroups II and III) were distinct from all CBC and most other CBS strains, whereas moderately and weakly aggressive CBS strains shared common epitopes with ti strains, *X. c. dieffenbachiae*, and several other pathovars.

Characterization of antigens detected with MAbs. The A1 MAb (clone 147-7) gave bright confluent immunofluorescence, and a high density of the epitope was indicated by the uniform pattern of binding in immunoelectron microscopy (Fig. 3A). In contrast, binding of MAb A2 was not detected by immunofluorescence. In immunoelectron microscopy, MAb A2 bound to the polar flagellum of A2-positive strains (Fig. 3B), and in some microscopic fields, binding to detached flagella was seen (Fig. 3C).

Western blots of 20 CBC-A strains from Japan, South America, and Florida, analyzed either as whole cells boiled for 3 min in SDS-PAGE sample buffer or as heat extracts, all had a major broad band at an M_r of approximately 80,000, and a weakly binding band at an M_r of approximately 120,000 (Fig. 4). The M_r of the major band of strain XC62 was slightly lower than that of XC63. The major bands on immunoblots of similarly prepared A1-positive CBS and ti strains had molecular weights of approximately 60,000 (Fig. 4). Thus, the CBC-A and A1-positive CBS strains were easily distinguished from one another by their banding patterns. Some of the bands had ladderlike patterns, which, however, were more evident in phenol extracts (Fig. 5). Similar patterns and molecular weights also were obtained with preparations treated with 2-mercaptoethanol and/or proteinase K and RNase. Based on chemical characteristics (heat resistance, proteolytic enzyme resistance, extraction with hot phenol, and sedimentation at 80,000 *g*), and the ladderlike patterns

obtained in western blots, the epitopes detected with MAb A1 were LPS antigens.

No bands were seen with heat-extracted *X. c. manihotis*, an organism that bound A1 weakly; however, phenol extracts gave weak binding with MAb A1 at an M_r value of 60,000, indicating a pattern similar to the CBS and ti strains (data not shown). No binding of either heat extracts or LPS was observed with MAbs that did not react with these organisms. Furthermore, MAb A1 did not react with extracts of A1-negative strains. Gels silver-stained for LPS showed the same major LPS regions as did western blots (Fig. 6). The higher molecular weight LPS fractions of these and other xanthomonads we have analyzed in stained PAGE gels appear as smears, whereas control LPS preparations of *Salmonella typhosa* LPS have the ladderlike banding in the high as well as the low molecular weight fractions (Fig. 6).

All three CBC-B MAbs (B1, B2, B3) reacted with LPS epitopes (Fig. 7). In western blots the LPS of CBC-B strain XC64 (Fig. 7) and CBC-D strain XC90 (B2-positive) (not shown) had LPS patterns consisting of three regions; a smear at an approximate M_r of 120,000 and two bands at 90,000 and 60,000, respectively. Neither corresponded to the major 80,000 LPS band of CBC-A strains, but rather resembled those of A1-positive CBS and ti strains.

The C1 MAb specific for XC70 detected a heat-sensitive epitope that appeared to be associated with a protein moiety. No binding was detected with heat extracts or with cells boiled in sample buffer. Extracts were prepared by disruption of approximately 10^9 cells per milliliter in a French pressure cell and cleared by centrifugation. In western blots of unreduced, unheated extracts, two major bands were seen with molecular weights of approximately 98,000, and 64,000, respectively (Fig. 8A). After treatment with proteinase K for 6 h at 37 C, two bands at 92,000 and 58,000, respectively, were seen (Fig. 8A). Reduction with 5% 2-mercaptoethanol of either untreated or proteinase K-treated preparations did not alter these values. Binding was not destroyed by digestion of the antigen with either pronase, trypsin, papain, or pepsin, although differences in the concentrations of the digestion products were apparent. Because extracts boiled in sample buffer failed to bind MAb C1, the effect of temperature on binding of extracts in the presence or absence of SDS was determined. Equal volumes of extract either in PBS or sample buffer each were heated between 50 and 100 C for 3 min. In the absence of SDS, significant reduction of binding occurred only after heating at 100 C (Fig. 8B). In the presence of SDS, doublets were formed at 50–60 C, and binding was greatly reduced; at 70 C no binding was observed (Fig. 8C).

Immunoblots of heat extracts and phenol extracts of the Mexican strains T20–T28 revealed that the T1 and T2 MAbs also detected epitopes associated with LPS, and that the LPS

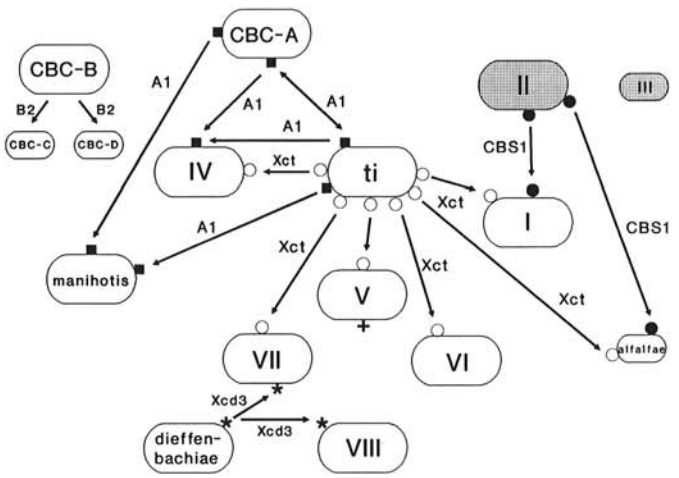

Fig. 2. Serological relationships among *Xanthomonas campestris* pv. *citri*, *X. c. citrumelo*, and other pathovars of *X. campestris* (*manihotis*, *dieffenbachiae*, *alfalfae*, and an unnamed pathovar of ti [*Cordyline terminalis*]). Ovals designating citrus bacterial canker (CBC)-A, CBC-B, CBC-C, and CBC-D are serological groups of the CBC pathogen. Roman numerals stand for the serological groups of *X. c. citrumelo*, eg., citrus bacterial spot (CBS) strains. Small ovals are serological groups represented by one to four strains. Large ovals are serological groups represented by five or more strains. Shaded ovals represent strongly aggressive strains. The arrow points from the strains used as immunizing antigen to the related epitope (symbol) on other strains detected by the indicated monoclonal antibody (MAb). The cross is an epitope that reacts with MAb CBS2.

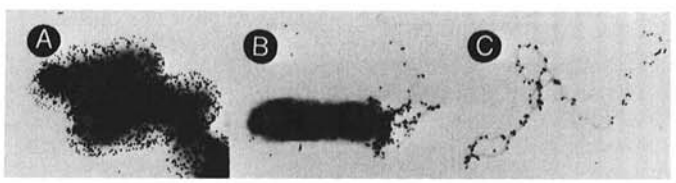

Fig. 3. Immunoelectron micrographs of *Xanthomonas campestris* pv. *citri* reacted with citrus bacterial canker (CBC)-A monoclonal antibodies (MAbs). **A**, MAb A1 + CBC-A strain XC63; **B** and **C**, MAb A2 + CBC-A strain XC62 (×31,250).

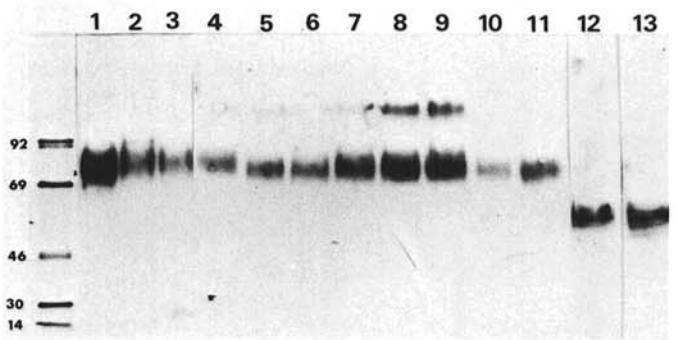

Fig. 4. Western blots of whole cells of *Xanthomonas campestris* pv. *citri* (lanes 1–11) and monoclonal antibody (MAb) A1-positive citrus bacterial spot (CBS) strains (lanes 12 and 13) reacted with MAb A1. Cells were boiled 3 min in SDS-PAGE sample buffer. Lanes 1–11 are CBC-A strains XC63, XC62, Xcc36, Xcc7, Xcc43, XC52, XC59, F134, F139, F142, and F212, respectively. Lanes 12 and 13 are CBS strains FP19 (moderately agressive) and LW10 (weakly aggressive), respectively. Molecular size standards ($\times 10^{-3}$) are on the left. The average molecular weight of the major bands of all citrus bacterial canker (CBC)-A strains is approximately 80,000; whereas, the average molecular weight of the major band patterns of all CBS strains is approximately 60,000.

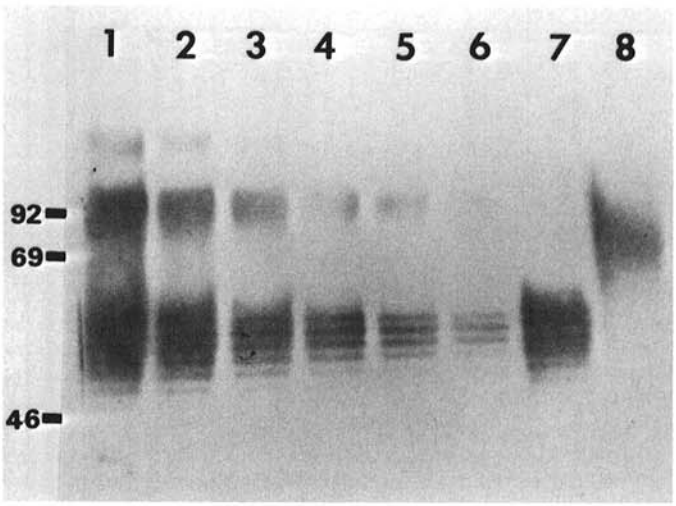

Fig. 5. Effect of dilution of lipopolysaccharides (LPS) on western blot patterns with monoclonal antibody (MAb) A1. LPS from citrus bacterial spot (CBS) strain F19 diluted 1:2 (lane 1); 1:4 (lane 2); 1:8 (lane 3); 1:16 (lane 4); 1:32 (lane 5); 1:64 (lane 6); undiluted heat extract of strain FP19 (lane 7); undiluted heat extract of CBC-A strain XC63 (lane 8). Molecular sizes ($\times 10^{-3}$) are indicated on the left.

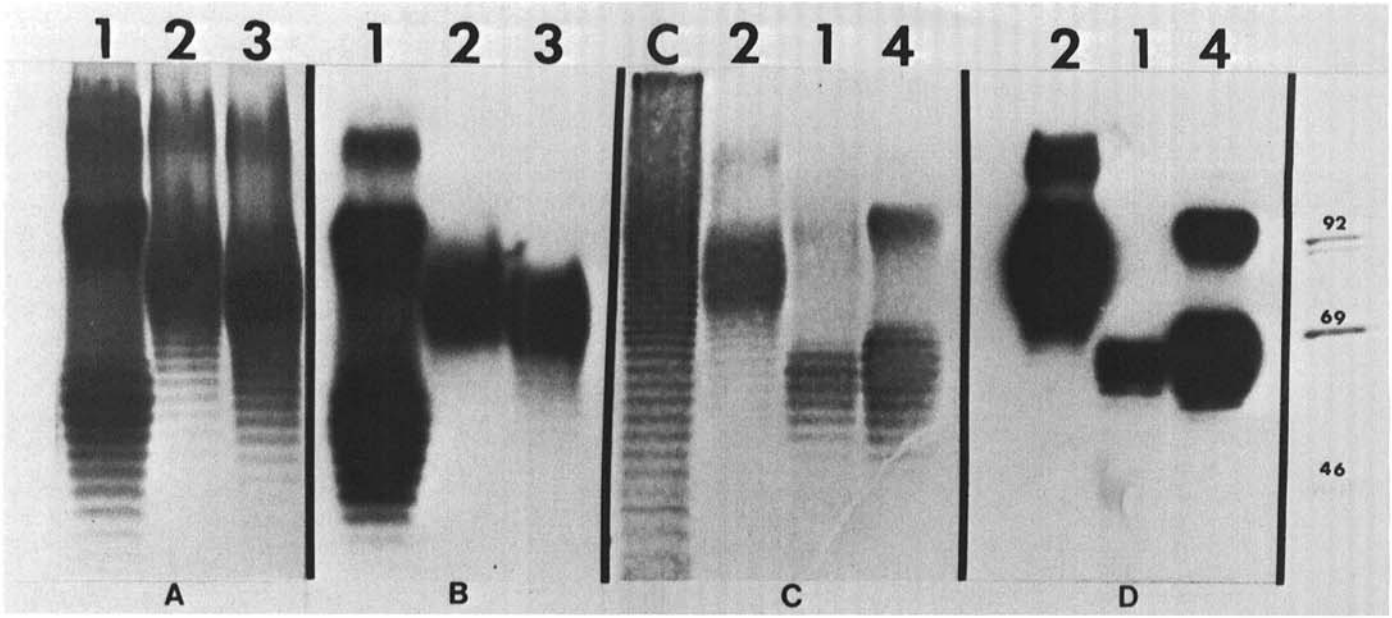

Fig. 6. Silver-stained SDS-PAGE gels (**A** and **C**) and corresponding western blots (**B** and **D**, respectively) of partially purified lipopolysaccharides (LPS). Lanes 1A–1D are the A1-positive citrus bacterial spot (CBS) strain FP19; lanes 2A–2D are citrus bacterial canker (CBC)-A strain XC62; lanes 3A and 3B are CBC-A strains XC63; lanes 4C and 4D are ti strain A910-2. Purified *Salmonella typhosa* LPS (Difco Laboratories, Detroit, MI) served as a control (lane C). The antibody was MAb A1, and molecular size standards ($\times 10^{-3}$) are on the right.

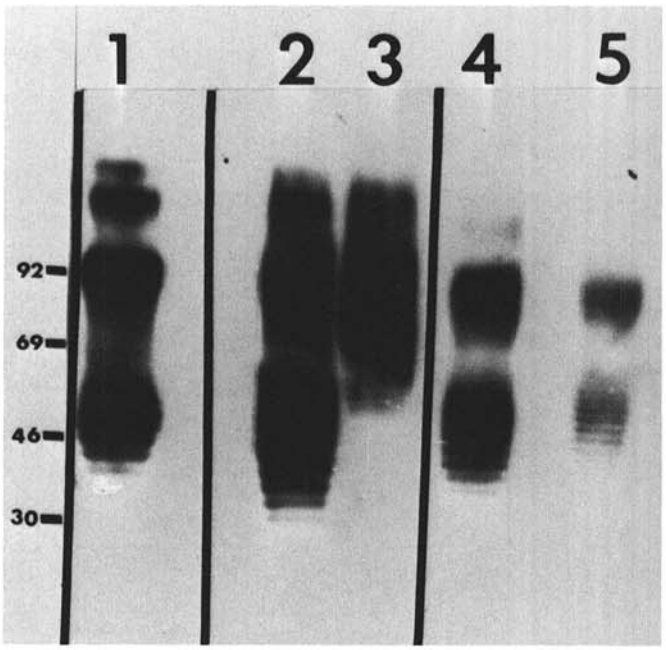

Fig. 7. Western blots of partially purified lipopolysaccharides (LPS) (lanes 2–5) of citrus bacterial canker (CBC)-B and CBC-C strains reacted with CBC-B monoclonal antibodies (MAbs). Lane 1, ti strain A910-2 + MAb A1 (control); lane 2, CBC-B strain Xcc90B + MAb B2; lane 3, CBC-C strain XC70 + MAb B2; lane 4, CBC-B strain XC64 + MAb B1; lane 5, CBC-B XC64 + MAb B3. Molecular sizes ($\times 10^{-3}$) are indicated on the left. The lower bands (ladderlike patterns) of CBC-B LPS (lanes 2, 4, and 5) corresponded to the major band (60,000) of the LPS of the ti strain (lane 1) and canker bacterial spot (CBS) strains. This band is missing for LPS of CBC-C strain XC70 (lane 3).

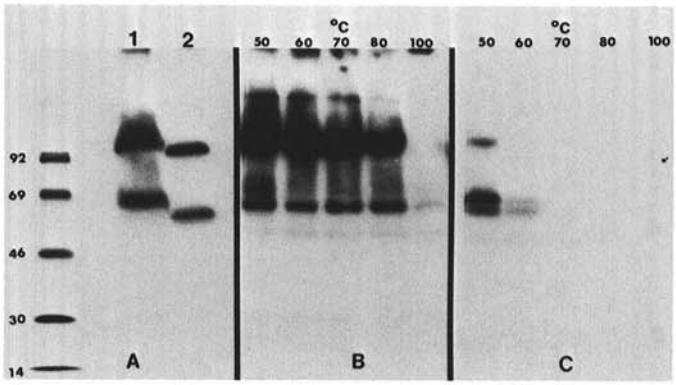

Fig. 8. Western blots of French pressure cell extracts of citrus bacterial canker (CBC)-C XC70 reacted with monoclonal antibody C1. **A,** Lane 1, unheated extract; lane 2, unheated extract treated with proteinase K for 6 h at 37 C. **B,** Extract in phosphate-buffered saline (PBS) without SDS, heated for 3 min at temperatures indicated. **C,** Extract in PBS containing 0.1% SDS heated for 3 min at temperatures indicated. Molecular size standards ($\times 10^{-3}$) are on the left.

patterns were similar to CBC-A strains; that is, a major band with an M_r of approximately 80,000. The pattern of heat extracts and phenol extracts of the CBC-D strain XC90 in western blots was the 60,000 type.

Monoclonal antibodies CBS1, CBS2, Xct, and Xcd3 were specific for LPS epitopes. As shown in Figure 9, western blots of heat extracts of weakly aggressive strains, FP2 and FP3, that reacted with MAb CBS2 had major ladderlike bands with M_r values of approximately 60,000 (lanes 1 and 2, respectively), and extracts of strongly aggressive strains VE1, VE16, and OC3 that reacted with MAb CBS1 (lanes 3, 4, and 5) had major bands with the same M_r values. Two weakly aggressive CBS strains, LW6 and LW16, that reacted with MAb A1 (lanes 6 and 7) also had major bands at 60,000, whereas CBC-A strains XC59 and XC62 (also reacted with MAb A1) had major bands at 80,000 (lanes 8 and 9). Extracts from 24 CBS strains (nine from Frostproof, six from Lake Wales, five from Venice, and four from Ocoee nurseries) blotted in a similar manner all had the major 60,000 band. In western blots of partially purified LPS (phenol extracts) of CBS strains (strongly or weakly aggressive), a major band with an M_r value of approximately 60,000 and weaker bands at 90,000 and 120,000 were obtained with MAbs CBS1 and CBS2. The specificities of MAbs CBS1 and CBS2 were verified because they did not react in western blots with strains that were negative in ELISA.

Several CBS strains were antigenically related to *X. c. dieffenbachiae* and to the ti strains; thus, it was of interest to determine

whether the LPS of the latter xanthomonads had a pattern similar to the LPS of CBS strains (major band at 60,000) or to the LPS of CBC strains (major band at 80,000). The LPS detected with MAb Xcd3 made to *X. c. dieffenbachiae* and the LPS of the ti strain resembled that of the CBS strains with a major band at 60,000 and weaker bands at 90,000 and 120,000.

The silver-stained patterns of CBS strains FP3, FP19, and VE16, ti strain A910-2, and *X. c. manihotis* strain HMB9 were essentially the same as the patterns obtained in western blots (Fig. 10). The usual 80,000 band was observed with CBC-A strain XC62, and the distinct banding pattern typical of LPS was observed for these strains as well as for purified LPS of *S. typhosa* (included as a control).

Rabbit-anti-CBC-A serum. In view of the specificity of the A1 MAbs, a re-evaluation of the polyclonal anti-CBC-A serum was made. Doubling dilutions from 1:50 through 1:6,400 of the rabbit serum were tested with numerous strains of *X. c. citri*, 108 xanthomonads of other *X. campestris* pathovars, and 89 non-xanthomonads used for screening MAbs. The rabbit-anti-CBC-A serum reacted with all MAb A1-positive CBS strains in serogroup IV. The antiserum had essentially the same titer to CBC-A strains XC62 and XC63, *X. c. manihotis*, ti strains, and to the MAb A1-positive CBS strains. Similar binding curves were obtained with other CBC-A strains from Argentina (Xcc7, Xcc36, Xcc49, Xcc62, Xcc63, Xcc60, Xcc70, and Xcc84A); however, lower binding curves were obtained with CBC-A strains XC59, Xcc43, and Xcc52 (data not shown). At a 1:100 dilution of antiserum, ELISA values above 0.3 were obtained for 44 of 108 strains of other *X. campestris* pathovars tested. Binding of the polyclonal antiserum to certain other strains of other *X. campestris* pathovars (*armoraciae*, *hederae*, *urticae*, *panax*, *begoniae*, *manihotis*, and strains from unnamed *X. campestris* pathovars from ti [*unpublished*] and onion [3]) was equal to binding to CBC-A strains XC59, Xcc43, and Xcc52. Except for a low reaction with one strain of *Pseudomonas solanacearum*, the rabbit antiserum failed to react at 1:50 dilution with all other nonxanthomonads.

DISCUSSION

Several significant observations on the antigenic relationships within and among strains of *X. c. citri* and *X. c. citrumelo* were made in this study. All CBC-A strains have a common epitope that is detected with MAb A1 and is associated with LPS. Group A strains have been reported to be serologically related to strains of *X. c. manihotis* (4,8); likewise MAb A1 reacted weakly to an LPS epitope of some strains of the latter pathovar. Furthermore, an identical MAb generated from a fusion after immunization with a ti strain bound to LPS antigens of all A1-positive strains. The sharing of an epitope with these other xanthomonads indicates that the A1 antigen probably is not related to a host-specificity factor responsible for the characteristic Asiatic canker disease. Although there is a close antigenic relationship between these bacteria, the characteristic LPS patterns of all CBC-A strains were different from the LPS banding patterns of CBS strains, ti strains, and *X. c. manihotis*.

Previous studies (8,9) with the polyclonal anti-CBC-A serum did not include testing a large number of other *X. campestris* pathovars. Although under the conditions of the present study the rabbit serum did not react with CBC-B or CBC-C strains, it did react significantly with individual strains of numerous other pathovars. The use of polyclonal antisera without other methods of confirmation for identifying A, B, and C strains and unknown strains isolated from suspected bacterial diseases of citrus (8,9), thus, is questionable. For example, the weakly virulent strains (T20–T28) associated with citrus bacteriosis in Mexico reacted with the rabbit-anti-CBC-A serum (24). Although the reaction was less than with the homologous strain (XC62), one cannot determine whether the reaction with an unknown strain is due to a low concentration of an homologous antigen(s) as illustrated by lower binding curves with CBS-A strains XC59, Xcc43, Xcc52, or due to a cross-reacting antigen(s) shared among numerous pathovars. In contrast, these weakly virulent Mexican strains did not react with group A, B, or C MAbs and were thereby distinguished from the virulent Mexican strain XC90, which reacted strongly with MAb B2.

Although CBS strains in serogroup IV could be distinguished from Asiatic canker strains by western blots of their LPS, neither MAb A1 nor the rabbit antiserum distinguished between these two groups. However, MAb Xct reacts with all A1-positive CBS strains (serogroup IV) but not with CBC-A strains and can be used to separate these groups. Analysis by PAGE and/or silver-staining of gels for LPS could be used for confirmation.

Because numerous A1-type MAbs were easily generated, apparently the CBC-A-specific LPS antigen is highly immunogenic. On the other hand, many attempts to produce an MAb specifically and universally reactive with all CBC-B strains failed. The antigens of CBC-B strains are heterogeneous in that at least three different MAbs specific for LPS epitopes were generated when the CBC-B strains were used as antigens. Whereas MAbs B1 and B3 reacted only with a few B strains, MAb B2 reacted with an LPS epitope

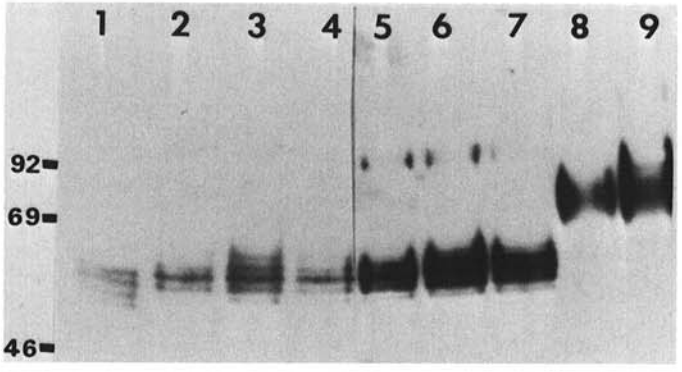

Fig. 9. Western blots of whole cells of citrus bacterial spot (CBS) strains reacted with monoclonal antibodies (MAb) CBS2 (lanes 1, 2), MAb CBS1 (lanes 3–5), and MAb A1 (lanes 6–9). Cells were boiled 3 min in SDS-PAGE sample buffer. Lanes 1 and 2 are CBS strains FP2, FP3 (weakly aggressive); lanes 3–5 are CBS strains VE1, VE16, and OC3 (strongly aggressive); lanes 6 and 7 are CBS strains LW6 and LW16 (weakly aggressive); lanes 8 and 9 are *Xanthomonas campestris* pv. *citri* citrus bacterial canker (CBC)-A strains XC62 and XC59, respectively. Molecular size standards ($\times 10^{-3}$) are indicated on the left. The average molecular weight of the major ladderlike pattern of all CBS strains is approximately 60,000, whereas the average molecular weight value of the major band of the CBC-A strains is approximately 80,000.

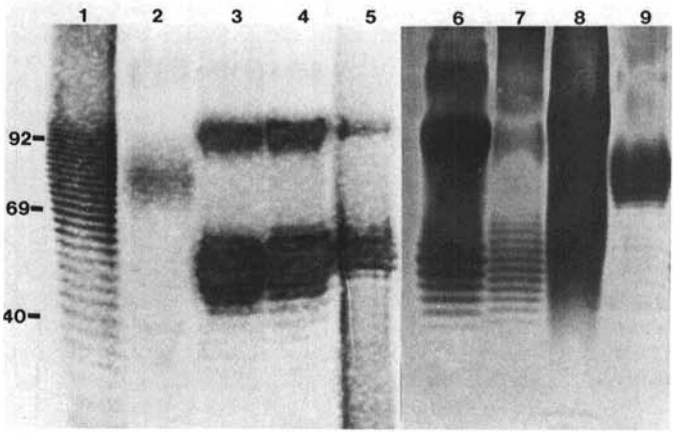

Fig. 10. Silver-stained SDS-PAGE gels of lipopolysaccharides (LPS) of xanthomonads. As a control, *Salmonella typhosa* LPS (Difco) is in lane 1. Xanthomonads are citrus bacterial canker (CBC)-A strain XC62 (lane 2); citrus bacterial spot (CBS) strain FP19 (lane 3); *Xanthomonas campestris* ti strain A910-A (lane 4); *X. c. manihotis* strain HMB9 (lane 5); moderately aggressive CBS strain FP19 (lane 6); weakly aggressive CBS strain FP3 (lane 7); strongly aggressive CBS strain VE16 (lane 8); CBC-A strain XC62 (lane 9). Molecular size standards ($\times 10^{-3}$) are indicated to the left.

of nearly all CBC-B strains, in addition to a CBC-C strain XC70 and CBC-D strain XC90 as reported earlier (2,6). Thus, there exists an antigenic relationship among groups B, C, and D. Similarly, RFLP patterns (11,12) formed two groups of *X. c. citri* strains corresponding to the A and B/C/D pathogenicity groups.

The serological heterogeneity of CBS strains recovered from 24 Florida nurseries contrasts with the uniformity of CBC-A strains. This heterogeneity also was reflected in differential host reactions by a limited number of CBS strains. Notably, MAb CBS1 detected most strongly aggressive and some moderately aggressive CBS strains but reacted with no other xanthomonads or other genera tested except one strain of *X. c. alfalfae*. Gabriel et al (11) presented evidence that some CBS strains (clonal group E2) are genetically related by RFLP patterns to *X. c. alfalfae*. Furthermore, all of the five strains included in clonal group E2 and pathogenic form E2 (pathogenic on citrus and alfalfa) (11) reacted in the present study with MAb CBS1 and were placed in serogroup II. In contrast, the three strains in clonal group E1 and pathogenic form E1 (11) reacted with MAb Xct as well as MAb CBS1 (serogroup I). Therefore, there was a relationship between surface antigens detected by MAbs and clonal groups delineated by RFLP analysis.

In the current study, most moderately to weakly aggressive strains shared an epitope with *X. campestris* strains from ti, whereas the strongly aggressive strains did not. In addition, many of the moderately and weakly aggressive strains isolated from citrus in Florida nurseries in 1984–1986 reacted with MAb Xcd3 generated to *X. c. dieffenbachiae*. Because MAb Xcd3 reacted with several other pathovars, we suspect that these moderately to weakly aggressive CBS strains represent a serologically and biologically diverse group of organisms that may be loosely related to epiphytic xanthomonads on a number of crops. In RFLP comparisons of strains of Florida group E (CBS) with strains of 26 other pathovars of *X. campestris*, Lazo et al (18) reported moderate similarity of CBS strains with *X. campestris* pathovars *alfalfae*, *phaseoli*, *cyamopsidis*, and *dieffenbachiae*. Xanthomonads from ti were not tested.

The specificity of MAb CBS1 was similar to that of a MAb (anti-4600) reported by Permar and Gottwald (20), based on comparisons of a limited number of strains. Nevertheless, the MAbs are not identical. Anti-4600 reacts with a heat-sensitive protein thought to be a flagellar H-antigen (20), whereas MAb CBS1 reacts with LPS.

Most of the *Xanthomonas* pathovar-specific epitopes that we have detected with MAbs have been associated with LPS, regardless of whether the epitope was found in all members of a pathovar (5,7) or in some but not all members of a pathovar (1,5). In addition, the *Xanthomonas*-specific epitope detected with MAb X11 (1) also is associated with LPS. Perhaps a renewed effort should be given to identifying and characterizing LPS of xanthomonads and other gram-negative phytopathogenic bacteria for taxonomic purposes and for identifying strains with different levels of virulence.

Despite the close antigenic relationships of XC70 and the Argentine B strains, a specific MAb (C1) was produced to XC70. It was interesting that the specific epitope in this case was not associated with LPS but apparently with a protein. The characteristics of this epitope are similar to the *X. c. oryzae* epitope detected with the pathovar-specific MAb Xco-1 (5; *unpublished data*). Both antigens were only partially sensitive to highly active proteolytic enzymes (proteinase K and pronase), apparently unaffected by reduction, and both epitopes are heat-sensitive in SDS solutions. The location in the cell and architecture of these antigens are being studied.

The MAb A2 specific for a flagellar antigen can be used for subgrouping CBC-A strains. Apparently this MAb binds with the CP1 bacteriophage receptor associated with the flagellum of phage-sensitive strains. All strains positive for MAb A2 are sensitive to bacteriophage CP1 and resistant to bacteriophage CP2 (9), except XC103 and XC83, which were not phage-tested. In contrast, all the other CBC-A strains (A2-negative) from Japan, Argentina, Brazil, and Paraguay were resistant to CP1 (9). It has been postulated that the chi phage of *Escherichia coli* (25) and the PBSI phage of *Bacillus subtilis* (21) attach to the flagellum and then slide along to the base of the flagellum where they inject their DNA. Perhaps a similar phenomenon occurs in phage infection of A2-positive strains of *X. c. citri*. Until it is shown that MAb A2 can block or modify CP1 phage infection, it is tentatively concluded that the CP1 phage site of adsorption is associated with the flagellum.

An important point emerging from our studies is the inverse relationship between the ease in generating taxon-specific MAbs and the heterogeneity of the host-range of the xanthomonad. Pathovar-specific MAbs were produced, and they reacted specifically with all strains of an *X. campestris* pathovar that infect relatively few genera of hosts, as was observed with pathovars *oryzae* (5) and *oryzicola* (5) which infect only *Oryza* and a few other grasses; pathovar *begoniae* (7) which infects only *Begonia*; pathovar *pelargonii* (7) which infects *Geranium* and *Pelargonium*; and pathovar *phaseoli* (*unpublished results*) which infects *Phaseolus* sp. On the other hand, for certain *X. campestris* pathovars that infect several host genera, such as *campestris*, *dieffenbachiae*, *vesicatoria*, and *citri*, no MAbs have been found that reacted with all strains of the respective pathovar, whereas panels of MAbs formed pathovar serogroups. Thus, the pathovars that have a narrow host range formed unique, homogeneous groups with MAbs, unlike those pathovars with a rather broad host range. Clearly a re-evaluation of the classification of some *X. campestris* pathovars is warranted when serological studies with MAbs are complemented with genetic and bacteriological analyses.

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