Neth. J. Pl. Path. 98 (1992) 81-94

Specificity of polyclonal and monoclonal antibodies for the identification of Xanthomonas campestris pv. campestris

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Accepted 10 December 1991

Abstract

Polyclonal and monoclonal antibodies (PCAs and MCAs), produced to whole cells and flagellar extracts of *Xanthomonas campestris* pv. *campestris* (Xcc), respectively, were tested for specificity. In immunofluorescence microscopy (IF) the three PCAs tested, reacted at low dilutions with all Xcc strains, some other xanthomonads and non-xanthomonads. At higher dilutions most cross-reactivity with non-xanthomonad strains disappeared. However, the cross-reactivity with strains of *X. c.* pv. *vesicatoria* (Xcv), *X. c.* pv. *amoraciae* (Xca) and *X. c.* pv. *phaseoli* var. fuscans (Xcpf) remained.

Six MCA-producing cell clones viz. 20H6, 2F4, 18G12, 10C5, 17C12 and 16B5 were selected for specificity tests with an enzyme immunoassay (EIA), IF and a dot-blot immunoassay (DBI). None of the MCAs reacted with all Xcc strains in IF and EIA. In DBI, only MCAs 17C12 and 16B5 reacted with all Xcc strains. All six MCAs tested, cross-reacted in one of either tests with other pathovars of *X. campestris*, such as Xcv or Xca. The MCAs were also tested in immunoblotting experiments using total bacterial extracts, cell envelope and flagellar extracts. MCAs 20H6, 2F4, 18G12 and 10C5 reacted with the lipopolysaccharide (LPS) of Xcc. MCAs 16B5 and 17C12 reacted with a 39 kilodalton and a 29 kilodalton protein, respectively.

It is concluded that the PCAs and MCAs discussed in this study may be used for routine identification and differentiation of (a group of) Xcc strains. The significance of the cross-reactions with other pathovars of *X. campestris* needs to be determined by testing seed lots.

Additional keywords: black rot, serology, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), proteinase K.

Introduction

Xanthomonas campestris pv. *campestris*, the causal agent of black rot in crucifers, is generally considered the most important disease of crucifers in the world (Williams, 1980). The use of healthy seeds is an important way to prevent the black rot disease. Methods to detect this pathogen in seeds are therefore needed. Serological techniques are potentially very sensitive and specific for detection and identification of seed-borne bacteria. Therefore high-quality antibodies are needed. Both polyclonal and monoclonal antibodies (PCAs and MCAs, respectively) are used in seed bacteriology

although the application of monoclonal antibodies is still limited (Franken and Van Vuurde, 1990). MCAs for *X. c.* pv. *campestris* (Xcc) were developed by Alvarez et al. (1985). These antibodies were tested for application in a radio-immunoassay and an enzyme immunoassay (EIA), and for identification and grouping of strains (Alvarez et al., 1985; Alvarez and Lou, 1985; Yuen et al., 1987). Haaheim et al. (1989) produced monoclonal antibodies that reacted with the exopolysaccharide xanthan from *X. campestris*.

The aim of this study was to evaluate the specificity of PCAs and MCAs, produced in our laboratory against Xcc.

Material and methods

Organisms and growth media. The strains employed in this study are listed in Table 1. All bacteria were cultured on a solid growth factor (GF) medium, unless otherwise stated, containing per l: 0.4 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.5 g $NH_4H_2PO_4$, 0.01 g $FeCl_3$, 1.0 g glucose, 3.0 g yeast extract and 15.0 g Bacto-agar. All strains were grown at 27-28 °C.

Enzyme immunoassay (EIA). Polystyrene microplates were coated for 30 min at 21 °C with poly-L-lysine, 0.1 mg ml^{-1}, in carbonate buffer, pH 9.6 (Clark and Adams, 1977). Plates were washed 3 times with phosphate-buffered saline (PBS), containing 0.1 % Tween 20 (PBST). Antigens were untreated or boiled cells at a concentration of 10^7 cells ml^{-1} and bacterial extracts (10.0, 1.0 and 0.1 μg protein per ml in PBS). Plates were incubated with antigen overnight at 4 °C and washed 3 times with PBST. Blocking took place by incubation with PBST, containing 0.5% bovine serum albumin, for 30 min at 37 °C. Antibodies from hybridoma supernatants (undiluted in culture medium) were added and incubated for 3 h at 4 °C; PCAs were not tested. Plates were washed 3 times with PBST. Goat-anti-mouse alkaline phosphatase-conjugate (GAM-AP) was added in a 1:1000 dilution in PBST, containing 0.1 % albumin, and incubated for 2 h at 37 °C. Plates were then washed 3 times with PBST and subsequently incubated with enzyme substrate (0.75 mg ml^{-1} p-nitrophenyl phosphate in 10% diethanolamine buffer (v/v), pH 9.8) for 0.5-1 h at 37 °C. Absorbance values were read at 405 nm.

Dot-blot immunoassay (DBI) and immunoblotting. The procedure for DBI was as described for EIA, with some modifications. One μl of antigen suspension was spotted onto nitrocellulose membranes (Schleicher & Schüll, 0.45 μm). The membranes were dried, blocked with PBS containing 0.5 % horse serum, washed three times, probed with antibodies from hybridoma supernatants (PCAs were not tested) for 2 h at 21 °C, washed three times and incubated with GAM-AP for 1 h at 21 °C. The reaction was visualized with 5 mg nitroblue tetrazolium in 5 ml deionized water to which 0.75 ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) solution (4 mg ml^{-1} in ethanol/acetone solution, 2:1) was added, and which was totally dissolved in 0.1M ethanolamine-4mM $MgCl_2$ buffer, pH 9.6. Immunoblotting of SDS-PAGE gels was carried out as described by Boonekamp et al. (1990); blots were developed with antibodies, GAM-conjugate and BCIP as described above for DBI.

Table 1. Bacterial strains employed for quality-testing of monoclonal and polyclonal antibodies produced against *Xanthomonas campestris* pv. *campestris*.

Bacteria	Strain designation	Received from[1]
Clavibacter michiganensis subsp. *michiganensis*	Cmm 542	4
Clavibacter michiganensis subsp. *insidiosus*	Cmi 533	4
Curtobacterium flaccumfaciens subsp. *flaccumfaciens*	Cff 547	4
Erwinia herbicola	Eher 732, Eher 1062, Eher 1063,	4
	Eher 83	2
Pseudomonas aeruginosa	Paer 60	4
Pseudomonas marginalis	Pmar 86/1118	2
Pseudomonas viridiflava	Pvir 540	4
Pseudomonas cichorii	Pcich 478	2
Pseudomonas syringae pv. *lachrymans*	Psl 520	4
Pseudomonas syringae pv. *maculicola*	Psm 154, Psm 155	4
Pseudomonas syringae pv. *phaseolicola*	Psp 570, Psp 111	4
Pseudomonas syringae pv. *pisi*	Pspi 518, Pspi 519	4
Pseudomonas syringae pv. *syringae*	Pss 1147, Pss 126	4, 5
Xanthomonas campestris pv. *amoraciae*	Xca 373, Xca 374	4
Xanthomonas campestris pv. *carotae*	Xcar	11
Xanthomonas campestris pv. *campestris*	Xcc 102, Xcc 297, Xcc 298, Xcc 363, Xcc 364, Xcc 365, Xcc 366, Xcc 367, Xcc 368, Xcc 369, Xcc 370, Xcc 371, Xcc 372[2], Xcc 557, Xcc 558, Xcc 559, Xcc 634, Xcc 671,	4
	Xcc 8-6, Xcc 2-24, Xcc 2-48, Xcc 4-4, Xcc 9-48, Xcc 9-4, Xcc 9-3ON, Xcc 9-3OZ,	1
	Xcc 3-31-8, Xcc 2-9-2, Xcc 8-19-1,	9
	Xcc BT-7, Xcc BT-8,	10
	Xcc 46-6, Xcc 117-26, Xcc 824-1	8
	Xcc 46 Cop.	7
	Xcc SG 11, Xcc SG 57	6
Xanthomonas campestris pv. *phaseoli*	Xcp 375, Xcp 510	4
Xanthomonas campestris pv. *phaseoli* var. fuscans	Xcpf 382, Xcpf 482	4
Xanthomonas campestris pv. *vesicatoria*	Xcv 512, Xcv 523	4
Xanthomonas maltophilia	Xm 1974, Xm 1975	3
unidentified	saprophyte 457	1

[1] 1 = Authors; 2 = Plant Protection Service, the Netherlands; 3 = National Culture Collection for Plant Pathogenic Bacteria, England; 4 = Research Institute for Plant Protection, the Netherlands; 5 = B. Rat, France; 6 = C. van Henten, Netherlands; 7 = F. Vinther, Denmark; 8 = P.H. Williams, Wisconsin, USA; 9 = D.L. Pinnow, Georgia, USA; 10 = N. Thaveechai, Thailand; 11 = T.L. Kuan, Washington, USA.

[2] Later designated as *X. c.* pv. *aberrans*.

Immunofluorescence microscopy (IF). Indirect IF was carried out as described by Van Vuurde et al. (1983). Antibodies from hybridoma culture supernatants were used undiluted. Secondary goat-anti rabbit antibodies, to which fluorescein-isothiocyanate was conjugated (Sigma), were used at a 1:100 dilution.

Preparation of bacterial extracts for the production of MCAs and quality testing of MCAs and PCAs. Flagellar extracts were prepared from cells in the early stationary phase ($\pm$ 10^8-10^9 cells ml^{-1}), using the shearing and differential centrifugation methods essentially as described by Martin and Savage (1985). In this procedure flagella were separated from cell debris by two differential centrifugation steps at 5000 *g* and 15 000 *g*. The two residual fractions containing the cell debris were used to prepare cell envelope extracts. These fractions were resuspended in a 50mM Tris - 2mM EDTA buffer, pH 8.5 and pooled. The pooled suspension was placed on ice and repeatedly sonicated in short bursts on ice with a Killings apparatus. After sonic disruption, large fragments were removed by centrifugation at 2000 *g* for 20 min at 4 °C. The supernatant fluid was centrifuged at 230 000 *g* (at r_{max}) for 2 h at 4 °C and the pellet was resuspended in 5 ml PBS. This suspension was called the cell envelope extract.

Total bacterial extracts were prepared from cells in the early stationary phase, as described by Kiredjian et al. (1986). Cells were suspended in sample treatment buffer with sodium-dodecyl sulphate (see below) and heated for 10 min at 100 °C. The suspension was centrifuged for 5 min at 13 000 *g*. The supernatant fluid was called the total bacterial extract.

Proteinase K treatment of cell envelope, flagellar and total bacterial extracts was done as described by De Weger et al. (1987).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE using reducing conditions was done as described by Laemmli (1970). A 4% stacking gel and 12.5% separating gel were used. Samples containing 5 - 10 μg protein were diluted with an equal volume of a 2x sample buffer. Gels were run at 200 V, and used for electroblotting to nitrocellulose membranes, staining with 0.025% (w/v) Coomassie Brilliant Blue R or staining with silver for the lipopolysaccharide (Tsai and Frasch, 1982).

Production of antibodies. PCAs were produced by H. Vruggink, IPO-DLO (Research Institute for Plant Protection, Wageningen, the Netherlands), against whole living cells. Cells were washed twice in PBS and centrifuged for 10 min at 6000 *g* prior to immunization. PCA 94 was produced against Xcc strain 367, PCA 111 against Xcc strain 102, PCA 113 against Xcc strain 365.

For production of MCAs, BALB/c mice were immunized three times, intraperitoneally, with flagellar extracts of strain Xcc 367 (20 μg). The first immunization was in Freund's complete adjuvant and the second, ten to fourteen days later, in Freund's incomplete adjuvant. Fourteen days after the second immunization the titre was determined by EIA. If necessary a third immunization of antigen in PBS was given.

Fusion and culturing of hybrid cell lines was done as described by Boonekamp et al. (1990).

Selection of hybridoma lines. Hybridoma lines were first screened in EIA with cell

envelope extracts and flagellar extracts of Xcc strains 367, 671 and 102 and afterwards with whole cells of a selected number of bacterial strains (Xcc 102, Xcc 367, Xcc 671, Xcv 523, Xcpf 382, Xcp 375, Psm 154, Psp 570, Cmm 542 and saprophyte 457, Table 1). A third screening was done with selected MCAs in EIA, DBI and IF using a wider range of bacterial genera and other Xcc isolates. For this screening, cells were heated for 15 min at 100 °C, prior to testing by EIA and DBI, to diminish background reactions of some bacteria. Isotyping of the MCAs was carried out using the Biorad mouse sub-isotyping kit in EIA and agar double diffusion.

Results

Specificity of PCAs in IF. The specificity of PCAs was studied in IF using dilutions of 1:100, 1:300, 1:900, 1:2700 and 1:8100. Strains of Xcc, other pathovars of *X. campestris* and several non-xanthomonads (whole cells) were tested. Table 2 shows that at dilution 1:100, PCAs 94, 111 and 113 cross-reacted with other pathovars of *X. campestris* (pv. *vesicatoria* (Xcv), pv. *amoraciae* (Xca) and pv. *phaseoli* var. fuscans (Xcpf)). PCAs 111 and 113 also cross-reacted with some non-xanthomonads at this dilution. The cross-reacting non-xanthomonad strains were: Psm 154 and 155, Eher 1062 and 1063, Pcich 478 and saprophyte 457, isolated from cabbage seed. At dilutions 1:900 most of the cross-reactions disappeared, but saprophyte 457 still cross-reacted

Table 2. Reactions of polyclonal antibodies prepared against *Xanthomonas campestris* pv. *campestris* with strains of various bacterial genera in immunofluorescence microscopy.

Designation of polyclonal antibodies	Bacteria[1]	Number of strains positive / Number of strains tested at three dilutions		
		1:100	1:900	≥1:8100
94	Xcc	37/37	27/37	7/37
	Xc pvs.	5/6	3/6	0/6
	other	0/22	0/22	0/22
111	Xcc	37/37	20/37	0/37
	Xc pvs.	6/6	0/6	0/6
	other	3/22	0/22	0/22
113	Xcc	37/37	24/37	12/37
	Xc pvs.	5/6	2/6	0/6
	other	4/22	1/22	0/22

[1] Xcc = *Xanthomonas campestris* pv. *campestris*; Xc pvs. = other pathovars of *X. campestris*: *X. c.* pv. *phaseoli* (two strains), *X. c.* pv. *phaseoli* var. *fuscans* (one strain) and *X. c.* pv. *vesicatoria* (two strains) and *X. c.* pv. *amoraciae* (one strain). Other are non-xanthomonads: *Pseudomonas syringae* pv. *phaseolicola* (2 strains), *P. s.* pv. *syringae* (2 strains), *P. s.* pv. *maculicola* (2 strains), *P. s.* pv. *pisi* (2 strains), *P. s.* pv. *lachrymans* (1 strain), *P. aeruginosa* (1 strain), *P. viridiflava* (1 strain), *P. cichorii* (1 strain), *P. marginalis* (1 strain), *Erwinia herbicola* (3 strains), *Clavibacter michiganensis* subsp. *michiganensis* (1 strain), *Cl. m.* subsp. *insidiosus* (1 strain), *Curtobacterium flaccumfaciens* subsp. *flaccumfaciens* (1 strain), *X. maltophilia* (2 strains) and saprophyte 457.

with PCA 113. However, at dilutions of 1:900 and 1:8100 none of the PCAs reacted anymore with all Xcc strains tested. Some strains of other pathovars of *X. campestris* (Xcv, Xca) reacted as well as strains of Xcc in IF.

The homologous strains Xcc 102, 365 and 367 each reacted strongest with their homologous antiserum. Xcc 102 reacted up to a dilution of 1:2700 with PCA 111 and up to 1:300 with PCAs 113 and 94. Xcc 365 reacted up to a dilution of 1:8100 with PCA 113, whereas it reacted up to 1:100 and 1:2700 with PCAs 111 and 94, respectively. Xcc 367 reacted up to a dilution of 1:8100 with PCA 94, whereas it reacted up to 1:300 and 1:900 with PCAs 111 and 113, respectively. Only a few strains reacted equally well with all three PCAs. For example, Xcc 671 reacted well up to a 1:900 dilution with all three PCAs.

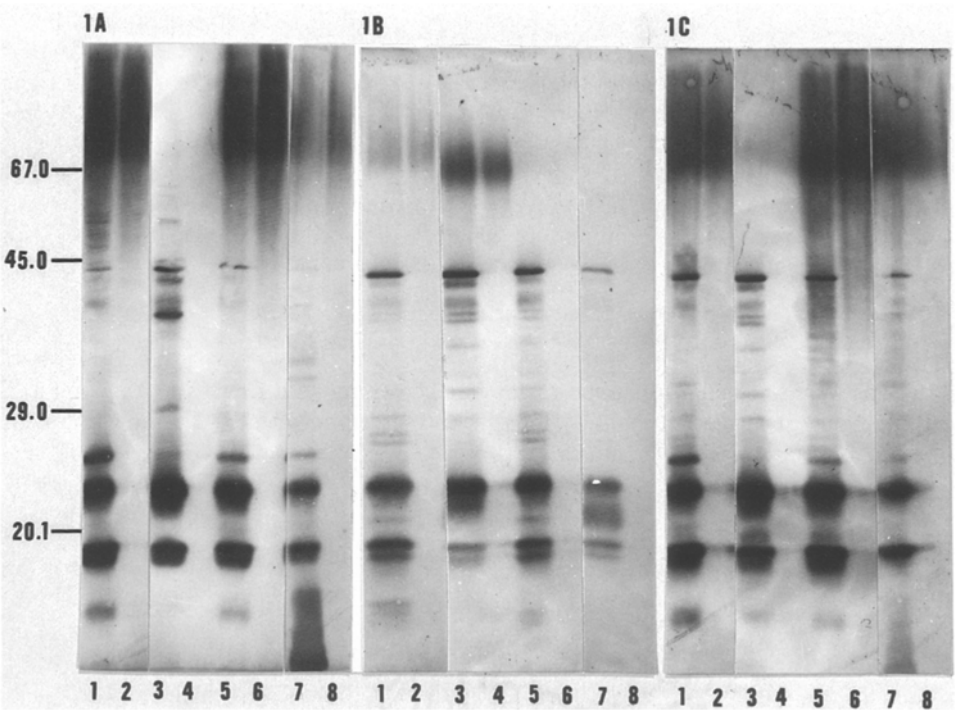

Fig. 1. Immunoblotting with polyclonal antibodies raised against *Xanthomonas campestris* pv. *campestris* to identify bands of cell envelope extracts, separated by SDS-PAGE.
A = immunoblot probed with PCA 94 (homologous strain Xcc 367).
B = immunoblot probed with PCA 111 (homologous strain Xcc 102).
C = immunoblot probed with PCA 113 (homologous strain Xcc 365).
Lanes 1,2: Cell envelope extracts of Xcc 367.
Lanes 3,4: Cell envelope extracts of Xcc 102.
Lanes 5,6: Cell envelope extracts of Xcc 365.
Lanes 7,8: Cell envelope extracts of saprophyte 457.
Uneven lanes: cell envelope extracts not treated with proteinase K.
Even lanes: cell envelope extracts treated with proteinase K.
Relative molecular masses (kilodaltons) of standard proteins are indicated to the left of the figures.

Reaction of PCAs in immunoblotting. The reactions of PCAs 94, 111 and 113 with antigens in cell envelope extracts of several Xcc strains and saprophyte 457 was studied by SDS-PAGE and immunoblotting (Fig. 1). The 44, 39, 25, 23, 18 and 17 kilodalton (kDa) bands of several Xcc strains reacted with the PCAs tested. Each PCA reacted strongest with antigenic components of the homologous strain in the region above 67 kDa. This smear was assumed to be the LPS since proteinase K treatment did not affect the reaction in immunoblotting (see even numbered lanes) and since LPS was stained in gels at the same position (data not shown). The strong reaction with the LPS of the homologous strains indicates that differences between the Xcc strains in the LPS are present. PCAs 94 and 113 also reacted relatively strong with the 23 and 18 kDa bands and the LPS of saprophyte 457 (lanes 7). Although PCA 94 did not cross-react significantly with saprophyte 457 in IF (Table 2), it reacted with the LPS of saprophyte 457 in this test, although weaker than with the LPS of the Xcc strains. On the other hand, PCA 111 did not clearly react with the LPS of saprophyte 457 although cross-reactions were found with saprophyte 457 at a 1:100 dilution in IF.

Characterization of flagellar extracts with PCAs. In SDS-PAGE, flagellar extracts seemed primarily to contain a 58 kDa protein and 44 kDa protein (Fig. 2). However, on immunoblots with PCAs 94, 111 and 113 bands were observed in the region of 15, 18, 23, 29 (weak reaction for PCA 113 and Xcc 367 and 671 only), 39, 44 and 58 kDa (Fig. 3). The reaction with the smear above 67 kDa indicates that LPS was present in the flagellar extracts.

Reaction of monoclonal antibodies in EIA, IF and DBI. Fourteen stable clones from 2 fusions were selected after subculturing and screening with EIA using flagellar and cell envelope extracts as antigens. After a second screening with EIA, using whole cells of 10 strains of several genera, six clones were selected on the basis of positive reactions

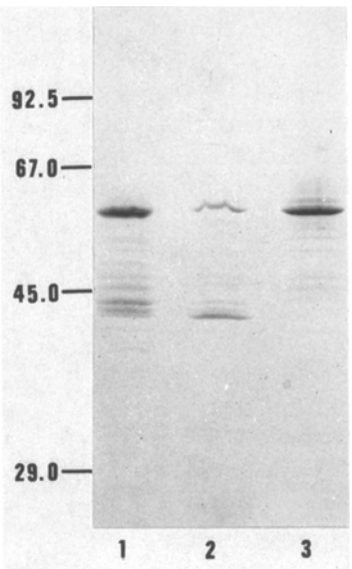

Fig. 2. SDS-polyacrylamide gel electrophoresis of flagellar extracts of several strains of *Xanthomonas campestris* pv. *campestris*. Gels are stained with Coomassie Brilliant Blue.
Lane 1 = flagellar extracts of Xcc 367.
Lane 2 = flagellar extracts of Xcc 102.
Lane 3 = flagellar extracts of Xcc 671.
Relative molecular masses (kilodaltons) of standard proteins are indicated to the left of the figure.

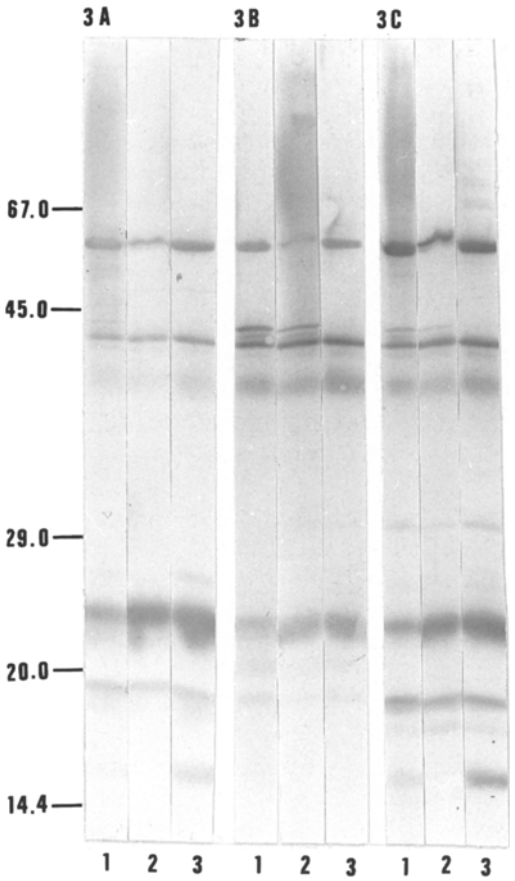

Fig. 3. Immunoblotting with polyclonal antibodies raised against *Xanthomonas campestris* pv. *campestris* to identify bands of flagellar extracts, separated by SDS-PAGE.
A = blot probed with PCA 94.
B = blot probed with PCA 111.
C = blot probed with PCA 113.
Lanes 1 contain flagellar extract of Xcc 367.
Lanes 2 contain flagellar extract of Xcc 102.
Lanes 3 contain flagellar extract of Xcc 671.
Relative molecular masses (kilodaltons) of standard proteins are indicated to the left of the figure.

with (some of the) Xcc strains and negative reactions with saprophyte 457. These clones were further tested in EIA, IF and DBI with a wider range of strains. Results are shown in Table 3. In EIA, DBI and IF, none of the six MCAs cross-reacted with non-xanthomonad strains. For MCAs 16B5 and 17C12, considerable differences were found between the serological assays used. All tested Xcc strains reacted in DBI whereas only some of the Xcc strains reacted in EIA and IF. All other xanthomonads (other pathovars of *X. campestris*) tested also reacted in DBI with MCAs 16B5 and 17C12, whereas in EIA and IF some strains of other xanthomonads gave negative reactions. For MCAs 20H6, 2F4, 18G12 and 10C5, only some of the Xcc strains reacted in EIA, DBI and IF. Other pathovars of *X. campestris* cross-reacted with MCAs 20H6, 2F4, 18G12 and 10C5 in all three serological tests.

The reactions in IF were studied in more detail. The total number of strains reacting in IF with the MCAs as well as the number of strains of which the whole cell wall was clearly stained in IF was recorded (Table 3). For MCA 16B5, good staining of the whole cell wall was observed for 20 out of 20 Xcc strains reacting in IF. For MCA 2F4, good staining of the whole cell wall was observed for only 9 Xcc strains; for 16 Xcc strains a partial staining of the cell wall was observed in IF. Also for the other MCAs,

Table 3. Reactions of monoclonal antibodies, produced against *Xanthomonas campestris* pv. *campestris*, with strains of various bacterial genera.

Monoclonal antibody	Antibody isotype	Bacteria[1]	Number of strains tested	Number of strains positive			
				EIA[2]	DBI[2]	IF	
						+[3]	±[4]
16B5	IgG2a	Xcc	37	18	37	20	0
		Xc pvs.	6	4	6	3	1
		other	15	0	0	0	0
17C12	IgG2a	Xcc	37	24	37	18	5
		Xc pvs.	6	5	6	3	0
		other	15	0	0	0	0
20H6	IgG1	Xcc	37	27	27	15	6
		Xc pvs.	6	2	1	1	1
		other	15	0	0	0	0
2F4	IgG3	Xcc	37	34	33	9	16
		Xc pvs.	6	2	2	2	0
		other	15	0	0	0	0
18G12	IgG3	Xcc	37	33	32	13	17
		Xc pvs.	6	2	2	2	0
		other	15	0	0	0	0
10C5	IgM	Xcc	37	33	26	13	15
		Xc pvs.	6	2	2	2	0
		other	15	0	0	0	0

[1] Xcc = *Xanthomonas campestris* pv. *campestris*, Xc pvs. = *X. c.* pv. *phaseoli* (2 strains), *X. c.* pv. *phaseoli* var. fuscans (2 strains), *X. c.* pv. *vesicatoria* (2 strains). Other strains are non-xanthomonads: *Pseudomonas syringae* pv. *phaseolicola* (2 strains), *P. s.* pv. *pisi* (2 strains), *P. s.* pv. *syringae* (2 strains), *P. s.* pv. *lachrymans* (1 strain), *P. s.* pv. *maculicola* (2 strains), *P. viridiflava* (1 strain), *P. aeruginosa* (1 strain), *Curtobacterium flaccumfaciens* subsp. *flaccumfaciens* (1 strain), *Clavibacter michiganensis* subsp. *michiganensis* (1 strain), *Cl. m.* subsp. *insidiosus* (1 strain) and saprophyte 457, isolated from cabbage seed.

[2] Test was conducted with heated cells.

[3] The number of strains of which the complete cell wall was well stained.

[4] The number of strains of which the cell wall was only partially stained.

only a part of the Xcc strains reacting in IF gave good staining of the whole cell wall. The staining quality (fluorescence intensity) in IF was the best for cells stained with MCA 20H6 (not shown). Some strains of other pathovars of *X. campestris*, such as Xcv, reacted also very well with the MCAs. Additional IF tests showed that MCAs 16B5, 17C12, 20H6, 2F4, 18G12 and 10C5 reacted well with the whole cell wall of, at least, one of the Xca strains tested.

Specificity of MCAs in immunoblotting. Total bacterial extracts of Xcc 367, 102 and 671, Xcar, Xcv 523, Xcp 375 and Xcpf 482 and a flagellar extract of Xcc 367, were

probed with several MCAs in immunoblotting. Two 'types' of reactions were found.

First, MCAs reacting with the LPS of the homologous Xcc strain 367, viz. MCAs 2F4, 18G12, 10C5 and 20H6 (proteinase K treatment of the extracts did not eliminate the reaction of these MCAs). Only, the blot for MCA 20H6 is shown in Fig. 4A and demonstrates a reaction with the LPS of a flagellar and total bacterial extract of Xcc strain 367 (resp. lane 1 and 2, the region above 58.1 kDa). A reaction in the same region was also found in cell envelope extracts (data not shown). MCA 20H6 did not show a reaction in the LPS region of the other strains tested in immunoblotting (lanes 3-8). MCAs 2F4, 18G12 and 10C5, however, also demonstrated a weak reaction with the LPS of Xcv 523 (not shown). MCA 2F4 also reacted with the LPS of Xcc strain 671 (not shown).

Second, MCAs reacting with protein epitopes, viz. MCAs 16B5 and 17C12 (protei-

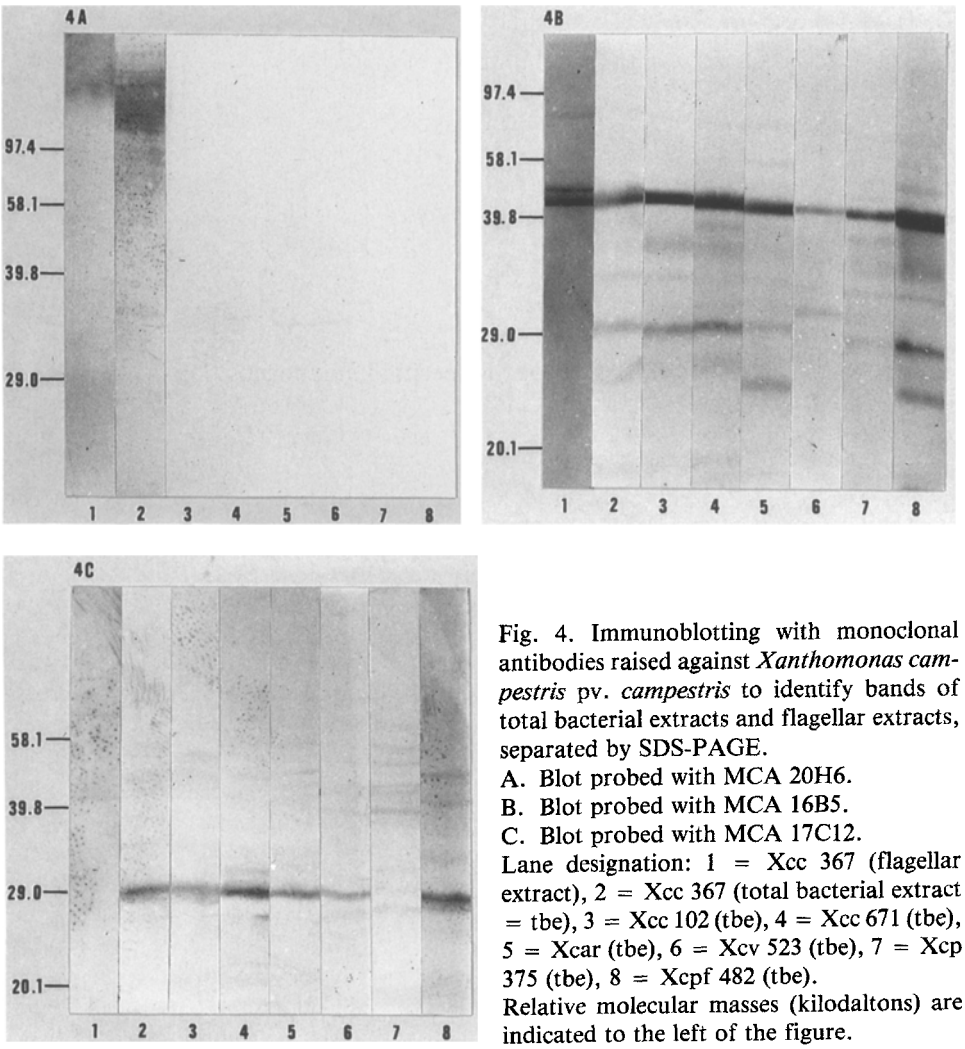

Fig. 4. Immunoblotting with monoclonal antibodies raised against *Xanthomonas campestris* pv. *campestris* to identify bands of total bacterial extracts and flagellar extracts, separated by SDS-PAGE.
A. Blot probed with MCA 20H6.
B. Blot probed with MCA 16B5.
C. Blot probed with MCA 17C12.
Lane designation: 1 = Xcc 367 (flagellar extract), 2 = Xcc 367 (total bacterial extract = tbe), 3 = Xcc 102 (tbe), 4 = Xcc 671 (tbe), 5 = Xcar (tbe), 6 = Xcv 523 (tbe), 7 = Xcp 375 (tbe), 8 = Xcpf 482 (tbe).
Relative molecular masses (kilodaltons) are indicated to the left of the figure.

nase K treatment of the extracts eliminated the reaction of the MCAs). MCA 16B5 (Fig. 4B) reacted with a major c. 39 kDa band of Xcc 367, Xcc 102, Xcc 671, Xcar, Xcv 523, Xcp 375 and Xcpf 482 (lanes 2-8). The reaction with this band also occurred in flagellar extracts (lane 1, Fig. 4B) and cell envelope extracts of Xcc strain 367 (not shown). A minor band was sometimes noted at 29 kDa (Xcc 367, Xcc 102, Xcc 671, Xcar and Xcpf 482) and 23 kDa (Xcar and Xcpf 482). MCA 17C12 (Fig. 4C) reacted with a major band at 29 kDa of Xcc 367, Xcc 102, Xcc 671, Xcar, Xcv 523 and Xcpf 482 (resp. lanes 2-6 and 8). For Xcp 375 no reaction was found with MCA 17C12. A reaction with a 29 kDa band was not detected in flagellar extracts of Xcc strain 367 (lane 1, Fig. 4C), but was detected in cell envelope extracts (not shown).

Discussion

The specificity of PCAs in IF and immunoblotting. Xcc strains 367, 102 and 365 reacted variably with the PCAs in IF. Also in SDS-PAGE and immunoblotting differences in reactions of PCAs 94, 111 and 113 with the LPS of these strains were found. This indicated that LPS patterns may be unique for strains of Xcc as is known for *Pseudomonas* strains (De Weger et al., 1987) and that the LPS may contain epitopes, that are unique for certain Xcc strains or group of Xcc strains.

The preparation and characterization of flagellar extracts used for immunization. For immunization of mice, crude extracts were used as immunogens because low-antibody titres were obtained in mice immunized with purified LPS. The same phenomenon was found by De Boer and McNaughton (1987) for the LPS from *Erwinia carotovora* subsp. *atroseptica*, serogroup I. Flagellar extracts contained both the LPS and protein bands which could possibly act as specific immunogens (Fig. 3).

SDS-PAGE profiles of flagellar extracts (Fig. 2) showed two major protein bands, viz. a 44 kDa band, that is believed to contain the heat modifiable major outer membrane protein A of Xcc (Minsavage and Schaad, 1983) and a 58 kDa band which may contain the protein subunits of the flagella (flagellin) of Xcc. This, however, needs further confirmation.

The production and selection of MCAs produced against Xcc. In this study MCAs were produced against flagellar extracts of Xcc strain 367. Immunization with cell envelope extracts of Xcc strains were also done but they did not yield hybridomas, that produced antibodies reacting strongly with Xcc strains. Moreover, many cross-reactions were obtained in EIA and DBI. Therefore, we did not proceed with cell envelope extracts as immunogens.

Although the MCAs discussed in this study were produced against flagellar extracts, we did not find good staining of flagella in IF. Some explanations may be given. Possibly, in initial selections using EIA the antigen may have been denatured, which could have led to not selecting suitable clones. As EIA was found to be more sensitive than indirect IF for detecting positive hybridoma supernatants (Jones and Van Vuurde, 1989), we used EIA for initial selections. Still, we did not obtain clones producing antibodies reacting with the 44 or 58 kDA bands (Fig. 2). Since motility was present in all strains tested prior to preparation of flagellar extracts, which indicates presence of flagella, we believe that the protein antigens used were poorly immuno-

genic, possibly due to the presence of highly repeating determinants (e.g. flagellin) which predominantly yields low affinity IgM antibodies (Roitt, 1988). We aimed at selecting high affinity IgG antibodies.

The results presented in Table 3 show that when selecting for suitable clones, great differences appear between serological tests, as was found for MCAs 16B5 and 17C12. A possible explanation is that the exposition of the epitope was variable and strain dependent in EIA and IF. In DBI the exposition of the epitope is probably less variable and not strain-dependent, which could make DBI therefore more suited for selection of antibodies against typical antigens. The reaction of MCAs was studied in a selection procedure with cells boiled for 15 min at 100 °C to diminish non-specific background reaction of Xcc strains (Table 3), possibly due to endogenous phosphatase activity (El-Sharkawy and Huisingh, 1971). Background reactions occasionally occurred for strains of Xcc and Xcp but not for strains of Xcv and all strains of other bacterial species tested. Boiling extracts did not significantly change the reactivity of the antibodies indicated in Table 3 compared to earlier selections with whole, unboiled cells in the DBI.

The characterization and specificity of MCAs produced to Xcc. In this study, MCAs (20H6, 18G12, 2F4, 10C5) were obtained, that reacted specifically with the LPS of a group of Xcc strains and some strains of narrowly related pathovars of *X. campestris*. Reactions with non-xanthomonads were not detected. None of our LPS-specific MCAs were pathovar-specific or reacted with all Xcc strains. Similar results were found for Xcc by Alvarez et al. (1985). The results obtained with MCAs confirmed observations done in immunoblotting experiments with PCAs and cell envelope extracts (Fig. 1), i.e. LPS may contain epitopes unique for a group of Xcc strains and related strains of other pathovars. LPS-specific antibodies for plant pathogenic bacteria were produced earlier by Benedict et al. (1989) for *X. c.* pv. *oryzae* and pv. *oryzicola* and by De Boer and McNaughton (1987) for *Erwinia carotovora* subsp. *atroseptica*, serogroup I. Benedict et al. (1990) even found MCAs specific for the LPS of all strains of *X. c.* pv. *begoniae* and pv. *pelargonii*.

MCAs (17C12 and 16B5) were obtained that reacted with proteins of Xcc and of related pathovars of *X. campestris*. They did not cross-react with non-xanthomonads. The nature of the homologous epitopes is not known. However, the major 39 kDa and 29 kDa band to which MCA 16B5 and MCA 17C12, respectively, reacted on immunoblots, could be the major protein bands of outer membrane extracts identified by Minsavage and Schaad (1983) and Laakso et al. (1990) in SDS-PAGE. Since the 39 kDa and 29 kDa bands were present in cell envelope extracts, and MCAs 16B5 and 17C12 generally gave good reactions in IF, it is assumed that these proteins are (thermostable) surface antigens often present in outer membranes of Xcc. A reaction of MCA 17C12 with the 29 kDa band was not found in flagellar extracts of Xcc strain 367 (Fig. 4C) although this band was shown to be present in flagellar extracts (Fig. 3, weak reaction of PCA 113 with this band in extracts of Xcc 367 in immunoblotting). This was probably due to the low concentration of this band in the flagellar extract. A reaction of MCA 17C12 with flagellar extracts was obtained in DBI indicating that the homologous antigen was present in the flagellar extract.

At some dilutions of the PCAs cross-reactions with Xcv as well as with Xca are possible. The MCAs discussed here are also able to cross-react with Xcv or Xca. Also

Schaad (1978), Alvarez et al. (1985), and Alvarez and Lou (1985) found a serological relationship of Xcc with Xcv and/or Xca. Since Xcv is host-specific for tomato, this cross-reaction will not be a problem in the detection of Xcc in crucifer seeds. Xca can affect crucifers and therefore the significance of the cross-reaction with this bacterium needs to be investigated. To assess the problems occurring with MCAs in the detection of Xcc in crucifer seeds, serological assays should be done in parallel with dilution-plating assays. This will provide information on the specificity of the MCAs and the sensitivity of serological tests with MCAs when applied to plant extracts.

We conclude that the monoclonal and polyclonal antibodies discussed in this study, may be used to differentiate and identify (groups of) Xcc strains in several serological tests. Studies are now in progress to investigate the application of the monoclonal and polyclonal antibodies for the detection of Xcc in crucifer seeds.

Acknowledgements

Thanks are due to P.S. van der Zouwen and M. Poppelier for excellent technical assistance. The critical comments of prof. dr ir J. Dekker and dr R. Bino are gratefully acknowledged.

Thanks are also due to IPO-DLO (Research Institute for Plant Protection) for kindly providing polyclonal antisera. The cooperation of IPO-DLO, PD (Plant Protection Service, Wageningen), and drs C. van Henten, T.L. Kuan, D.L. Pinnow, B. Rat, N. Thaveechai, F. Vinther and P.H. Williams in providing cultures is also gratefully acknowledged.

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