Serological, Pathological, and Genetic Diversity Among Strains of *Xanthomonas campestris* Infecting Crucifers

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ABSTRACT

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Monoclonal antibodies (MAbs) specific for strains of *Xanthomonas campestris* pv. *campestris* (Xcc) and/or *X. campestris* pv. *armoraciae* (Xca) were evaluated to determine their reliability for pathogen identification. Reactivity patterns of 1,023 bacterial isolates from crucifer leaves, seeds, weeds, and alternate crops were compared with original host symptoms; and reactivity patterns of 498 of these isolates were compared with pathogenicity tests. Six MAbs were selected that distinguished strains of Xcc and Xca from nearly all other bacteria tested. However, Xcc and Xca strains were not serologically distinguished as groups from each other, despite numerous attempts to do so. Ninety-nine percent of the Xcc and Xca strains tested reacted with one or more of the Xcc/Xca-specific MAbs. Three MAbs separated all the Xcc (and some Xca) strains into three serological groups. A subset of 12 Xcc and seven Xca strains representing the serological and pathological diversity found in these two pathovars was further evaluated by restriction fragment length polymorphism (RFLP). Strains of both pathovars were heterogeneous by RFLP, and the RFLP patterns of the seven Xca strains overlapped with patterns of the Xcc strains. However, the 12 Xcc strains formed three identifiable groups (C1, C2, and C3), which corresponded to the three serological groups (S1, S2, and S3). The type strain of the species *X. campestris* (Xcc528^T) and all members of group S3/C3 caused a distinctive blight symptom that later developed into black rot. Strains in the other two Xcc groups caused typical black rot symptoms but not blight. The panel of antibodies can be used for rapid identification of Xcc and Xca pathogens in field and seed assays.

Additional keywords: black vein, diagnosis, leaf spot.

Black rot, a serious disease of crucifers that occurs worldwide, is characterized by V-shaped lesions on leaf margins and blackened veins associated with systemic movement of the pathogen in the plant (41). The causal organism is *Xanthomonas campestris* pv. *campestris* (strains designated Xcc). *Xanthomonas campestris* pv. *armoraciae* (strains designated Xca) causes leaf spot and hydathode necrosis on crucifers and on other hosts (6). The genetic diversity of strains within these pathovars has not been well-studied, even though pathogenic variation has been noted (21). Strains ideally are placed in different pathovars on the basis of their host range and distinctive disease phenotype (7,10), but in practice strains are often assigned to a pathovar on the basis of the host plant from which they were first isolated (36). Since some pathovars have a host range that involves different families (7) and overlapping host ranges, occurrence of a strain of *X. campestris* on a particular host should be only one criterion used to classify the pathogen.

Several methods have been used with varying degrees of success to differentiate *X. campestris* pathovars or subgroups without conducting pathogenicity tests. These include DNA–DNA hybridization (19,32), serology (2,5,11,17,38), phage typing (16,26), electrophoretic analysis of membrane proteins (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) (31), gas chromatographic analysis of fatty acids (27), DNA digests (18,24), and restriction fragment length polymorphism (RFLP) analysis (13–15,17,24,25). Of these procedures, SDS-PAGE and serology have been used in an effort to determine differences among strains of Xcc that cause black rot of crucifers and strains of Xca that cause leaf spot.

A high degree of similarity was found among Xcc strains tested by the SDS-PAGE procedure (31,38). Serological differences among strains Xcc were detected with monoclonal antibodies (MAbs), which were used to divide 200 strains into six groups (2). Strains that reacted with different MAbs spread at different rates from disease foci in field plots (43). Some evidence of the relatedness of Xcc to Xca was obtained when two strains of Xca reacted with two MAbs that previously had reacted only with Xcc and not with 192 strains of other species or pathovars of *Xanthomonas* (2). Although Xcc was serologically heterogeneous, three Xcc/Xca-specific MAbs (X9, X13, and X17) reacted with nearly all of 200 strains tested (2). These MAbs thus appeared to be useful for rapid identification of Xcc in seed and plant samples. Nevertheless, the failure of these three MAbs to detect all pathogenic strains caused concern in the seed industry because it reduces the reliability of the antibody tests. Furthermore, the reaction of some Xca strains with these MAbs made them less than pathovar-specific.

The initial purpose of this study was to evaluate a new and larger panel of MAbs for the ability to identify and distinguish strains of Xcc and Xca from each other and from all other bacteria. A total of 521 Xcc and Xca strains, isolated from 1,023 plant samples and seed extracts of diverse origins, were compared by serology and by symptoms induced on inoculated cabbage plants. During the course of the study, the serological and pathological diversity observed among strains comprising the two pathovars prompted us to evaluate the genetic diversity of selected strains that represented distinct groups encountered through serological and pathogenicity assays.

MATERIAL AND METHODS

Bacterial strains. The 521 Xcc and Xca strains derived from 1,023 isolations of pathogenic and saprophytic bacteria used in this study are listed in Table 1. Strains were tested with MAbs in three phases: 1) 595 strains were evaluated with a panel of MAbs already available from previous research; 2) 329 additional strains were evaluated with these MAbs plus additional MAbs generated to previously MAb-negative strains; and 3) 32 of the above strains plus 99 additional strains were used to compare MAb reactivity patterns with virulence ratings of strains following inoculation onto cabbage seedlings and larger plants. Other *X. campestris* strains tested (not in Table 1) included two strains of *X. campestris* pv. *aberrans* (ICPB 163 and PDDCC 4805) and one strain of *X. campestris* pv. *incanae* (PDDCC 574). Cultures were maintained on YDC (1% yeast extract, 2% dextrose, and 2% fine-powdered calcium carbonate) or lima bean agar (Difco) media at 24–28 C. For long-term storage, cultures were lyophilized or stored in LB (28) broth with 20% glycerol at −20 or −80 C.

Pathogenicity tests. Three different pathogenicity tests were used: spray, notched-leaf, and excised-cotyledon (R. Morrison and W. Wiebe, *personal communication*) assays. Spray inoculations were performed in an attempt to reproduce natural infections through hydathodes and stomates. Notch inoculations were used to facilitate rapid characterization of large numbers of cultures. If results were unclear, strains were retested by spray inoculation. The excised-cotyledon assay of young seedlings was used as a quantitative measure of virulence. To prepare inoculum, cultures were grown overnight on YDC agar and resuspended in sterile saline to an approximate concentration of 2×10^8 cfu/ml as determined by viable plate counts converted to a spectrophotometric reading of $A_{600} = 0.1$ optical density (OD).

For spray inoculations, 6-wk-old cabbage (*Brassica oleracea* L. var. *capitata* L. cv. C-G Hybrid) plants were inoculated by spraying to runoff. Three plants were inoculated per strain. Inoculated plants were enclosed in plastic bags and incubated for 16 h on a laboratory bench under Gro-Lux lamps (approximately 24 C). Plants were subsequently removed from the bags and placed in the greenhouse for 21 days. Symptoms were recorded at weekly intervals.

For notched-leaf assays, 6-wk-old C-G Hybrid cabbage plants were inoculated by notching the leaf tips with a razor blade and applying inoculum (a single overnight colony suspended in 1 ml of sterile saline). Plants were incubated for 16 h in plastic bags, then placed in a greenhouse for 21 days as described above. Symptoms were recorded at weekly intervals.

The excised-cotyledon assay was conducted as follows. Seedlings were grown in the greenhouse and inoculated 8 to 10 days after planting when the first true leaf was just visible and the cotyledons were well expanded. One cotyledon was excised from each seedling by cutting as close to the stem as possible. The seedling was inoculated by applying a mass of bacteria from a colony to the wounded surface with a sterile cotton swab. Three

TABLE 1. Sources of *Xanthomonas campestris* pv. *campestris* (Xcc) and *X. campestris* pv. *armoraciae* (Xca) strains used in this study

Isolates tested[a]	Xcc and Xca strains[b]	Origin	Location	Source[c] or reference
First evaluation and strain isolation				
371	128	Cabbage, broccoli, weed, lettuce leaves	Hawaii	This study
224	224	Cabbage, broccoli, cauliflower, weed, collard leaves	Georgia	R. Gitaitis
595	352			
Second evaluation and strain isolation				
118	18	Cabbage and other Brassica seed	New York	This study
211	67	Cabbage and broccoli seed	California	This study
329	85			
Third evaluation (*Xanthomonas* only)				
(32)[d]	(32)	Atypical strains from first and second evaluations (includes GAC-17, GAC-20, G3-38A, GAC-137, G2-12, G2-17, G3-27)	Hawaii	This study
21	14	Cabbage, broccoli, cauliflower, Brussel sprouts (seed and leaves)	California	R. Morrison
24	24	Cabbage, broccoli, cauliflower leaves	Panama	R. Alvarez
30	22	Cabbage, broccoli, cauliflower, rape, black mustard, other crucifer seed	California	This study
3	3	Cabbage, mustard seed	Nepal	D. Shakya
3	3	Cabbage leaves (includes PHW-117)	Wisconsin	P. Williams
1	1	Cabbage leaf (H-M)	Wisconsin	This study
1	1	Cabbage leaf (X3 or Cabaret)	Florida	This study
7	7	Raddish, collard, broccoli leaves[e]	Georgia	R. Gitaitis
2	2	Cabbage seed (417, 756)	E. Asia	(2)
1	1	Broccoli leaf (A342)	Hawaii	(26)
1	1	*X. c. campestris* (XC-114)	N. Carolina	(43)
1	1	*X. c. campestris* (A249)	Hawaii	(2)
3	3	*X. c. armoraciae* (XLS2, XLS6, XLS10)	N. Carolina	L. L. Black
1	1	*X. c. campestris* (Xcc528[T])	United Kingdom	ATCC 33913
131	116			
1,023	521	Total strains		

[a] Many bacteria, particularly those isolated from seed samples, were not xanthomonads. Other bacteria were *Xanthomonas* by colony morphology and reaction with *Xanthomonas*-specific MAbs X1 and/or X11.

[b] Xanthomonads that caused black rot or leaf spot of cabbage.

[c] ATCC, American Type Culture Collection, Rockville, MD 20852; R. Alvarez, Facultad de Agronomia, University of Panama, Chiriqui, Panama; L. L. Black, Louisiana State University, Baton Rouge, LA 70803; R. Gitaitis, Coastal Plains Experiment Station, Tifton, GA 31793; R. H. Morrison, Sakata Seed America, Inc, Salinas, CA 93907; D. D. Shakya, Institute of Science and Technology, Kirtipur, Tribhuvan University, Kathmandu, Nepal; P. Williams, Department of Plant Pathology, University of Wisconsin, Madison 53706.

[d] These 32 strains were from the first and second evaluations and are counted only once in the total strain count.

[e] These strains were received as *X. campestris* pv. *raphani*.

plants were inoculated per strain for each test, and three cabbage varieties (C-G, Tastie, and Copenhagen Market) were tested in separate experiments. For each bioassay, a set of control strains with known reactions was included. Plants were maintained in the greenhouse, with day and night temperatures between 24–35 C and 19–24 C, respectively. Plants were scored at 4, 7, and 14 days after inoculation.

For the excised-cotyledon assay, plants that developed black stem lesions by day 4–7 were classified as leaf spotting (Xca) strains, because in separate studies all such strains produced typical leaf spot lesions when spray inoculated onto cabbage leaves. Plants that showed no stem lesions on days 4–7 were rated at day 14 on a scale of 0–9 based on the following severity scale: 0 = plant healthy, no stunting, foliar symptoms, or darkening of vascular traces at the inoculation site; 1 = slight vascular darkening evident at the inoculation site; 3 = plant slightly stunted, one or more true leaves with a pale mottle but no distinct leaf lesions, vascular darkening evident in stem and occasionally in a lower leaf petiole; 5 = plant somewhat stunted, one or more true leaves with pale mottle and a small indistinct black rot lesion, vascular darkening extending from the stem into the leaf petiole; 7 = plant clearly stunted, well-developed typical black rot lesion (tissue collapse, chlorosis, vein blackening) in one or more true leaves, vascular discoloration extensive, developing into new growth; and 9 = plant nearly dead or dead, extreme stunting, necrosis of tissues and systemic vascular discoloration. Even-numbered values were used for plants that fell between the above classes.

A disease severity index (DSI) was calculated for each black rot strain as follows:

$$\Sigma(\text{Number of plants in class} \times \text{severity class}) \div \text{total number of plants}$$

Based on the DSI, the virulence of strains was classed as follows: 0–1 = avirulent, 1.1–3 = weakly virulent, 3.1–6 = moderately virulent, 6.1–8 = virulent, and 8.1–9 = highly virulent.

First evaluation of MAbs and Xcc/Xca isolation. In Maui, Hawaii, 128 presumptive strains of *Xanthomonas* were recovered from 371 samples taken from leaves of symptomless crucifers, crucifers showing a range of symptoms, weeds, and lettuce planted as an alternate crop in fields previously planted with crucifers. Leaf samples (approximately 5 mm diameter) were excised and placed in 1 ml of sterile distilled water, and 100-μl aliquots were assayed with a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (3). Bacteria were isolated from samples showing positive ELISA readings by dilution streaking from the original tube onto FS medium (43) to test for starch hydrolysis. We also obtained 224 lyophilized, presumptive *Xanthomonas* strains that were isolated from crucifers in Tifton, Georgia, and tested positive for sensitivity to *Xanthomonas* phage by R. Gitaitis (*personal communication*). All strains were checked for purity by two successive transfers on tetrazolium chloride (TZC) (22) medium modified to contain 0.001% TZC, then maintained on YDC.

Nine MAbs previously generated to identify and group 200 strains of Xcc (2) were evaluated with the 352 presumptive *Xanthomonas* strains described above. Serotyping was done with three Xcc/Xca-specific MAbs (X9, X13, and X17), four MAbs that are not Xcc/Xca-specific (X2, X3, X4, and X16), and two genus-specific MAbs (X1 and X11). Strains were transferred to YGA (0.5% yeast extract, 2% glycerol [w/v], 0.1% K_2HPO_4, 0.05% $MgSO_4$) agar medium, harvested, and dried onto microtiter plates for ELISA (2). The indirect ELISA used to type the bacterial strains in this study was the same as described earlier (43). The following code was used to indicate net ELISA readings above background: 0 = 0–0.05, 1 = 0.051–0.1, 2 = 0.101–0.2, and 3 = greater than 0.201. For qualitative evaluations, only ratings 0.1 above background were considered positive. Presumptive xanthomonads that were isolated from crucifers exhibiting typical black rot symptoms and testing positive with MAbs X1 and X11, and at least one of MAbs X9, X13, or X17 were considered to be Xcc or Xca strains and were not tested for pathogenicity in this first evaluation. All presumptive xanthomonads that were negative by ELISA for all Xcc/Xca-specific MAbs (X9, X13, and X17) were re-assayed, and if still negative, were tested for pathogenicity by spray inoculations, along with two positive controls. All presumptive xanthomonads recovered from crucifers with atypical symptoms or from weeds or lettuce were also tested for pathogenicity by spray inoculations.

Generation and selection of new MAbs. Two strains that produced distinctive symptoms on crucifers and that also were negative for all of the previously generated Xcc/Xca-specific MAbs were selected for generation of new MAbs. Strain G2-12 produced severe blight symptoms on cabbage seedlings, and strain G3-27 produced leaf spot, characteristic of Xca. A third strain from North Carolina (XC114) was selected because of its aggressive spread in field experiments (43). BALB/c mice were individually immunized with suspensions containing washed, formalin-killed cells of these strains at approximately 1×10^8 cfu/ml. Antibody-secreting hybridomas were successively screened, first with a panel of six and then with 12 known Xcc and Xca strains (1). After the selection of desired clones, hybridomas were recloned, and culture supernatants were screened for reactivity with 89 Xcc strains from Hawaii, 266 Xcc strains from Georgia, 21 Xcc strains from California, and 11 Xcc strains from Thailand (2). The same hybridomas were also screened using 103 strains from 21 other pathovars of *X. campestris*, 14 strains of *X. oryzae*, three strains of *X. albilineans*, and 84 strains of 17 other genera of bacterial plant pathogens and nonpathogens (2,4). Ascitic fluids were prepared for selected antibodies as described (1).

Second evaluation of MAbs. MAbs were evaluated with 329 strains obtained from crucifer seeds during routine seed assays and provided to us by researchers from seed companies (Table 1). Aliquots (100 μl) prepared from contaminated seed extracts were placed directly into duplicate wells of a microtiter plate and tested by ELISA using *Xanthomonas*-specific MAbs X1 and X11 (2). Colonies were simultaneously isolated from the seed extracts by dilution streaking onto modified TZC and FS media. Saprophytes and presumptive xanthomonads were cultured for pathogenicity testing by notch inoculation and serotyping. Each strain was transferred to YGA medium and reacted with a panel of MAbs to determine the serotype with a panel of six Xcc/Xca-specific MAbs (X9, X13, X17, X21, A11, and B35) plus X1 and X11. Plants with questionable reactions were reevaluated by spray inoculation as described earlier.

Suspected *X. maltophilia* strains were tested for the following determinative tests (8,20,35,37): gelatinase; starch hydrolysis; nitrate reduction; esculin hydrolysis; cytochrome C oxidase; oxidation/fermentation; acid from glucose, lactose, and salicin; and accumulation of β-hydroxybutyrate. Growth at 37 C; growth on 0.1% TZC; growth requirement for methionine; use of asparagine as sole carbon and nitrogen sources; and growth on glucose, fructose, sucrose, trehalose, maltose, and cellobiose were also tested.

Third evaluation of MAbs. Thirty-two presumptive Xcc or Xca strains from the first and second evaluations plus 99 additional presumptive or known *Xanthomonas* strains isolated from seed samples in California, Florida, Georgia, Hawaii, Louisiana, North Carolina, Wisconsin, East Asia, Nepal, Panama, and the United Kingdom (Table 1) were tested with the six Xcc/Xca-specific MAbs plus X1 and X11. MAb reactivity patterns were compared with pathogenicity tests of each strain following plant inoculation using spray and notch-inoculation methods. Sixty-seven strains were then retested and rated for virulence using the excised cotyledon assay.

DNA manipulations and similarity coefficients. Procedures for extraction of DNA from bacterial cell cultures, digestion with restriction endonucleases (*Eco*RI and *Bam*HI), agarose gel electrophoresis, Southern blotting on nylon membranes, hybridization under high-stringency conditions, and autoradiographs were as described (13). Three probes were used: pUFT-1 (formerly Xct-1), pUFT-11, and pUFA-704 (14). Autoradiographs were scanned with a Gilford Response II spectrophotometer (Gilford Instru-

ments, Oberlin, OH) equipped with an autoradiograph scanner. Comparative data for each probe/enzyme combination were totaled, and the similarity coefficient between two strains was calculated using described methods (13). Cluster analysis by the unweighted average pair group method (34) was performed using the Clustan program (42).

RESULTS

Black rot, blight, and leaf spot of crucifers. Some strains of Xcc caused typical systemic black rot symptoms, and others caused extensive necrosis of leaf tissue more characteristic of a blight (Fig. 1). Blight symptoms on cabbage were characterized by necrosis and a sudden collapse of large areas of leaf mesophyll, usually in advance of black vein symptoms. In spray inoculations, blight symptoms developed 1–2 wk after hydathode infection. Symptoms of blight resembled the sudden tissue collapse that occurs when a pathogen is infiltrated into a large region of the mesophyll of a nonhost plant (i.e., a hypersensitive response), except that the initial onset of symptoms was considerably delayed. Once symptoms began, however, the foliar necrosis was quite rapid, and symptoms appeared to be more like a plant response to mesophyll invasion than a plant response to a xylem-invading pathogen like Xcc. Blighted regions were typically tan colored, with a distinct border initially defining regions of diseased and healthy tissue. A few days later, the vascular tissues in the healthy regions of blighted leaves darkened to form black veins, and leaves eventually developed symptoms typical of black rot.

The blight symptom was sometimes preceded by the development of pinpoint water-soaked spots around stomata. The transient pinpoint water-soaked spots induced by these strains was easily distinguished from the necrotic leaf spot induced by strains of Xca. Some severe black rot strains also induced pinpoint water-soaking symptoms on seedlings prior to development of black rot. The blight symptoms could not be distinguished from severe black rot either by spraying seedlings or by the seedling bioassay. However, following spray inoculation of larger plants (at least six true leaves from 5 to 10 cm long) the blight strains produced broad expanding necrotic lesions and mesophyll collapse, characteristic of the blight symptoms first observed in the field. In contrast, with Xca strains there was no observable variation in symptoms due to age of leaves at the time of inoculation.

Fig. 1. Cabbage plants exhibiting symptoms of blight 2 wk after spray inoculation with *Xanthomonas campestris* pv. *campestris* G2-12.

First evaluation of MAbs and isolation of Xcc and Xca strains from leaves. The *Xanthomonas*-specific MAb X1 reacted with all 352 raised, mucoid colonies that hydrolyzed starch on FS medium and had characteristic morphology and pigmentation of *Xanthomonas* on TZC and YDC media. Of these, 348 strains (98%) reacted with MAb X11; and 326 (92%) reacted with previously identified Xcc/Xca-specific MAbs X9, X13, and X17.

All 26 *Xanthomonas* strains that failed to react with MAbs X9, X13, and X17 gave mild to severe pathogenic reactions on crucifers using both spray and notched-leaf inoculation assays. Two of these nonreactive strains (G2-12 and G2-17) produced a rapid necrosis or blight of leaf tissue (Fig 1). Both strains were isolated from patches of tan colored necrotic crucifer leaf tissue. Thirteen of the 26 nonreactive strains induced typical black rot symptoms. Two nonreactive strains produced necrotic lesions on cabbage plants typical of leaf spot caused by Xca (6). The remaining nine nonreactive strains produced only a blackening of the hydathodes on notch inoculation.

Three strains isolated from mildly necrotic or symptomless leaves of lettuce growing in a field bordering infected cabbage plants showed MAb reactivity patterns characteristic of Xcc (strong reactions with X9 and X17). These strains produced black rot when inoculated on cabbage seedlings but produced only minimal necrosis or no symptoms on lettuce. In contrast, the remaining 61 strains isolated from necrotic or water-soaked lesions of lettuce reacted only with genus-specific MAbs X1 and X11 and four non-pathovar-specific MAbs, X2, X3, X4, and X16. These strains produced water-soaked marginal lesions on lettuce characteristic of *X. campestris* pv. *vitians* but produced no symptoms on cabbage. The four non-pathovar-specific MAbs separated the Xcc strains into several subgroups. They also showed differential reactions with presumptive *X. c. vitians* and Xca strains, indicating that these pathovars also are serologically heterogeneous.

Generation and selection of new MAbs. Since not all of the Xcc and Xca strains reacted with Xcc/Xca-specific MAbs X9, X13, and X17, and none of these MAbs separated Xcc from Xca, new antibodies were generated in an attempt to overcome these limitations. Three antibodies were selected that reacted with strains of Xcc and Xca, but with no other pathovars, species, or genera tested. MAb X21 (clone 88.45.1.3, isotype IgG_1) reacted with most strains of Xcc and a few Xca strains. MAb A11 (clone W10.A11.2.1, isotype IgG_3) reacted only with blight strains of Xcc, including the two that failed to react with any other Xcc/Xca-specific MAbs. MAb B35 (clone W11.B35.3.1, isotype IgG_3) reacted with nearly all of the Xca strains.

Second evaluation of MAbs and isolation of Xcc and Xca strains from seeds. Independent pathogenicity and serological tests were conducted on all 329 strains isolated from seeds (Table 2). Genus-specific MAbs X1 and X11 reacted with 107/329 samples from seed extracts. Of these 107 presumptive xanthomonads, 85 strains had colony morphologies typical of Xcc, and 83 of these strains were pathogenic. The 22 strains with atypical colony morphologies were subjected to further bacteriological tests (see below). Eighty-four of 85 strains with typical Xcc colony morphology reacted with the Xcc/Xca-specific MAbs, and two of the 84 (2%) were nonpathogenic; one of the 85 presumptive xanthomonads (1%) did not react with these MAbs but gave a weakly virulent reaction on cabbage. Therefore, the six Xcc/Xca MAbs gave approximately 2% false positive and 1% false negative reactions in these tests of Xcc and Xca strains from seeds. MAbs A11 and X13 were Xcc-specific, but they did not react with a majority of the Xcc strains. No individual MAbs reacted with all Xcc, Xca, or Xcc/Xca strains; but a reaction with two or more MAbs was characteristic of typical black rot strains. Eight strains that reacted with only one MAb (usually X17) were weakly virulent or gave no reaction. Although these MAbs distinguished the Xcc/Xca strains from other bacteria tested, no MAb combinations were found that distinguished the two pathovars from each other.

Twenty-two of the 107 X1/X11-positive colonies from cabbage and broccoli seed were identified as *Xanthomonas maltophilia* (*Stenotrophomonas maltophilia* nom. nov. Palleroni & Bradbury) by fatty acid analysis (M. Sasser, *personal communication*) and/

or determinative biochemical tests. None of the *X. maltophilia* strains reacted with any of the Xcc/Xca-specific MAbs. From the remaining 222 (X1/X11-negative) samples, a variety of flat or mucoid, light red to pink colonies were isolated from TZC medium, and none of these reacted with the Xcc/Xca-specific MAbs. One of these 222 isolates caused mild blackening of veins on spray and notch inoculation; the rest were nonpathogenic.

Third evaluation of MAbs. Of 131 *Xanthomonas* strains tested, 121 reacted with one or more Xcc/Xca-specific MAbs, and 116 strains were pathogenic by spray inoculation and/or were rated virulent in the seedling bioassay (Table 3). Three major MAb reactivity patterns were observed; and on this basis, strains were separated into three serological groups. The first group of 84 strains was defined by a positive reaction with MAb X9. Most of these strains had positive reactions to X21 and X17, and some to MAb B35. Both Xcc and Xca strains were in this group, as well as 5/84 (6%) of xanthomonads that were not pathogenic to crucifers. The second group of five strains was defined by a strong positive reaction with MAb X13; these strains also reacted to X21 and X17. This group included only Xcc strains that caused typical black rot. The third group of 10 strains was defined by a positive reaction with MAb A11 and a negative reaction with X21. This group included only Xcc strains that caused blight. Twenty-one of the 22 strains that reacted with only one MAb (X17, X21, or B35) were either Xca strains or Xcc strains that were weakly virulent in the seedling bioassay (Table 3). One strain in this last group was nonpathogenic. A total of 6/121 strains that reacted with the Xcc/Xca-specific MAbs was nonpathogenic, yielding 5% false positive Xcc/Xca MAb reactions. Ten *Xanthomonas* strains failed to react with any of the Xcc/Xca-specific MAbs, and one of these was slightly pathogenic to cabbage; therefore, 1/116 strains pathogenic to cabbage did not react with any of the Xcc/Xca-specific Mabs, yielding 0.9% false negative Xcc/Xca MAb reactions.

Following numerous attempts to produce antibodies that could distinguish Xcc from Xca strains, no suitable antibodies were found. Xca strains showed very weak to no reactions with MAbs X13 and A11, but several gave strong reactions with MAbs X9 and X21, which also reacted with a majority of Xcc strains tested. MAb B35 gave strong reactions with most Xca strains and weaker to no reaction with most Xcc strains. Strains isolated from radish and identified as *X. campestris* pv. *raphani* were not distinguished from known Xca strains by MAb or by pathogenicity tests. By contrast with Xcc, all known Xca strains and strains identified as *X. c. raphani* produced a distinctive necrotic region that girdled the stem of cabbage seedlings at the inoculation point, and the seedling collapsed 4–7 days after inoculation. All of the strains that produced necrotic stem girdling caused necrotic leaf spots on cabbage seedlings when spray inoculated. When notch inoculated, these strains produced darkened areas restricted to the inoculation site.

The two *X. c. aberrans* strains tested reacted with MAb X9, and the *X. c. incanae* strain reacted with MAb B35. Both *X. c. aberrans* strains produced blackened areas around the hydathodes of cabbage leaves following spray inoculation. The two *X. c. aberrans* strains were distinguished from Xcc and Xca strains by failure to react with more than MAb X9 alone (refer to Table 3). The sole extant *X. c. incanae* strain (PDCC574) of which we are aware was not distinguished from some weakly virulent Xca and Xcc strains and produced no symptoms on cabbage.

Characteristics of strains selected for genetic analysis. Nineteen representative strains were selected for genetic studies based on the MAb reactivity patterns and the symptoms they induced on cabbage. Three typical black rot strains (A249, PHW-117, and H-M) represented strains from Hawaii, Louisiana, and Wisconsin, respectively. Two additional Hawaiian strains (GAC-17 and GAC-20) were initially isolated from cabbage seedlings that showed only mild chlorosis of primary leaves. On reinoculation into cabbage, they produced typical black rot symptoms. One Hawaiian strain (GAC-137) represented a group of three unusual strains that were isolated from mildly necrotic leaves of lettuce but had serotypes characteristic of Xcc and produced black rot on cabbage in pathogenicity tests. Strains XC-114 and X3 from North Carolina and Florida, respectively, represented a group of strains that were serologically distinguished by positive reactions with MAb X13. Three Hawaiian strains (G2-12, G2-17, and G3-38A) were selected because they were initially isolated from cabbage leaves showing unusual tan necrotic patches resembling mesophyll collapse. On reinoculation into cabbage, two of these strains (G2-12 and G2-17) consistently reproduced the mesophyll collapse or blightlike symptoms on cabbage plants. These strains were A11-positive and X21-negative, whereas strain G3-38A showed the reverse MAb reactivity pattern and produced typical black rot. Xca strains from Hawaii (A342 and G3-27) were isolated from broccoli and cabbage plants, respectively, having a range of black rot symptoms as well as necrotic leaf spots. These strains were associated with blackened necrotic tissues around veins and hydathodes. On

TABLE 2. Reactivity patterns of eight monoclonal antibodies (MAbs) with 329 strains isolated from crucifer seed

Reactivity with MAbs		Strains (no.)	Plant reaction in pathogenicity tests[b]
Genus-specific	Xcc/Xca-specific[a]		
X1, X11	X9, X17, X21, B35	1	Typical black rot
X1, X11	X9, X17, X21, B35	3	Leaf spot
X1, X11	X9, X17, X21	58	Typical black rot
X1, X11	X9, X17, X21	3	Black necrotic lesions, weakly virulent
X1, X11	X13, X17, X21	4	Typical black rot
X1, X11	A11, X17	5	Severe necrosis, blight
X1, X11	X21	1	Black necrotic lesions, weakly virulent
X1, X11	X17	7	Black necrotic lesions, weakly virulent
X1, X11	X17	2	No reaction
X1, X11	All negative	1	Black veins[c]
X1, X11	All negative	22	No reaction[d]
Both negative	All negative	222	No reaction
Total		329	

[a]Xcc = *Xanthomonas campestris* pv. *campestris*; Xca = *X. campestris* pv. *armoraciae*.
[b]Cabbage plants were first tested by notch inoculation; plants with questionable symptoms were retested by spray inoculation.
[c]Colonies identified as *X. campestris* by biochemical tests.
[d]Colonies identified by biochemical tests and fatty acid analysis as *X. maltophilia* (renamed *Stenotrophomonas maltophilia*; 33).

TABLE 3. Pathogenicity tests and reactivity patterns using Xcc/Xca-specific MAbs on 131 presumptive or known *X. campestris* pv. *campestris* (Xcc) and *X. campestris* pv. *armoraciae* (Xca) strains isolated from crucifer seeds

	Reactions with two or more MAbs		Reaction with one MAb		
	X9 and X17, X21 and/or B35	X13, X17, and X21	All	X17, X21, or B35	No MAb reaction
Total strains reacting	84	5	10	22	10
Strains pathogenic by spray inoculation	79[a]	5	10[b]	21[c]	1
Average virulence index (Xca strains not included)	6.8 (±1.3)	7.2 (± 0.9)	7.5 (± 0.6)	1.8 (± 0.6)	0.2 (± 0.4)

[a]Twelve strains reacted with B35 in addition to MAbs X9 and X17; nine of these gave leaf spot, three gave black rot.
[b]Six strains also reacted with X17; all A11 positive strains, including Xcc528[T], caused blight symptoms.
[c]Eleven strains reacted only with B35, nine with X17, one with X21; plants reactions were atypical and weakly virulent.

inoculation into cabbage seedlings, these strains and two from Asia (756 and 417) produced necrotic stem girdling; and when sprayed onto foliage, they produced the typical leaf spot symptoms. The other Xca strains (XLS2, XLS6, and XLS10) were isolated in Louisiana from young cabbage plants showing characteristic leaf spot symptoms. They all produced leaf spot on spray inoculation, darkened areas around the inoculation site on notch inoculation, and necrotic stem girdling in the excised-cotyledon seedling assay.

Comparison of MAb reactivity patterns with RFLP patterns. The RFLP patterns for strains of both pathovars are shown in Figure 2, except for strains Xcc528^T and G2-17 of Xcc, which were examined on separate blots and only in comparisons with G2-12, X3, and GAC17. Visual inspection of the RFLP patterns revealed that there were distinctly different clusters or groups of strains within Xcc. Furthermore, although some bands appeared conserved within each pathovar, some of these same bands were also common to both pathovars, indicating that these two pathovars were phylogenetically close. Similarity coefficients of the combined data from all strains are presented in Table 4. A cluster analysis using the combined data from Table 4 revealed a high level of heterogeneity between and within both pathovars (Fig. 3). Strains within Xcc were heterogeneous but formed distinct clusters that were more related to each other than to strains of Xca. Strains of Xca also were heterogeneous but were distinguished as a group from Xcc at RFLP similarity levels of approximately 40% (Fig. 3). Of the seven Xca strains tested, only XLS2 and XLS6 appeared to be clonally related. Despite the high level of heterogeneity observed within Xca, all seven strains tested induced identical symptoms.

The 12 Xcc strains formed three identifiable RFLP groups, designated C1, C2, and C3; and these corresponded to serogroups S1, S2, and S3, identified by MAbs X9, X13, and A11, respectively (Table 5). The type strain of the species *X. campestris,* Xcc528^T, was in the C3 group, reacted with MAb A11, and produced a blightlike symptom on cabbage plants.

DISCUSSION

In previous studies, we described six serogroups of Xcc based on classification with three Xcc/Xca-specific MAbs and seven less specific MAbs (2). In the current analysis of a much larger collection of strains, we again encountered examples of all six serogroups. However, by ignoring differences produced by nonspecific MAbs, we have redefined the groups based solely on reactivity with six Xcc/Xca-specific MAbs and have thereby

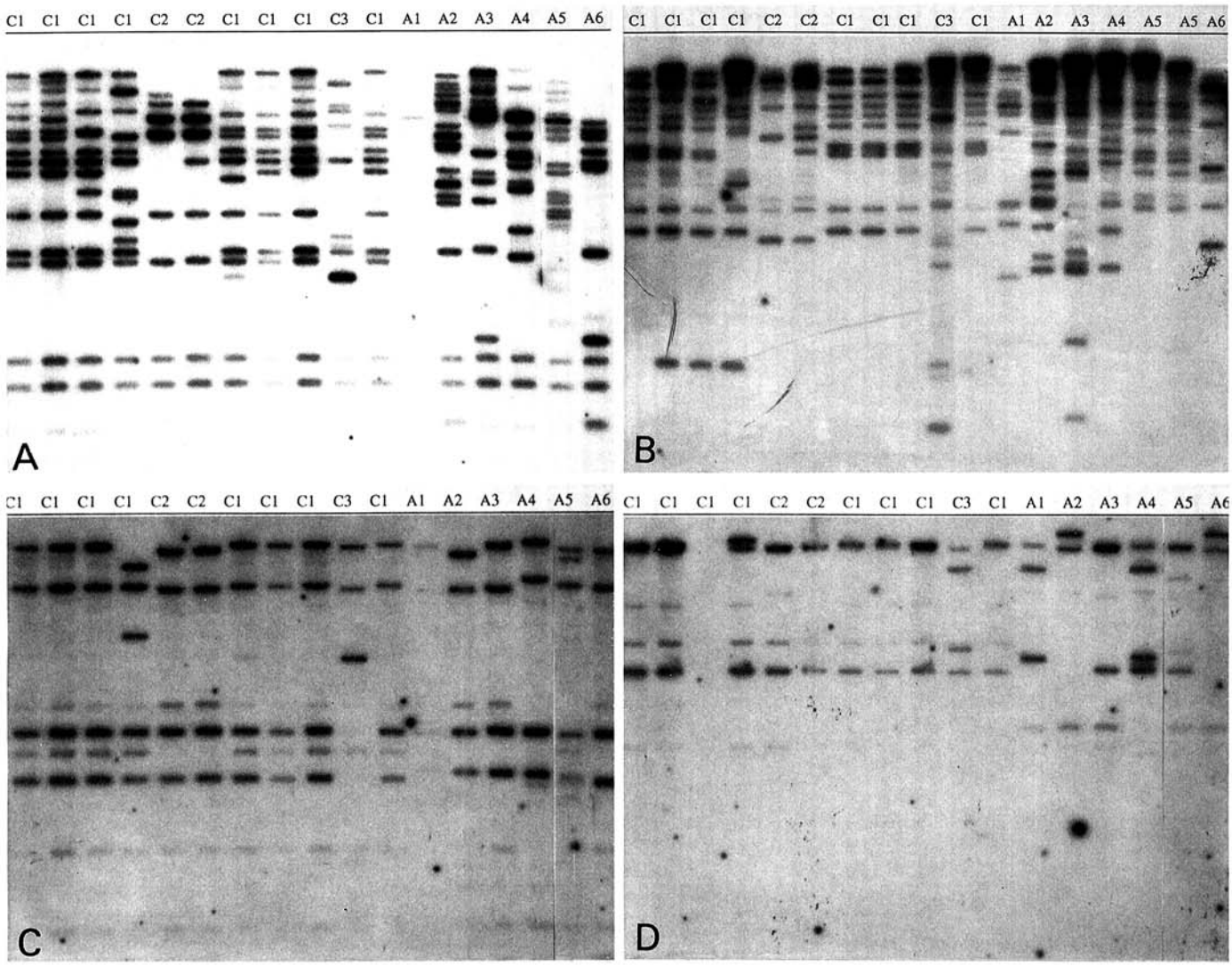

Fig. 2. Restriction fragment length polymorphism (RFLP) analyses using total DNA of strains of *Xanthomonas campestris* pv. *campestris* and *X. campestris* pv. *armoraciae.* DNA in blots **A and C** were digested with *Eco*RI; DNA in blots **B and D** were digested with *Bam*HI. DNA in blots **A and B** were probed with pUFT-1; DNA in blots **C and D** were probed with pUFT-11 and pUFA-704. From left to right, strains in **B** are: H-M, PHW-117, GAC-137, A249, X3, XC114, GAC-20, GAC-17, G3-38A, G2-12, GAC-17, A342, G3-27, 417, 756, XLS2, XLS6, and XLS10. Strain order in **A, C, and D** is the same, except that strain XLS2 is missing, and in **D** only, GAC-137 is missing. The labels at the top of each lane indicates the RFLP group.

reduced the six original groups to three. Group S1 comprises those strains that showed strong reactions with MAb X9 and earlier had been divided into four groups based on reactions with non-pathovar-specific MAbs (X3, X4, and X15) (2). Group S2 comprises all strains that react with X13 (equivalent to previous group 5). Group S3 is a new group based on reactivity with the recently generated MAb A11. The original group 6 was eliminated because it comprises only 2% of the Xcc strains that reacted with the genus-specific MAb X1, but failed to react with the genus-specific MAb X11. The new MAb X21 detects a common epitope shared by the majority of the Xcc strains tested. Only strains in group S3 (defined by positive reactions with MAb A11) lacked this epitope.

The non-pathovar-specific MAbs detected essential differences in closely related wild-type strains and were useful for tracing such strains in epidemiological studies (43). However, for rapid identification of the black rot/leaf spot pathogens, a mixture of Xcc/Xca-specific MAbs would suffice. If the distinction between Xcc and Xca is critical, final identification would depend on pathogenicity tests or genetic analysis.

Analysis of RFLP patterns of 19 representative strains confirmed that both pathovars were genetically heterogeneous. The groups formed by RFLP analysis corresponded well to the groups formed by serological and pathogenicity tests. All strains in RFLP group C1 were included in serogroup S1 and caused typical black rot symptoms. Serogroup S1 also included nine Xca strains,

TABLE 4. Similarity coefficients of the combined data from Figure 2

	Xanthomonas campestris pv. *campestris* similarity group[a]												*X. campestris* pv. *armoraciae* similarity group						
Strain	C1 H-M	C1 PHW-117	C1 GAC-137	C1 A249	C2 X3	C2 XC-114	C1 GAC-20	C1 GAC-17	C1 G3-38A	C3 G2-12	C3 G2-17	C3 Xcc528	A1 A342	A2 G3-27	A3 417	A4 756	A5 XLS2	A5 XLS6	A6 XLS10
H-M	...	*97*	(*96*)	*77*	52	59	*100*	*100*	*100*	40	(ND)	(ND)	32	26	33	32	(47)	41	40
PHW-117	...	...	(*93*)	*78*	60	57	*89*	*94*	*91*	40	(ND)	(ND)	31	22	34	30	(50)	44	37
GAC-137	...	...	...	(*81*)	(42)	(48)	(*85*)	(*82*)	(*84*)	(39)	(ND)	(ND)	(45)	(26)	(37)	(25)	(57)	(41)	(39)
A249	...	...	...	...	49	53	*79*	*76*	*76*	39	(ND)	(ND)	36	35	41	49	(62)	59	36
X3	...	...	...	...	...	*86*	56	47	56	33	(ND)	(ND)	41	49	50	37	(45)	46	34
XC-114	...	...	...	...	...	...	60	60	61	39	(ND)	(ND)	34	40	44	36	(38)	52	53
GAC-20	...	...	...	...	...	...	...	*97*	*94*	41	(ND)	(ND)	36	23	37	30	(47)	47	41
GAC-17	...	...	...	...	...	...	...	...	*100*	39	(ND)	(ND)	41	33	39	32	(47)	49	44
G3-38A	...	...	...	...	...	...	...	...	...	37	(ND)	(ND)	41	31	37	36	(47)	51	38
G2-12	...	...	...	...	...	...	...	...	...	...	(*93*)	(*71*)	35	36	43	38	(40)	29	32
G2-17	...	...	...	...	...	...	...	...	...	...	...	(*74*)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)
Xcc528	...	...	...	...	...	...	...	...	...	...	...	...	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)
A342	...	...	...	...	...	...	...	...	...	...	...	...	...	40	42	49	(58)	56	40
G3-27	...	...	...	...	...	...	...	...	...	...	...	...	...	...	56	38	(44)	46	46
417	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45	(54)	64	45
756	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	(62)	52	33
XLS2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	(*92*)	(27)
XLS6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	62

[a]Restriction fragment length polymorphism similarity groups were determined by including strains with ≥ 70% similarity, indicating a high degree of phylogenetic relatedness. Parentheses indicate partial data set. ND = not determined.

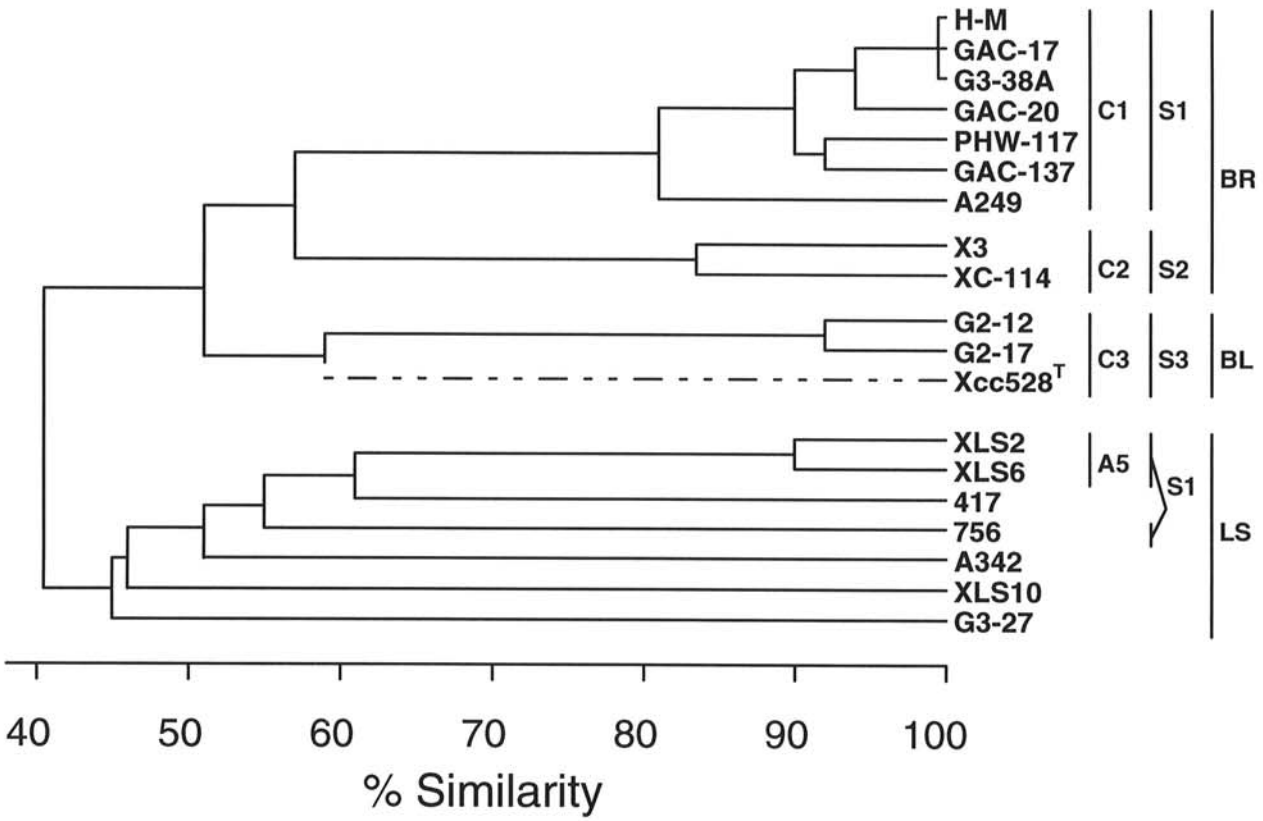

Fig. 3. Dendrogram calculated on the basis of the data in Table 4, including data not shown of comparisons of G2-17 and *Xanthomonas campestris* pv. *campestris* 528^T. First set of vertical lines at right of dendrogram indicate restriction fragment length polymorphism (RFLP) groups C1, C2, C3, and A5; second set of vertical lines indicate serological groups S1, S2, and S3; and third set of vertical lines indicate pathogenicity groups: BR = black rot, BL = blight, and LS = leaf spot. Dashed line indicates similarity comparisons were based on limited strain comparisons.

including 756, XLS2, and XLS6; therefore, the epitope recognized by MAb X9 was not pathovar-specific.

The two RFLP group C2 strains exhibited more severe symptoms than any of the strains in the C1 group. Both strains were in serogroup 2 and reacted with MAb X13. However, both in this study and in an earlier study (2), not all strains that reacted with MAb X13 were measurably more aggressive than strains that reacted with MAb X9. Therefore, the epitope recognized by MAb X13 is probably not associated with a virulence factor. Although the two strains appear clonally related, more comparative work with a larger number of strains is needed to determine if there is any epidemiological significance to RFLP group C1 and C2 strains.

The three RFLP group C3 strains were all in serogroup S3, which includes only A11-positive, X21-negative strains. Most serogroup S3 strains produced distinctive blightlike symptoms during early stages of black rot; and as such, these strains appear to be an aggressive variant of Xcc. The mesophyll collapse characteristic of blight was clearly distinguished from the leaf spots caused by Xca, and MAb A11 did not react with any of the latter strains tested. A single 5.4-kb DNA fragment cloned from Xcc528 was recently found to confer partial blight symptoms to Xca strains; this DNA fragment hybridizes only to the C3 group strains and was not found in any other *Xanthomonas* strain tested (9). The three strains able to cause blight therefore are associated with a specific epitope and a disease-specific virulence factor (blight) cloned on a 5.4-kb DNA insert. However, it is unlikely that the epitope recognized by MAb A11 is encoded by or affected by genes on the 5.4-kb DNA fragment. Rather, the epitope and the blight gene(s) are likely to remain clonally linked due to a lack of recombination (linkage disequilibrium). Low rates of recombination have been reported or deduced in many microbial pathogens (40), including plant pathogens (12).

The conservation of epitopes and DNA fragments within two different pathovars of crucifers obscured the apparent distinctions between Xcc and Xca that were based on pathogenicity tests. The fact that these two pathovars share common hosts, epitopes, and RFLP markers that distinguish these two pathovars from all other *Xanthomonas* strains may indicate that strains of these two pathovars may be more closely related to each other than to strains of other pathovars currently placed in species *X. campestris*. This idea is consistent with the known phylogenetic distance of *X. campestris* Xcc528^T from many other members currently placed in the same species (15,19,32,36). Many pathovars are in fact highly clonal groups of strains (14), and representative strains from these groups are sufficiently phylogenetically distinct from Xcc528^T to warrant separation from the species defined by this type strain.

Logically, the subgroup of *X. campestris* strains that cause blight on crucifers represent a pathogenic variant that might be used to name a new pathovar (refer 10). However, separation of blight strains into a separate pathovar of *X. campestris* might be confusing at this time, since there is only inconsistent application of any standard for naming pathovars, and even the concept of the species *X. campestris* is controversial (for example, refer 36). For example, we found no basis in our serological, genetic, and pathogenicity studies for the separation of *X. c. raphani* strains from *X. c. armoraciae*. In agreement with Black and Machmud (6), the data generated in this study confirm that these pathovars are synonymous. The pathogenic variant name *armoraciae* (30) evidently has priority over *raphani* (39).

We are aware of only a single extant *X. c. incanae* strain, PDDC574. The bacterium was described as a pathological variant of *Phytomonas campestris* Kendrick & Baker (23). The pathogen caused severe stem lesions and stem girdling of *Methiola incana* (garden stocks) but was not pathogenic to cabbage. PDDC574 reacted with MAb B35, which reacted with most Xca strains. PDDC574 may therefore be closely related to Xca, which also causes stem lesions and girdling, but of cabbage. Additional strains of *X. c. incanae* are needed to investigate this potential relationship.

In this study, the two *X. c. aberrans* strains were found to be related to some Xcc/Xca strains, but were also distinguished from all Xcc/Xca strains used. It is possible that strains of *X. c. aberrans* are low-virulence mutants of Xcc or Xca. Although *X. c. aberrans* strains are true pathogenic variants (and therefore merit independent pathovar status), such weakly virulent strains are often found in association with outbreaks of black rot and may be incapable of spread on crucifers in the field. Field studies would be required to answer this question definitively.

For practical purposes, panels of MAbs provide a simple diagnostic tool that is well-suited for rapid identification of large numbers of strains in field experiments and seed assays. Despite the genetic heterogeneity found among the Xcc and Xca strains, an MAb panel can be reliably used if the distinction between Xcc and Xca is not critical to the problem at hand. Clearly, the lack of clonality within these two pathovars may complicate application of diagnostic DNA-based tools, such as DNA probes or primers. On the other hand, the use of the polymerase chain

TABLE 5. Reactivity of monoclonal antibodies (MAbs) with selected strains of *Xanthomonas campestris* pv. *campestris* (Xcc) and *X. campestris* pv. *armoraciae* (Xca) and grouped according to serology restriction fragment length polymorphisms (RFLPs) and symptoms on cabbage

Strain	MAbs						Sero group	RFLP group	Symptom type[a]
	X9	X13	X17	X21	A11	B35			
Xcc A249	3[b]	0	3	3	0	1	S1	C1	BR
Xcc PHW-117	3	0	0	3	0	1	S1	C1	BR
Xcc H-M	3	0	0	3	0	1	S1	C1	BR
Xcc GAC-17	3	0	3	3	0	1	S1	C1	BR
Xcc GAC-20	3	0	3	3	0	2	S1	C1	BR
Xcc G3-38A	3	0	3	3	0	2	S1	C1	BR
Xcc GAC-137	3	0	3	3	0	1	S1	C1	BR
Xcc XC-114	0	3	3	3	0	1	S2	C2	BR
Xcc X3	0	3	3	3	0	1	S2	C2	BR
Xcc G2-12	0	1	0	0	3	0	S3	C3	BL
Xcc G2-17	0	1	0	0	3	0	S3	C3	BL
Xcc 528^T	0	0	1	0	3	0	S3	C3	BL
Xca A342	0	1	0	3	0	3	...	A1	LS
Xca G3-27	0	1	0	1	0	3	...	A2	LS
Xca 417	0	0	1	0	0	3	...	A3	LS
Xca 756	3	1	3	3	0	3	S1	A4	LS
Xca XLS2	3	1	3	3	1	2	S1	A5	LS
Xca XLS6	3	1	2	3	0	2	S1	A5	LS
Xca XLS10	0	0	2	1	1	3	...	A6	LS

[a]BR = typical black rot; BL = blight; LS = leaf spot.
[b]Code for net ELISA above background: 3, > 0.201; 2, from 0.101 to 0.2; 1, from 0.51 to 0.1; 0, from 0 to 0.05. Readings of 0 and 1 were considered negative.

reaction has increased the sensitivity of DNA primers well beyond the sensitivity of antibody assays (29). As familiarity with DNA primer technology increases, the technique of choice may depend entirely on the level of sensitivity and specificity desired in the test.

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