Physiology and Biochemistry

Serological and Electrophoretic Analysis of a Membrane Protein Extract of *Xanthomonas campestris* pv. *campestris* from Thailand

N. Thaveechai and N. W. Schaad

Department of Plant, Soil and Entomological Sciences, University of Idaho, Moscow 83843. Present address of the first author: Department of Plant Pathology, Kasetsart University, Bangkok, Thailand.

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ABSTRACT

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LiCl-extracted membrane proteins of 35 strains of *Xanthomonas campestris* pv. *campestris* from Thailand were characterized by serology and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antisera were made to membrane proteins of seven strains. When membrane proteins of homologous strains were tested by Ouchterlony double diffusion, a single major line of precipitin always resulted. With some strains, up to three minor lines of precipitin also resulted. Two to four other minor lines of precipitin were present in immunoelectrophoresis. By testing with the major common line of precipitin, the strains of *X. c.* pv. *campestris* were grouped into three serovars—I, I-A, and II. When tested by immunofluorescent microscopy, all strains of *X. c.* pv. *campestris* were positive with immunoglobulin G to membrane proteins of the seven strains. None of 20 other xanthomonads or 11 other species reacted with the seven antisera by Ouchterlony double diffusion except four strains of *X. campestris* pv. *vesicatoria* and one strain of *X. campestris* pv. *incanae* (which cross-reacted in Ouchterlony double diffusion). All 24 bacteria were immunofluorescent negative except four strains of *X. c.* pv. *vesicatoria* and one strain each of *X. c.* pv. *incanae* and *X. campestris* pv. *manihotis*. SDS-PAGE profiles of membrane protein of *X. c.* pv. *campestris* were distinct from all the other bacteria tested. These results suggest that the membrane proteins of *X. c.* pv. *campestris* are distinct and therefore useful for identification and taxonomy.

Additional key words: bacteria, black rot.

Xanthomonas campestris pv. *campestris*, the causal agent of black rot of crucifers, is the most destructive seedborne pathogen of crucifers worldwide (25). In Thailand (20), several cruciferous crops such as cabbage (*Brassica oleracea* L. var. *capitata* L.), cauliflower (*B. oleracea* L. var. *botrytis* L.), Chinese cabbage (*B. pekinensis* Rupr.), and broccoli (*B. oleracea* L. var. *italica* Plenck) are of economic importance and subject to black rot. Research on epidemiology of black rot has received little attention in Thailand; however, the pathogen does survive in infected debris in soil (23). A method of rapid detection and identification of *X. c.* pv. *campestris* would greatly help development of control measures.

Serology is one of the most useful tools available for identification and detection of phytopathogenic bacteria (18). Rapid serological tests such as immunofluorescence (1,17,24) and enzyme-linked immunosorbent assay (ELISA) (6,15) are well adapted for diagnosis. However, these techniques require highly specific antisera for precise results. Antisera against several different immunogens of *X. c.* pv. *campestris* have been studied, but none are specific enough in immunofluorescence tests to differentiate *X. c.* pv. *campestris* from other xanthomonads (24). In one study (24), strains of *X. c.* pv. *campestris* from different hosts and geographical regions were grouped into four serovars based on antisera to ribosome preparations. Because ribosome extraction requires equipment not readily available everywhere, LiCl-

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TABLE 1. Bacterial strains used in the study of membrane proteins of *Xanthomonas campestris* pv. *campestris*

Strain no.	Received as: Name	Strain	Source	Location	Host of origin
	Xanthomonas campestris				
BT-1,2,20,25,26,28	pv. *campestris*	Original	1[a]	Thailand	Cauliflower (*Brassica oleracea* L. var. *botrytis* L.)
BT-3,4	pv. *campestris*	Original	1	Thailand	Kale (*B. oleracea* var. *acephala* DC)
BT-5 to 11, 15 to 19,23,24,27	pv. *campestris*	Original	1	Thailand	Cabbage (*B. oleracea* var. *capitata* L.)
BT-12,13,21,22	pv. *campestris*	Original	1	Thailand	Broccoli (*B. oleracea* var. *italica* Plenck)
BT-14	pv. *campestris*	Original	1	Thailand	Chinese cabbage (*B. oleracea* var. *pekinensis* Rupr.)
BT-29,30	pv. *campestris*	Original	1	Thailand	Cabbage, seed
BT-31	pv. *campestris*	12-1	2	Thailand	Cauliflower
BT-32	pv. *campestris*	13-1	2	Thailand	Kale
BT-33	pv. *campestris*	81-5	2	Thailand	Chinese cabbage
BT-34,35	pv. *campestris*	91-1,2	2	Thailand	Chinese radish (*Raphanus sativus* L. var. *longipinnatus*)
B-1	pv. *campestris*	BBS	3	California	Brussels sprouts (*B. oleracea* var. *gemmifera*)
B-12	pv. *campestris*	Original	4	Georgia	Cabbage
B-18	pv. *campestris*	Original	4	Florida	Cabbage, field soil
B-24	pv. *campestris*	-	5	Oregon	Broccoli
B-33	pv. *campestris*	13	6	N. Zealand	Brussels sprouts
B-65	pv. *campestris*	Original	4	Brazil	Cabbage
B-82	pv. *campestris*	-	7	Australia	Cauliflower
B-85	pv. *campestris*	Original	4	Georgia	Peppergrass (*Lepidium viginicum* L.)
B-87,88	pv. *campestris*	Original	4	California	Black mustard (*B. nigra* (L.) Kock)
B-90,94	pv. *campestris*	Original	4	California	Field mustard (*B. campestris* L.)
B-98	pv. *campestris*	Original	4	California	White top (*Cardaria pubescens* (C. A. Mey) Roll.)
B-107	pv. *campestris*	Original	4	California	Cauliflower, seed
B-127	pv. *campestris*	31	8	California	Radish (*R. sativus* L.), weed
B-130	pv. *campestris*	39	8	California	Black mustard
B-132	pv. *campestris*	42	8	Australia	Cauliflower, seed
B-133	pv. *campestris*	44	8	California	Wild radish (*R. raphanus* L.)
B-135	pv. *campestris*	46	8	California	Charlock (*B. kaber* (D.C.) Wheeler)
B-139	pv. *campestris*	51	8	California	*B. geniculata* (Desf.) J. Ball
B-145	pv. *campestris*	A342	30	Hawaii	Broccoli
B-497	pv. *malvacearum*	-	9	Brazil	Cotton (*Gossypium hirsutum* L.), seed
B-930,931	pv. *malvacearum*	056-1,8	2	Thailand	Cotton
B-487	pv. *manihotis*	Xm-58	10	Brazil	Cassava (*Manihot esculenta* Crantz.)
B-932	pv. *manihotis*	MbI-L	11	Thailand	Cassava
B-444	pv. *oryzae*	PXO-79	12	Philippines	Rice (*Oryza sativa* L.)
B-919	pv. *oryzae*	TB-20	2	Thailand	Rice
B-496	pv. *phaseoli*	P-60	13	Canada	Bean (*Phaseolus vulgaris* L.)
B-702	pv. *phaseoli*	1208	14	Brazil	Bean
B-490	var. *fuscans*	XP-18	15	California	Bean
B-495	var. *fuscans*	R-10	16	Michigan	Bean
B-933	var. *sojensis*	054-1	2	Thailand	Soybean (*Glycine max* (L.) Merr.)
B-428	pv. *translucens*	549	17	N. Dakota	Wheat (*Triticum aestivum* L.)
B-433	pv. *translucens*	Original	18	Georgia	Triticale (*Triticosecale* sp. Whittmack)
B-909	var. *oryzicola*	TS 8203	2	Thailand	Rice
B-934	pv. *cerealis*	Xt-8	19	Minnesota	Wild rice (*Zizania aquatica* L.)
B-202	pv. *vesicatoria*		5	Oregon	Tomato (*Lycopersicon esculentum* Mill.)
B-218	pv. *vesicatoria*	549	20	Delaware	Tomato
B-260	pv. *vesicatoria*	VB-1	2	Thailand	Tomato
B-261	pv. *vesicatoria*	97-IF	2	Thailand	Chili pepper (*Capsicum frutescens* L.)
B-935	pv. *begoniae*	XB-8	29	New York	Begonia (*Begonia* sp.)
B-936	pv. *incanae*	XI-3	29	New York	*Matthiola incanae* L.
B-937	pv. *pelargonii*	XP-39	29	New York	*Pelargonium* sp.
B-938	pv. *vitians*	XV-29	29	New York	*Lactuca* sp.
C-7	*Pseudomonas syringae* pv. *syringae*	B-3	21	California	Peach (*Prunus persica* (L.) Batsch)
C-21	pv. *coronafaciens*	Original	18	Georgia	Oat (*Avena sativa* L.)
C-199	pv. *phaseolicola*	-	22	Idaho	Bean (*Phaseolus vulgarus* L.)
C-198	pv. *tomato*	Field 8	23	Georgia	Tomato (*Lycopersicon esculentum* L.)
C-51	*P. solanacearum*	K-60	24	N. Carolina	Tomato
C-158	*P. solanacearum*	51	24	Sri Lanka	Potato *Solanum tuberosum* L.)
A-B6	*Agrobacterium tumefaciens*	B6	5	Oregon	Tomato
A-4	*Erwinia carotovora* subsp. *carotovora*	EC-105	25	Missouri	Potato
A-310	*E. chrysanthemi*	B-102	26	Florida	African violet (*Santpaulia ionantha* Wendel)
D-2	*Escherichia coli*	01-6	27	Georgia	Pig
E-26	*Salmonella typhimurium*	RI-A	28	Illinois	Attunuated

[a] Source names and locations: 1 = N. W. Schaad and N. Thaveechai, Moscow, ID; 2 = S. Chuenchitt, Bangkok, Thailand; 3 = R. G. Grogan, Davis, CA; 4 = N. W. Schaad, Moscow, ID; 5 = L. M. Moore, Corvallis, OR; 6 = D. Dye, Auckland, New Zealand; 7 = D. S. Trimboli, Narromine, Australia; 8 = R. H. Morrison, Woodland, CA; 9 = P. S. Randhawa, Beltsville, MD; 10 = A. Takatsu, Brasilia, Brazil; 11 = T. Jarupat, Bangkok, Thailand; 12 = T. W. Mew, Manila, Philippines; 13 = J. W. Sheppard, Ottawa, Canada; 14 = S. K. Mohan, Londrina, Brazil; 15 = M. P. Starr, ICPB, Davis, CA; 16 = A. W. Saettler, East Lansing, MI; 17 = J. Otta, Brookings, SD; 18 = B. M. Cunfer, Experiment, GA; 19 = R. L. Bowden, Moscow, ID; 20 = M. Sasser, Willmington, DE; 21 = H. English, Davis, CA; 22 = J. W. Guthrie, Moscow, ID; 23 = S. M. McCarter, Athens, GA; 24 = A. Kelman, Madison, WI; 25 = R. N. Goodman, Columbia, MO; 26 = J. Miller, Gainesville, FL; 27 = W. Ewing, CDC, Atlanta, GA; 28 = N. Bigley, Chicago, IL; 29 = R. S. Dicky, Ithaca, NY; 30 = A. Alvarez, Honolulu, HI.

extracted membrane proteins (8,27) (an immunogen that is more easily extracted) was chosen for this study.

LiCl-extracted membrane proteins are highly specific and useful for identification of *Neisseria meningitidis* (8) as well as several plant pathogens including *Erwinia chrysanthemi* (27), *Pseudomonas solanacearum* (19), and the phony peach bacterium (3).

The purpose of this study was to determine the serological relationships among strains of *X. c.* pv. *campestris* from Thailand and other closely and distantly related bacterial species in order to determine the specificity of the membrane protein immunogens. Profiles of membrane proteins of different xanthomonads and other bacterial species were compared to define any specfic peptide bands by SDS-PAGE.

MATERIALS AND METHODS

Bacterial strains. Ninety-two strains representing 25 different bacteria were used in this study (Table 1). Of 56 strains of *X. c.* pv. *campestris*, 35 were from Thailand. Strains BT-1 through BT-28 were isolated from various cruciferous plants in Thailand (20). Bacterial cultures were maintained on slants of yeast extract-dextrose-calcium carbonate (YDC) agar (26) at 4 C.

Purity of cultures was checked every 2 mo, at the time of transfer, by streaking them on YDC plates. Strains BT-1 through BT-28 and strains B-1 through B-107 of *X. c.* pv. *campestris* were tested for pathogenicity on cabbage seedlings as previously described (20). Other strains of *X. c.* pv. *campestris* (B-127 through B-145) and pathovars of *X. campestris* were not tested for pathogenicity.

Preparation of membrane proteins. Bacteria were grown by adding 7 ml of 24-hr-old inoculum in 523 medium (10) to 1.5 L of 523 medium in 2.8-L Fernbach flasks and incubating the culture in a rotary environmental shaker at 30 C for 24 hr. Cultures in the mid- to late-exponential phase of growth were harvested and cell membrane proteins were extracted with lice as described (27). Extracts were stored at 4–5 C until use.

Preparation of antisera. Membrane proteins from strains BT-1, BT-4, BT-6, BT-16, BT-17, BT-21, and BT-27 of *X. c.* pv. *campestris* were injected into New Zealand white rabbits as described (27). Preimmune sera were obtained from rabbits 1 wk before the first injection. One milliliter of each preparation of membrane proteins was emulsified with an equal volume of Freund's incomplete adjuvant (Difco Co., Detroit, MI). Rabbits were injected intraperitoneally at 10-day intervals with three graded injections containing 0.2, 0.4, and 0.6 mg of protein, respectively. Protein was determined by using the method of Lowry et al (12) with bovine serum albumin as a standard. The rabbits were bled from a marginal ear vein 1, 2, and 3 wk after the last injection. After serum processing, antisera were divided and stored at −20 C for long-term storage and at 4 C for immediate use.

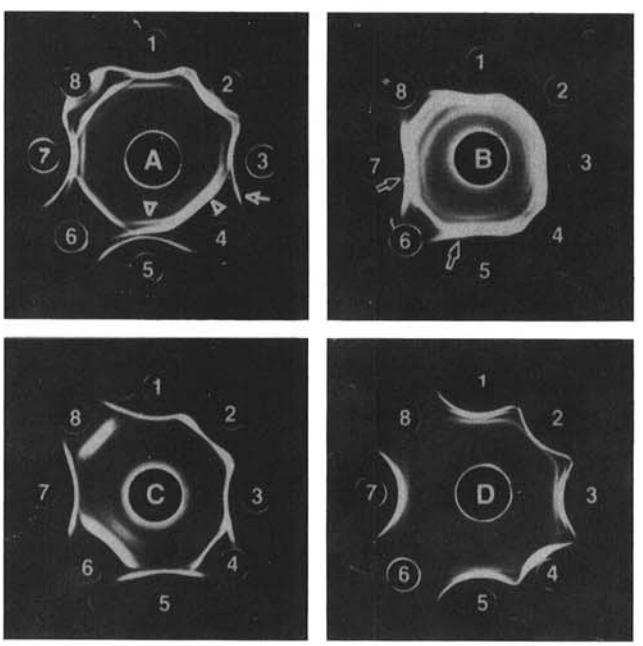

Fig. 1. Ouchterlony double diffusion patterns of membrane proteins of different serovars of *Xanthomonas campestris* pv. *campestris* and several other bacteria. Center wells contained antiserum to membrane proteins of strains: A and D, BT-16 (Ser. I); B, BT-4 (Ser. IA); and C, BT-27 (Ser II). Outer wells 1, 3, 5, and 7 of each pattern contained homologous membrane proteins. Outer wells of A contained membrane proteins of strains: 2, BT-1; 4, BT-4; 6, BT-27; and 8, BT-33. Outer wells of B contained membrane proteins of strains: 2, BT-34; 4, BT-1; 6, BT-27; and 8, BT-33. Outer wells of C contained membrane proteins of strains: 2, BT-21; 4, BT-1; 6, BT-4; and 8, BT-33. Outer wells of D contained membrane proteins of strains: 2, B-145; 4, B-936; 6, C-7; and 8, B-85. Note specific precipitin lines (arrows in pattern A, well 3 and in pattern B, wells 6 and 7) and nonspecific precipitin lines (triangle in pattern A, wells 4 and 5).

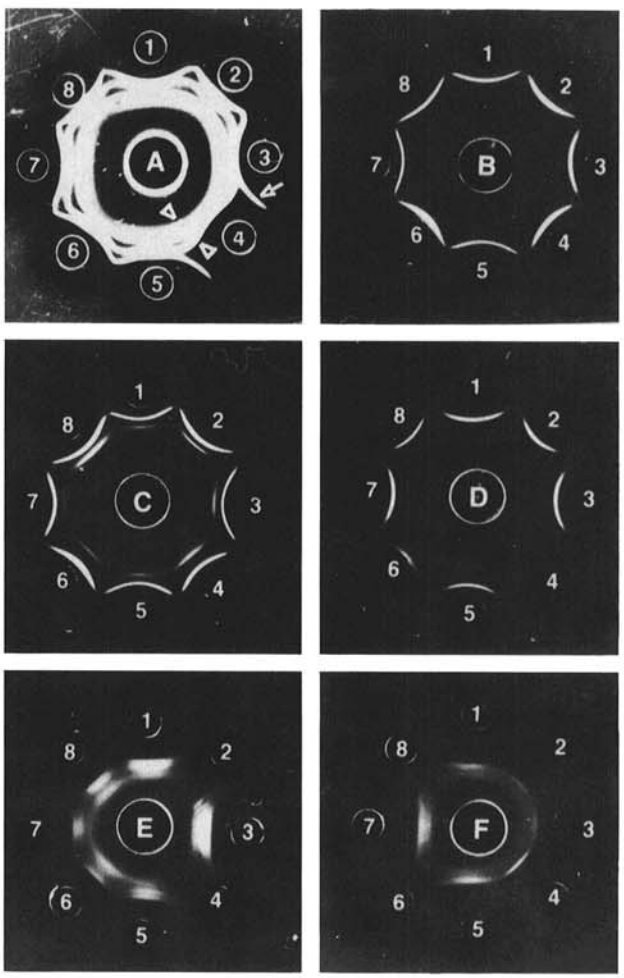

Fig. 2. Immunodiffusion patterns of absorbed antiserum to membrane proteins of *Xanthomonas campestris* pv. *campestris* tested against membrane proteins (antigen) of the three serovars and *X. campestris* pv. *vesicatoria*. Center wells of patterns: A contained unabsorbed antiserum of BT-16, B contained antiserum of BT-16 absorbed with membrane proteins of BT-9, C contained antiserum of BT-16 absorbed with membrane proteins of BT-27, D contained antiserum of BT-16 absorbed with membrane proteins of B-202, E contained antiserum of BT-27 absorbed with of BT-4, and F contained antiserum of BT-4 absorbed with membrane proteins of B-202. Outer wells 1, 3, 5, and 7 of each pattern contained homologous membrane protein. Outer wells of A contained membrane proteins of strains: 2, BT-1; 4, BT-9; 6, BT-6; and 8, BT-33. Outer wells of B and C contained membrane proteins of strains: 2, BT-1; 4, B-202; 6, BT-6; and 8, BT-33. Outer wells of D contained membrane proteins of strains: 2, BT-1; 4, BT-27; 6, B-261; and 8, B-202. Outer wells of E contained membrane proteins of strains: 2, BT-16; 4, BT-4, 6; BT-17; and 8, BT-202. Outer wells of F contained membrane proteins of strains: 2, BT-16; 4, BT-34, 6; BT-27; and 8, B-202. In pattern A specific (arrow) and nonspecific (triangle) precipitin lines are marked.

Immunoglobulin G (IgG) was prepared from crude antiserum by ion exchange column chromatography by using DEAE-Sephadex A-50 resins (Pharmacia Fine Chemicals, Pascataway, NJ) packed in C-16 (bed 1.6× 15 cm) glass columns. The IgG was contained in the void volume fraction. The IgG fractions were precipitated overnight at 4 C with ammonium sulfate (50% of saturation) (13). The suspension was centrifuged at 10,000 *g* for 10 min. The pellet containing IgG was dissolved with 0.01% aqueous thimerosal to original volume and stored at 4–5 C.

Absorption of antisera was carried out as previously described (27). The sera to membrane proteins were absorbed with membrane proteins of strains of the same and different serovars, including some strains showing cross reactions.

Serology. Titers of antisera were determined by micro-agglutination. Fourfold dilutions of antisera were made from 1:4 to 1:4,096 with 0.5 ml of saline solution as diluent. After placing 10 μl of each antiserum dilution on a 35-mm-diameter × 10-mm-high plastic petri dish, 10 μl of a cell suspension containing about 10^8–10^9 colony-forming units (cfu) per milliliter was added. Preimmune sera and saline solution were used as controls. Plates were incubated at 4 C overnight and observed for agglutination. Each antiserum was tested against antigen of homologous and heterologous strains.

Ouchterlony double diffusion tests were conducted in 35-mm-diameter × 10-mm-high plastic petri plates containing 15 ml of 0.75% agarose-trypan blue medium (16). A pattern of wells with a center well 5 mm in diameter surrounded by eight peripheral wells each 3 mm in diameter and 5 mm from the center well was made with a stainless steel gel puncher (Bio-Rad Laboratories, Richmond, CA). The center well was filled with 10 μl of antiserum and each peripheral well was filled with 5 μl of antigen containing 3 mg of protein per milliliter (12). Plates were placed in a humidity chamber at 4–5 C and observed after 2–3 days. Results were

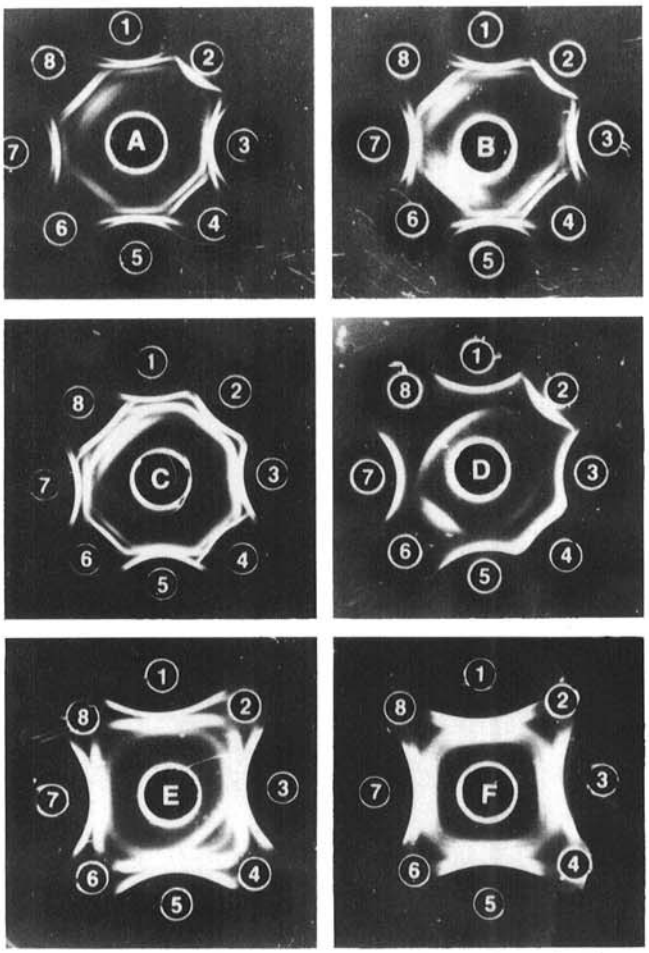

Fig. 3. Comparative Ouchterlony double diffusion reactions of antisera to membrane proteins of Xanthomonas campestris pv. campestris against membrane proteins (antigens) of strains of *X. campestris* pv. *campestris* from cultivated and weed hosts and several other bacteria. Center wells contained 10 μl of antiserum to membrane proteins of strains: A and B, BT-1; C and E, BT-4; D, BT-27; and F, BT-17. Outer wells contained 5 μl of membrane protein antigens. Wells 1, 3, 5, and 7 of each pattern contained homologous membrane proteins. Outer wells of A contained membrane proteins of strains: 2, B-1; 4, B-12; 6, B-33; and 8, D-90. Outer wells of C contained membrane proteins of strains: 2, B-24; 4, 8-18; 6, B-82; and 8, B-98. Outer wells of C contained membrane proteins of strains: 2, B-1; 4, B-18; 6, B-82; and 8, B-98. Outer wells of D contained membrane proteins of strains: 2, B-12; 4, B-1, 6; B-33; and 8, B-90. Outer wells of E contained membrane proteins of strains: 2, B-932; 4, B-930; 6, B-496; and 8, B-433. Outer wells of F contained membrane proteins of strains 2, C-21; 4, C-51; 6, A-310; and 8, A-B6.

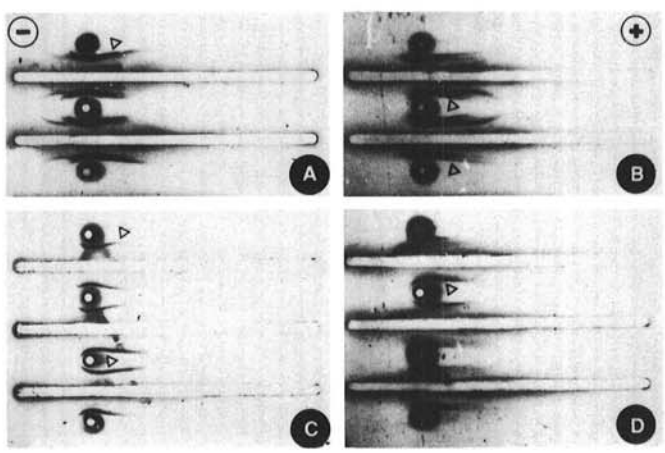

Fig. 4. Immunoelectrophoresis of membrane proteins of three different serovars of *Xanthomonas campestris* pv. *campestris*. Antiserum troughs contained 50 μl of antiserum to membrane proteins of strains: gel A, BT-6; gel B, BT-17; gel C, BT-27; and gel D, BT-4. Sample wells from upper to lower contained one μl of membrane proteins of strains: gel A—BT-4, BT-6, and BT-27; gel B—BT-1, BT-17, and BT-4; gel C—BT-4, BT-1, BT-27, and BT-4; gel D—BT-1, BT-27, BT-4, and BT-1. Open triangle denotes specific immunoprecipitate.

TABLE 2. Strains of *Xanthomonas campestris* pv. *campestris* from Thailand grouped into serovars based upon Ouchterlony double diffusion reactions between antisera to membrane proteins

		Antiserum						
	Antigen	Serovar I			Serovar I-A	Serovar II		
Serovar	Strain	BT-1	BT-6	BT-16	BT-4	BT-17	BT-21	BT-27
I	BT-1,2,5,6,7,10,11,12,13, 14,15,16,18,19,20,23,24, 25,26,29,30,31,32,33	++[a]	++	++	++	+	+	+
I-A	BT-4,9,34,35	–	–	–	++	+	+	+
II	BT-3,8,17,21,22,27,28	–	–	–	±	++	++	++

[a] Symbols: ++ = reaction of complete fusion (identity), + = reaction of partial fusion (partial identity), ± = reaction of noninteraction (nonidentity), and – = no reaction (no precipitin line) (4).

recorded as reactions of identity (complete fusion), partial identity (partial fusion), or nonidentity (noninteraction) (4).

For immunoelectrophoresis, a modification of the method of Yakrus and Schaad (27) was used. A 1% (w/v) agarose support medium was prepared by dissolving 1 g of SeaKem HGT agarose (FMC Corp., Rockland, ME) in 1 ml of 1% aqueous thimerosal and 99 ml of buffer A (5) containing 80 mM tris, 40 mM sodium acetate, and 1 mM Na_2 EDTA, pH 8.6. Gels were formed on GelBond support films (FMC Corp.) in immuno-agaroslide molds (Millipore Corp., Bedford, MA 01730). One-microliter samples of membrane protein dissolved in water were added with a 10-μl Hamilton syringe. One well was filled with 0.1% (w/v)

TABLE 3. Ouchterlony double diffusion reactions between antisera to membrane proteins from strains of *Xanthomonas campestris* pv. *campestris* from Thailand and membrane protein antigens of *X. campestris* pv. *campestris* and other bacteria

Membrane protein antigen from		Antisera						
		Serovar I			Serovar I-A	Serovar II		
Pathovar or species		BT-1	BT-6	BT-16	BT-4	BT-17	BT-21	BT-27
Xanthomonas campestris								
pv. *campestris*	BT-1,24,107,145	++[a]	++	++	++	+	+	+
	BT-4, B-1	−	−	−	++	+	+	+
	BT-27	−	−	−	±	++	++	++
	B-12,18,65	+	+	+	±	++	++	++
	B-90,98	+	+	+	+	+	+	+
	B-88	+	+	+	+	−	+	+
	B-87	+	−	++	+	−	+	+
	B-130,133,135	+	+	+	+	(+)	(+)	−
	B-33,82,85,94,127,132,139	−	−	−	−	−	−	−
pv. *vesicatoria*	B-202	++	++	++	+	++	++	++
	B-218	+	(+)	+	+	−	−	−
	B-260	+	+	+	+	−	(+)	−
	B-261	−	(+)	+	(+)	−	(+)	−
pv. *incanae B-936,937,938,934*		++	++	++	+	−	+	−
pv. *pelargonii* B-937, pv. *vitians* B-938		−	−	−	−	−	−	−
pv. *cerealis* B-934, pv. *begoniae* B-975		−	−	−	−	−	−	−
pv. *malvacearum* B-497,B-930,B-931		−	−	−	−	−	−	−
pv. *manihotis* B-487,B-932		−	−	−	−	−	−	−
pv. *oryzae* B-932,B-441, pv. *phaseoli* B-702,B-490, B-495,B-933		−	−	−	−	−	−	−
pv. *translucens* B-428,B-433,B-909		−	−	−	−	−	−	−
Pseudomonas syringae								
pv. *syringae* C-7, pv. *coronafaciens* C-21		−	−	−	−	−	−	−
pv. *phaseolicola* C-199, pv. *tomato* C-198		−	−	−	−	−	−	−
P. solanacearum C-51		−	−	−	−	−	−	−
P. solanacearum C-158		−	(+)	(+)	(+)	−	−	−
Erwinia carotovora var. *carotovora* A-4		−	−	−	−	−	−	−
E. chrysanthemi A-310		−	−	−	−	−	−	−
Agrobacterium tumefaciens A-B6		−	−	−	−	−	−	−
Escherichia coli D-2		−	−	−	−	−	−	−
Salmonella typhimurium E-26		−	−	−	−	−	−	−

[a] Symbols: ++ = reaction of complete fusion (identity), + = reaction of partial fusion (partial identity), ± = reaction of noninteraction (nonidentity), (+) = questionable reaction of partial fusion since precipitin lines were faint or not completely fused to form a true spur, − = no reaction (no precipitin line) (4).

TABLE 4. Ouchterlony double diffusion reactions between absorbed antiserum of *Xanthomonas campestris* pv. *campestris* and membrane proteins (antigens) of *X. c.* pv. *campestris* serovars I, I-A, and II and *X. c.* pv. *vesicatoria* (B-202)

Antiserum to:		Absorbed with:		Antigen						
				Serovar I		Serovar I-A		Serovar II		
Serovar	Strain	Strain	Serovar	BT-1	BT-16	BT-4	BT-9	BT-17	BT-27	B-202
Ser I	BT-16	None		++[a]	++	−	−	−	−	++
		BT-1	Ser I	−	−	−	−	−	−	−
		BT-9	Ser I-A	++	++	−	−	−	−	++
		BT-27	Ser II	++	++	−	−	−	−	++
		B-202		++	++	−	−	−	−	−
Ser I-A	BT-4	None		++	++	++	++	±	±	++
		BT-16	Ser I	−	−	−	−	−	−	−
		BT-9	Ser I-A	−	−	−	−	−	−	−
		BT-27	Ser II	++	++	++	++	−	−	++
		B-202		++	++	++	++	−	−	−
Ser II	BT-27	None		+	+	+	+	++	++	++
		BT-16	Ser I	−	−	−	−	++	++	++
		BT-4	Ser I-A	−	−	−	−	++	++	++
		BT-17	Ser II	−	−	−	−	−	−	−
		B-202		−	−	−	−	−	−	−

[a] Symbols: ++ = reaction of complete fusion (identity), + = reaction of partial fusion (partial identity), ± = reaction of noninteraction (nonidentity), and − = no reaction (no precipitin line).

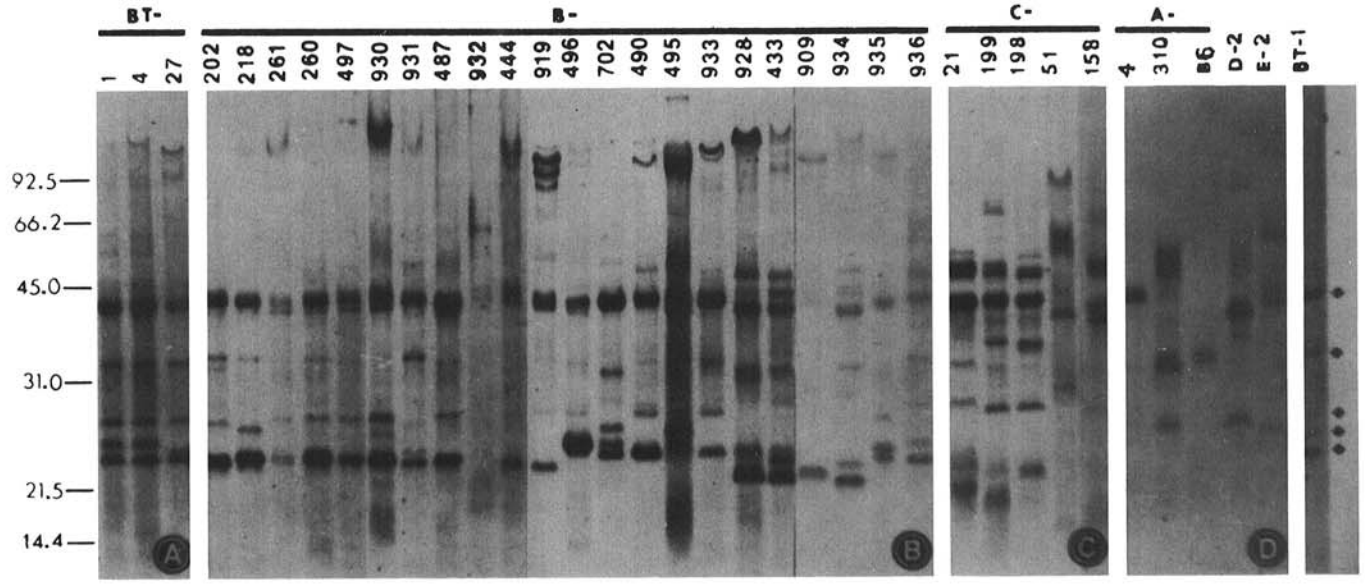

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of membrane proteins of strains of *Xanthomonas campestris* pv. *campestris*: **A,** strains representing serovars I, I-A, and II (left to right); **B,** strains of serovar I; **C,** strains of serovar I-A; **D,** strains of serovar II; and **E,** strains from different hosts and geographical regions. Strain numbers are listed at the top of each lane. Samples containing 10 μg of protein per milliliter, were heated at 100 C in Laemmli (11) sample buffer for 3 min before being applied to the sample wells. Relative molecular masses (kDas) of standard proteins are indicated to the left of the figure. Dark diamonds on the right denote major peptides.

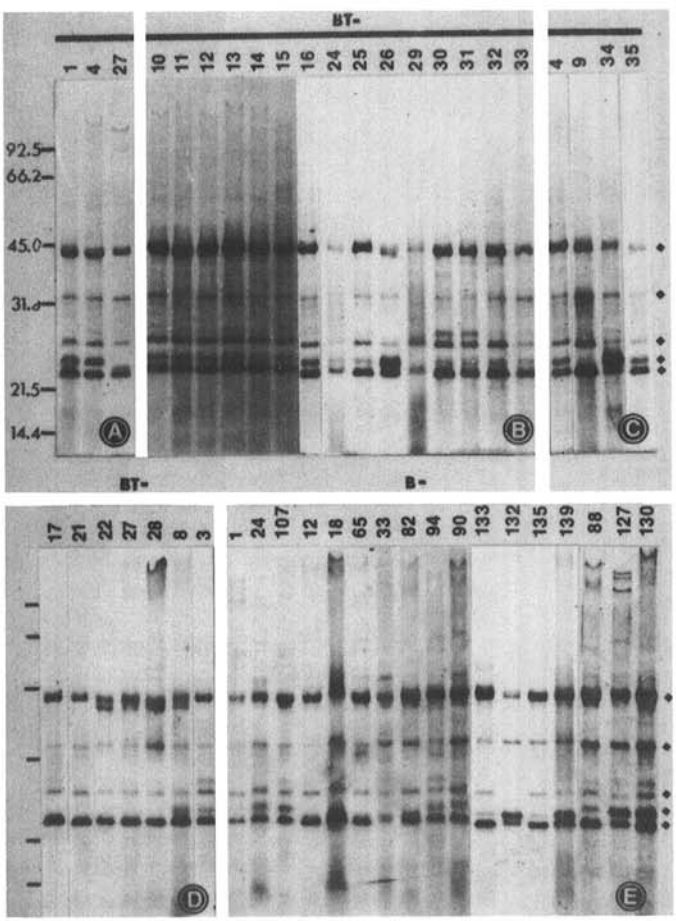

Fig. 6. Comparative sodium dodecyl sulfate-polyacrylamide electrophoresis SDS-PAGE profiles of membrane proteins of **A,** *Xanthomonas campestris* pv. *campestris*; **B,**other xanthomonads; **C,** pseudomonads; and **D,** five other bacteria. Strain numbers are given at the top of each lane. Ten μl of sample containing 10 μg of protein was applied to each lane. Samples were heated at 100 C in Laemmli (11) sample buffer for 3 min before being applied to the sample well. Relative molecular masses (kDas) of standard proteins are indicated at the left of the figure and the relative molecular masses of the major peptides are marked as dark diamonds.

bromophenol blue as a tracking dye. A horizontal electrophoresis cell (Buchler Instruments, Fort Lee, NJ) kept at 10 C and was used with buffer A as the electrophoresis buffer. The gels were electrophoresed at a constant 10 V/cm until the tracking dye had migrated 40 mm toward the anode. The gels were immediately transferred to a humidity chamber for 10 min at room temperature and flooded with 50 μl of appropriate antiserum or normal serum. Diffusion was allowed to occur overnight at 4–5 C. The gels were stained with 0.05% Coomassie brilliant blue R250 (Sigma Chemical Co., St. Louis, MO) as previously described (2).

Indirect immunofluorescence tests were performed (17) with IgG of BT-1, BT-4, and BT-27. Preimmune sera were used as controls. Block titration tests were used to determine optimum ratios between IgGs and goat anti-rabbit affinity purified IgG heavy and light chain conjugated to dichlorotriazinylaminofluorescein (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Stained preparations were examined under epifluorescence with a ×100 Neofluar objective fitted to a Zeiss microscope. A 50-W mercury lamp was used as the exciting light source.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The discontinuous SDS-PAGE system of Laemmli (12) was performed in a Bio-Rad Protean dual slab cell. Acrylamide concentrations of stacking and resolving gels were 4.5% and 10.0%, respectively. Gels (37.5 to 1 or 32.0 to 1 ratio of acrylamide-to-Acrylaide [FMC Corp.]) were cast on GelBond PAG film (FMC Corp.) as described by the manufacturer. Stock samples were adjusted to 2 mg of protein. For electrophoresis, 50 μl of sample were mixed with 50 μl of sample buffer and heated at 100 C for 3 min (7). Five microliters of tracking dye was added to each sample mixture. The samples containing 5–10 μg of protein were overlayed onto the sample well of preelectrophoresed (1 hr) gels and electrophoresed at a constant current of 20 mA/gel for 2 hr at 10 C. Gels were stained in 0.05% Coomassie brilliant blue R250 (Sigma Chemical Co.). Molecular weights of peptides were estimated by comparing their band positions with those of Bio-Rad low-molecular-weight protein standards (7).

Similarity coefficient of the composite relative mobility (R_f) values (21) of *X. c.* pv. *campestris* and other bacteria were determined from an average R_f value of each peptide obtained from triplicate gels. The resulting coefficients were used to determine relationships among the strains as previously described (14,22).

Absolute migration distances of peptide bands were not exactly reproducible because of variation in preparation of gels and

conditions of electrophoresis. To aid in comparison of peptide bands of different gels, protein of *X. c.* pv. *campestris* strain BT-1 was incorporated into each gel at wells 2, 10, and 19. The R_f of each strain could then be compared by using a correction factor (21).

RESULTS

Titers of antisera. Titers of antisera to the seven homologous strains of *X. c.* pv. *campestris* ranged from 1/16 to 1/1,024 depending upon the strain. All antisera cross agglutinated to some degree with cells of heterologous strains, but no agglutination occurred with preimmune sera or saline.

Ouchterlony double diffusion tests. All antisera to membrane proteins of the seven homologous strains resulted in one major line of precipitin and from none to three minor lines of precipitin (Fig. 1A, B, and D, wells 1, 3, 5, and 7) when tested against homologous membrane proteins. Antiserum to strain BT-27 produced a single major precipitin line of complete fusion against homologous membrane proteins (Fig. 1C, wells 1, 3, 5, and 7). However, this antiserum showed additional minor lines of precipitin when reacted with heterologous strains (Fig. 1C, well 6). The major precipitin line near the antigen well was defined as a strain-specific precipitin line (Fig. 1A, wells 1, 3, 5, and 7 and Fig. 2A, wells 1, 2, 3, 5, 6, 7, and 8). One to several minor nonspecific precipitin lines usually formed closest to the antiserum well (Figs. 1A and 2A, wells 4) Based on the seven antisera and the strain-specific line of precipitin, 35 strains of *X. c.* pv. *campestris* from Thailand grouped into three serovars (Ser) (Ser I, Ser I-A, and Ser II) containing 24, four, and seven strains, respectively (Table 2). Strains of Ser I and Ser I-A resulted in a reaction of partial identity, (Fig. 1C, wells 4, 6, and 8) when reacted with antisera to Ser II. In contrast, reciprocal tests with antisera to Ser I and membrane proteins of Ser I-A and Ser II resulted in three and two minor lines of precipitin, respectively, but no specific line of precipitin (Fig. 1A, wells 4 and 6). Ser I strains were serologically identical (Fig. 1, well 4) and distinct from strains of Ser II (Fig. 1B, well 6) when tested against antisera to Ser I-A.

Of the 36 other bacteria tested, only *X. campestris* pv. *vesicatoria* and *X. campestris* pv. *incanae* reacted (Table 3; Fig. 1D, well 4; and Fig. 2D, wells 6 and 8). Those strains isolated from cruciferous weeds cross reacted (true spur) (Fig. 3A to D, well 8). Strains B-33, B-82, B-85, B-94, B-127, B-132, and B-139 failed to react with any of the seven antisera (Fig. 1D, well 8 and Fig. 3A to D, well 6). Except for four strains of *X. c.* pv. *vesicatoria* (Fig. 2D, wells 6 and 8) and *X. c.* pv. *incanae* strain B-396 (Fig. 1D, well 4), no membrane proteins of other bacteria reacted with antisera to membrane proteins of *X. c.* pv. *campestris* (Table 3, Fig. 3E and F).

Specific antibodies of all serovars were completely eliminated after absorption with membrane proteins of the same serovar but not after absorption with the membrane proteins of a different serovar. An exception was antiserum of Ser I-A absorbed with BT-16 (Table 4); Fig. 2B, C, and E) or *X. c.* pv. *vesicatoria* strain B-202 (Fig. 2D and F). However, membrane proteins of BT-16 and B-202 removed specific antibodies from antisera of BT-4 (Ser I-A) and BT-27 (Ser II), respectively. In addition, antibodies responsible for nonidentity (Ser I-A) or partial identity (Ser II) were eliminated after being absorbed with membrane proteins of B-202 (Table 4; Fig. 2F, wells 6 and 8) or heterologous membrane proteins (Table 4 and Fig. 2E, wells 2 and 4).

Indirect immunofluorescence tests. Among 18 strains of *X. c.* pv. *campestris*, 14 strains were immunofluorescent positive against IgG of each of the three serovars of *X. c.* pv. *campestris* (Table 5). Three of the four strains which gave a positive reaction with only one or two of the three serovars were isolated from weeds (Table 1). In general, no consistent difference in intensity of fluorescence was observed among serovars (Table 5). Four strains of *X. c.* pv. *vesicatoria* and one strain each of *X. c.* pv. *incanae* and *X. campestris* pv. *manihotis* were fluorescent positive with IgG to two of the three serovars (Table 5). However, no fluorescence was noted with the other 14 xanthomonads and 10 miscellaneous bacteria to any of the IgGs tested.

Immunoelectrophoresis. Depending on the antiserum, two to four immunogens were detected in immunoelectrophoresis by using crude antisera to membrane proteins (Fig. 4). With antiserum to membrane proteins of Ser I, one immunogen (basic) migrated toward the cathode, whereas the other three immunogens (neutral) remained near the sample well (Fig. 4A). In contrast, antisera of Ser II resulted in one acidic immunogen and three neutral immunogens. However, with heterologous membrane proteins, an acidic immunogen was not observed (Fig. 4B, upper and lower sample wells).

With antiserum of BT-27 two neutral immunogens were observed (Fig. 4C third well from top). A similar result was observed with antiserum to BT-4 (Fig. 4D). Whether in homologous or heterologous systems, the neutral immunogens were always present. The specific line of precipitin observed in Ouchterlony double diffusion reactions was identified as the neutral immunogen (Fig. 4, triangle). In some tests, the specific immunogen formed very close to the second neutral band and, therefore, was very difficult to distinguish. Immunoelectrophoresis of IgG resulted in the removal of some nonspecific bands and often reduced the intensity of most immunoprecipitates.

Comparison of SDS-PAGE profiles of membrane proteins. SDS-PAGE of membrane proteins of *X. c.* pv. *campestris* and most of the other bacteria that were tested contained four to five major

TABLE 5. Indirect immunofluorescent staining of cells of *Xanthomonas campestris* pv. *campestris* and other bacteria with immunoglubulin G (IgG) of each serovar

Cells from:		IgG Serovar I	IgG Serovar I-A	IgG Serovar II
Pathovar or species		(BT-1)	(BT-4)	(BT-27)
Xanthomonas campestris				
pv. *campestris*	BT-1,16	4[a]	2+	4+
	BT-4,34	2+	3+	4+
	BT-17,27	2+	4+	4+
	B-1	2+	4+	4+
	B-18,24,65	3+	4+	4+
	B-90	2+	3+	1+
	B-33,94,127	1+	2+	3+
	B-130,133	−	1+	2+
	B-85,132	−	1+	−
pv. *vesicatoria*	B-202	2+	1+	4+
	B-218,260,261	2+	1+	−
pv. *incanae*	B-936	2+	1+	1+
pv. *manihotis*	B-932	−	3+	2+
	B-497	−	−	−
pv. *vitians*	B-938	−	−	−
pv. *cerealis*	B-934	−	−	−
pv. *begoniae*	B-935	−	−	−
pv. *malvacearum*	B-497	−	−	−
pv. *pelargonii*	B-937	−	−	−
pv. *oryzae*	B-444,919	−	−	−
pv. *phaseoli*	B-496,490,933	−	−	−
pv. *translucens*	B-428,433,909	−	−	−
Pseudomonas syringae				
pv. *syringae*	C-7	−	−	−
pv. *coronafaciens*	C-21	−	−	−
pv. *phaseolicola*	C-199	−	−	−
pv. *tomato*	C-198	−	−	−
P. solanacearum	C-51	−	−	−
Erwinia carotovora var. *carotovora*	A-4	−	−	−
E. chrysanthemi	A-310	−	−	−
Agrobacterium tumefaciens	A-B6	−	−	−
Escherichia coli	D-2	−	−	−
Salmonella typhimurium	E-26	−	−	−

[a] Symbols: 4+ = strong fluorescence, 1+ = weak fluorescence, − = no fluorescence. Dilutions of IgG and conjugated fluorescence antibody of each serovar were as follows: IgG of Ser I and Ser I-A: DTAF conjugate = 1/128:1/128 and IgG of Ser II: DTAF conjugate = 1/64:1/64.

and several minor peptides (Figs. 5 and 6). The major peptide patterns of *X. c.* pv. *campestris* contained four or five major peptides with relative molecular masses of 44, 34, 29, 25, and 23.4 kDa (Fig. 5B to D). Most variations in the profiles occurred in the minor peptides with molecular masses of 46 to 120 kDa and the major 25-kDa peptide band. No peptide pattern difference was observed between Ser I and Ser I-A but these two serovars were distinctively different from Ser II (Fig. 5A). For example, the 25-kDa peptide of Ser II was missing from all but strains BT-3 and BT-8 (Fig. 5D). An increased amount of the 25-kDa peptide was observed with BT-26 (Fig. 5B) and BT-34 (Fig. 5C) when compared to other Ser I and I-A strains. Cruciferous weed strains B-85 and B-132 (Fig. 5E) contained less of the 44-kDa peptide and more of the 25-kDa peptide. Peptide patterns of *X. c.* pv. *campestris* (Fig. 5 and 6A) were very similar, whereas those of the other bacteria (Fig. 6B to D) were very dissimilar. Among the other bacteria, peptides of *X. c.* pv. *vesicatoria* (B-202, 218, 260, and 261) and *X. c.* pv. *incanae* (B-936) were most similar to *X. c.* pv. *campestris* (Fig. 6B). The predominant major 44-kDa peptide was common to most xanthomonads. Most differences between membrane proteins of *X. c.* pv. *campestris* and other xanthomonads occurred in the 23- to 34-kDa sizes. A coefficient of similarity of 80% was average among strains of *X. c.* pv. *campestris* isolated from plants of cultivated crops, as compared to 47% for strains isolated from weeds (Table 6). All other bacteria, except *X. c.* pv. *vesicatoria* and the unidentified strain of *Xanthomonas*, had coefficients of similarity to *X. c.* pv. *campestris* of 36% or less. The coefficients of similarity of *X. c.* pv. *vesicatoria* and the unidentified *Xanthomonas* were 43 and 49%, respectively (Table 6).

DISCUSSION

Membrane proteins of *X. c.* pv. *campestris* contain a subspecies-specific immunogen. A similar specificity of membrane proteins has been reported for *P. solanacearum* (20) and *E. chrysanthemi* (27). By using antisera to membrane proteins in comparative Ouchterlony double diffusion tests, 36 strains of *X. c.* pv. *campestris* from Thailand were grouped into three serovars (Table 3). Ser I-A was designated as a subserovar of Ser I because strains of Ser I resulted in reactions of identity with Ser I-A antisera, whereas a nonspecific precipitation band occurred when strains of Ser I-A reacted against antisera of Ser I (Table 3). There was no correlation between serovars and symptoms on crucifers (23), but there was a positive correlation between serovars and host of origin. Strains from weeds were distinct from strains from cultivated crucifers (Table 3 and Fig. 3). Furthermore, the specificity of antisera to membrane proteins was similar to the specificity of ribosomal antisera for Ser I and Ser II (23). Perhaps those strains most frequently associated with black rot in Thailand and the United States belong to the same serovar (Ser I). However, many cultivars grown in the United States and Thailand originated in Japan or Taiwan. Since most crucifer weed strains failed to react with antisera to membrane proteins of *X. c.* pv. *campestris* (Table 3), they were not typable. Whether these crucifer weed strains are true pathovars of *X. c.* pv. *campestris* or of a different species or subspecies can not be determined without further investigation.

The cross-reaction of *X. c.* pv. *vesicatoria* with antisera to membrane proteins of *X. c.* pv. *campestris* agrees with previous reports on studies of antisera to ribosomes (17,24). On the other hand, the serological relatedness of *X. c.* pv. *incanae* to *X. c.* pv. *campestris* has not been previously reported. Since *X. c.* pv. *incanae* infects *Matthiola incanae* L., a cruciferous plant, *X. c.* pv. *incanae* should be considered a true pathovar of *X. c.* pv. *campestris*. Our results also support *X. c.* pv. *vesicatoria* being a true pathovar of *X. c.* pv. *campestris*, however, the other serologically distinguishable xanthomonads are most likely not pathovars but subspecies or species.

Cross-absorption studies supported grouping of 35 strains of *X. c.* pv. *campestris* from Thailand into three serovars. The failure of *X. c.* pv. *vesicatoria* (B-202) to absorb specific antibody of BT-16 antiserum (Ser I) or BT-4 antiserum (Ser I-A) and positive absorption of BT-27 antiserum (Ser II) with B-202 suggests that B-202 is closely related to Ser II.

The strong fluorescence staining of B-202 with BT-27 antiserum correlated well with Ouchterlony double diffusion tests with absorbed antiserum (Fig. 2). The cross-reaction of *X. c.* pv. *manihotis* in immunofluorescence tests were not expected since no precipitation bands occurred in Ouchterlony double diffusion tests. This strain may share a trace amount of common antigen with Ser I-A and Ser II detected only by the more sensitive immunofluorescence tests.

Jones et al (9) separated *P. syringae* pv. *tomato* into serogroups by immunofluorescent tests with antisera to whole cells. However, our studies using an optimum antiserum and conjugate ratio

TABLE 6. Average similarities within and between taxons of SDS-PAGE profiles of membrane proteins[a]

Taxon		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Xanthomonas campestris pv. *campestris*																
A. Typical strains[b]	(42)[c]	80														
B. Atypical strains[d]	(14)	47	46													
C. pv. *vesicatoria*	(4)	43	32	44												
D. Other pathovars[e]	(20)	36	31	21	31											
E. Unidentified	(1)	49	32	21	42	100										
F. *Pseudomonas syringae* pv. *syringae*	(1)	0	2	0	3	0	100									
G. pv. *coronafaciens*	(1)	16	7	13	17	25	0	100								
H. pv. *phaseolicola* (1)		6	10	13	4	0	0	25	100							
I. pv. *tomato* (1)		6	10	13	4	0	0	0	75	100						
J. *P. solanacearum*	(1)	0	0	0	0	0	16.5	0	0	0	100					
K. *Erwinia carotovora* subsp. *carotovora*	(1)	30	0	15	37	40	0	0	0	0	0	100				
L. *E. chrysanthemi*	(1)	0	11	6	13	0	25	0	0	0	0	40	100			
M. *Agrobacterium tumefaciens*	(1)	23	16	19	5	0	0	0	0	0	0	0	0	100		
N. *Escherichia coli*	(1)	6	9	6	1	0	0	25	25	25	12.5	0	0	0	100	
O. *Salmonella typhimurium*	(1)	23	30	38	13	0	20	0	0	0	0	0	25	33	0	100
Taxon		(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)	(K)	(L)	(M)	(N)	(O)

[a] Similarity based upon major and minor polypeptide bands.
[b] Resulted in typical yellowing and black veins of cabbage seedlings following inoculations.
[c] Numbers in parentheses are numbers of strains tested.
[d] Resulted in localized black lesions, no yellowing or black veins.
[e] Representing 10 pathovars of *X. c.* pv. *campestris*.

obtained from block titration tests (17,18) failed to distinguish any serovars. These results emphasize that identifying strains by immunofluorescence tests is of questionable value (17). In overall immunofluorescent reactions, IgG of membrane protein antiserum was either equally as specific or slightly more specific than ribosomal antiserum. According to the results of immunoelectrophoresis, some nonspecific antibodies were removed during IgG preparation. Therefore, it is suggested that IgG be used in immunofluorescence tests to eliminate some of the nonspecific straining.

Membrane proteins of *X. c.* pv. *campestris* are easily distinguished from other bacteria by using SDS-PAGE profiles. Our results agree with Minsavage and Schaad (14) in that membrane proteins of *X. c.* pv. *campestris* are unique and distinct. Peptide profiles of membrane proteins of typical strains from cultivated crops were all very similar, whereas those of strains from crucifer weeds varied somewhat. Coefficients of similarity between profiles of the various xanthomonads were much higher with *X. c.* pv. *campestris* than with pseomonads and the other bacteria that were tested. Among the xanthomonads, profiles of *X. c.* pv. *vesicatoria* and *X. c.* pv. *incanae* were closest to *X. c.* pv. *campestris*. We found a close relationship between similarity of SDS-PAGE peptide profiles and serology.

SDS-PAGE profiles of Ser I and Ser I-A strains were identical. Furthermore, the profiles of Ser I and Ser I-A were distinguished from profiles of Ser II by the absence of the 25-kDa band in profiles of most Ser II strains. On the other hand, two strains of Ser II (BT-8 and BT-28) contained the 25-kDa peptide (Fig. 5). These results suggest that peptide profiles do not dictate serological activities. A similar result occurred with membrane protein studies of *E. chrysanthemi* (27). In contrast, the 41-kDa peptide of membrane extracts of *N. meningitidis* was correlated with the specific serotype antigen (8).

Results of this study demonstrate that membrane proteins contain an immunogen of high specificity at the subspecies level. The SDS-PAGE profiles of membrane proteins showed that the bacteria tested possessed very few major membrane peptides. Furthermore, these peptides were unique within taxonomic groups. This suggests that membrane proteins may be useful for identification of phytopathogenic bacteria.

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