Characterization of Membrane Proteins of *Xanthomonas campestris* pv. *campestris*

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ABSTRACT

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Total envelope proteins of *Xanthomonas campestris* pv. *campestris* (hereafter referred to in this abstract as *X. campestris*) were purified by differential centrifugation of cells that were disrupted in a French pressure cell. The proteins were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Proteins of total envelopes of *X. campestris* presented a unique pattern when compared with profiles of proteins of other Gram-negative rod-shaped bacteria, including other pathovars of *X. campestris*. There were four major bands of 62, 44, 26, and 23 kdaltons and approximately 24 minor bands. Whereas no differences occurred in profiles of membranes of cells of different-aged cultures, differences were observed with a temperature shift from 30 to 37 C. Two proteins, the 44-kdalton major polypeptide and a 107-kdalton polypeptide, were found to be heat modifiable.

The cell envelope of Gram-negative bacteria is a metabolically important functional organelle of unique biological design. It is a complex, multilayered structure composed of a variety of structural molecules including proteins, phospholipids, and a lipopolysaccharide. Each structural layer of the envelope is characterized as a distinct zone with unique physiocochemical attributes (15). The cell surface components of the envelopes of bacterial pathogens, both plant (1,21,31) and animal (33), are known to play important roles in the processes of infection and determination of specific host-pathogen associations. A lipopolysaccharide (LPS) and proteins of Gram-negative bacteria are elements of the exposed surface that have been recognized in specific host-pathogen interactions. To understand the physiology of the host-pathogen relationship, it is necessary to identify and characterize the specific biochemical components involved.

Cell envelopes of Gram-negative bacteria are comprised of two phospholipid-protein bilayer membranes, the cytoplasmic or inner membrane and the outer membrane, with a layer of peptidoglycan in between (15). The characteristic functions of the inner membrane (IM) and outer membrane (OM) are the consequence of the particular composition molecular architecture of each membrane zone itself (13). Biochemical characterization of the cell envelope unveils the major importance of membrane proteins to both the structural and functional integrity of the organelle. To date, characterization of bacterial membranes has largely been confined to the Enterobacteriaceae and other bacteria of medical importance (15,18,25).

Little information is available describing the membrane proteins of phytopathogenic bacteria. The IM and OM of *Erwinia carotovora* have been isolated and partially characterized (26,32) and a total envelope protein profile has been shown for *Xanthomonas sinensis* (28). Proteins of the OM of *E. amylovora* (37) and the major heat-modifiable OM proteins of *Pseudomonas fluorescens*, a commonly encountered plant saprophyte, have been isolated and characterized (4). Dianese and Schaad (7) described a procedure for using a French pressure cell and a 45–70% sucrose density gradient centrifugation for isolating and separating the IM and OM of *X. campestris* pv. *campestris* (hereafter referred to in this paper as *X. campestris*) strain B-24. Succinate dehydrogenase activity was predominantly in the light density gradient fraction (largely IM) whereas xanthomonadin, a yellow brominated arylpolyene pigment (35), was found exclusively in the heavy fraction (largely OM). Six phospholipids were identified with three, lysophosphatidylethanolamine, phosphatidylethanolamine, and phosphatidylserine, predominating. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) resolved approximately 30 polypeptides in the total membrane fraction. The OM contained three major polypeptides (44, 26, and 23 kdaltons) whereas the IM contained only minor polypeptides.

The purpose of this study was to define the characteristic total membrane protein composition of several strains of *X. campestris* by SDS-PAGE and to describe and account for any variations in the typical protein profile associated with specific taxonomical and preparative differences.

MATERIALS AND METHODS

Bacterial strains and culture maintenance. Thirty-nine strains of *X. campestris* and 16 strains of other bacterial species or pathovars were used (Table 1). Stock cultures were maintained on slants of yeast extract-dextrose-calcium carbonate (YDC) agar as described (40).

Authenticity of strains of *Xanthomonas campestris*. Strains of *X. campestris* from several geographic regions were chosen for membrane extraction based on differences in virulence to cabbage, colony pigmentation, and host of origin. Colony morphology and growth characteristics of all strains were examined on YDC and SX agar (8). All strains were tested for protease activity by the plate overlay method (8). For pathogenicity tests, cultures were grown in liquid medium 523 (16) for 18 hr at 30 C on a tabletop shaker, and diluted 10^{-2} with sterile 0.85% NaCl (saline) as described (7). Three healthy seedlings of *Brassica oleracea* var. *capitata* 'Market Prize' (cabbage) at the two- to four-leaf stage were inoculated by injecting 0.1 ml using a 10-cc syringe fitted with a 0.51-mm (25-gauge) needle. Plants were left in the greenhouse and observed for symptom development for up to 14 days. Three plants were injected for each strain and the tests were repeated twice.

Growth of cells and isolation of total envelopes. Cultures were grown for 18 hr at 30 C on a New Brunswick G-25 rotary shaker in

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1-L Erlenmeyer flasks containing 440 ml of fresh 523 liquid medium and harvested by centrifugation. Total envelopes were obtained from pelleted and washed cells by differential centrifugation of cells disrupted in a French pressure cell as described (7), except that flagella were first removed in a Waring blender (6).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Methods were those as previously described. Briefly, total envelopes were solubilized in Laemmli sample buffer (17) by treatment at 100 C for 3 min, except when noted otherwise. Protein concentrations were determined by the method of Lowry (19), for samples in tris buffer or by a modification (20) of this procedure, for samples in tris buffer containing SDS (Sigma Chemical Co., St. Louis, MO 63178) or other detergents. SDS-PAGE was performed on a Bio-Rad model 221 electrophoresis unit at 15 C using the discontinuous system of Laemmli (17). Gels were removed from the electrophoresis apparatus, fixed in 12.5% trichloroacetic acid for 2–4 hr at room temperature, washed in distilled water, and stained overnight in 0.05% Coomassie Brilliant Blue R (Sigma Chemical Co.) in methanol:acetic acid:water (5:1:5, v/v). After destaining and photographing, gels were dried for permanent record on a slab gel dryer (Hoeffer Scientific Instruments, San Francisco, CA 94107). Molecule weights were calculated using the method of Weber and Osborn (42), using a Bio-Rad low-molecular-weight

TABLE 1. Strains used in the study of envelopes of *Xanthomonas campestris* pv. *campestris*

Laboratory strain no.	Received as: Name	Strain	Source	Location	Origin
B-1	pv. *campestris*	BBS	(1)[a]	California	*Brassica oleracea* var. *gemifera*
B-2	pv. *campestris*	Original		Georgia	var. *gongylodes*
B-3, 6	pv. *campestris*	Original		Georgia	var. *acephala*
B-4	pv. *campestris*	Original		Georgia	var. *italica*
B-18	pv. *campestris*	Original		Florida	Soil
B-24	pv. *campestris*		(2)	Oregon	var. *italica*
B-26	pv. *campestris*	Original		Georgia	*var. capitata*
B-28	pv. *campestris*	Original		Georgia	variant of B-24
B-30	pv. *campestris*	K-2	(3)	Germany	var. *rapa*
B-31	pv. *campestris*		(3)	Germany	variant of B-30
B-32	pv. *campestris*	8	(4)	New Zealand	var. *capitata*
B-33	pv. *campestris*	13	(4)	New Zealand	var. gemifera
B-36, 37, 67	pv. *campestris*	Original		Japan	var. *botrytis*[b]
B-65	pv. *campestris*	Original		Brazil	var. *capitata*
B-53, 70	pv. *campestris*	Original		Japan	var. *capitata*[b]
B-75	pv. *campestris*	Original		Washington	var. *capitata*
B-76	pv. *campestris*	Original		Florida	var. *capitata*
B-79	pv. *campestris*		(5)	Louisiana	*var. capitata*
B-82	pv. *campestris*		(6)	Australia	var. *botrytis*
B-85	pv. *campestris*	Original		Georgia	*Lepidium virginicum*
B-87	pv. *campestris*	Original		California	*B. nigra*
B-88	pv. *campestris*	Original		California	*B. nigra*
B-89	pv. *campestris*	Original		California	*Raphanus sativus*
B-90	pv. *campestris*	Original		California	*B. campestris*
B-92	pv. *campestris*	Original		California	*B. geniculata*
B-97	pv. *campestris*		(7)		Industrial strain
B-98	pv. *campestris*	Original		California	*Cardaria pubescens*[b]
B-107	pv. *campestris*	Original		California	var. *botrytis*[b]
B-110, 113, 114	pv. *campestris*	Original		California	var. *capitata*[b]
B-111, 112	pv. *campestris*	Original		Washington	var. *capitata*[b]
B-447	pv. *campestris*	102	(8)	Netherlands	var. *capitata*[b]
R-26	pv. *campestris*	Original		Georgia	B-24, Rif[r]
B-102	pv. *pruni*	XP-11	(9)		*Prunus persica*
B-210	pv. *vesicatoria*	68-4	(10)	Florida	*Lycopersicon esculentum*
B-412	pv. *malvacearum*	Xm-5	(9)	California	*Gossypium hirsutum*
B-430	pv. *translucens*	Original	(11)	Georgia	*Secale cereale*
B-444	pv. *oryzae*	PXO-79	(12)	Phillippines	*Oryza sativa*
B-460	pv. *phaseoli* (fuscans)	Xpf-16	(13)	Michigan	*Phaseolus vulgaris*
B-482	pv. *manihotis*	XM-5	(14)	Brazil	*Manihot esculenta*
C-7	*Pseudomonas syringae pv. syringae*	B-3	(15)	California	*Prunus persica*
C-107	pv. *coronafaciens*	Original		Georgia	*Secale cereale*
C-198	pv. *tomato*	3	(16)	Georgia	*Lycopersicon esculentum*
C-87	pv. *caryophylli*	B-1	(17)	New York	*Dianthus caryophyllus*
C-94	*P. fluorescens*	KC678	(18)		
C-158	*P. solanacearum*	51	(19)	Cylon	*Solanum tuberosum*
	Agrobacterium tumefaciens	AB6	(2)	Oregon	*Lycopersicon esculentum*
A-310	*Erwinia chrysanthemi*	B102	(20)	Florida	*Saintpaulia ionantha*
D-2	*Escherichia coli*	U5-41	(21)		Pig

[a] Source names and locations: 1 = R. G. Grogan, CA; 2 = L. Moore, OR; 3 = K. Rudolph, Germany; 4 = D. Dye, New Zealand; 5 = L. Black, LA; 6 = D. Trimboli, Australia; 7 = Northern Regional Research Laboratory; 8 = J. van Vruggink, the Netherlands; 9 = W. Schnathorst, CA; 10 = R. Stall, FL; 11 = B. Cunfer, GA; 12 = T. Mew, The Phillippines; 13 = W. Saettler, MI; 14 = A. Takatsu, Brazil; 15 = H. English, CA; 16 = S. McCarter, GA; 17 = R. Dickey, NY; 18 = R. E. Weaver, Center for Disease Control, Atlanta, GA; 19 = L. Sequiera, Wisconsin; 20 = J. Miller, Florida; and 21 = W. Ewing, Center for Disease Control, Atlanta.

[b] Isolated from seeds.

standard protein solution.

Comparison of total membrane polypeptide profiles of strains of *X. campestris* and other bacteria. R_f values for each protein band were calculated by dividing the distance from the origin by the distance from the origin to the solvent front marked by bromophenol blue. Composite R_f values were analyzed with a computer by the similarity coefficient method of Jaccard (34) by R. R. Colwell, University of Maryland. This program computes only similarity coefficients based on +/+ matches between two organisms (11) and similarities are calculated after computation of average linkage.

Effect of age of culture and temperature of growth on membrane polypeptide profiles. Cultures of *X. campestris* strains B-1 and B-24 were grown for membrane extraction as previously described but with changes in time and temperature of incubation before harvest of the cells. Cells were harvested and membranes extracted from 12-, 18-, 24-, and 72-hr cultures of strain B-1 grown at 30 C. Extracts of strain B-24 were prepared from 18- and 24-hr cultures grown at 30 C. A 24-hr culture was extracted for both strains grown at 37 C. Electrophoresis was performed as described above.

Separation of inner and outer membranes by sucrose density gradient centrifugation. Total envelopes of *X. campestris* strain B-24 were separated into inner, intermediate, and outer membrane fractions by using 45–70% sucrose step gradients (7). Briefly, the gradients were fractionated and collected using a ISCO gradient fractionator, UA-5 monitor, and automatic peak separator. Each band fraction was pooled, dialyzed against tris buffer, and the membranes were collected by centrifugation. Pellets were resuspended in 1 ml of tris buffer overnight at 4 C. Protein concentration was determined and the samples were stored at −20 C until used. Fractions were prepared for electrophoresis as described above. Coomassie Brilliant Blue-stained gels were scanned with a Photovolt densitometer (Photovolt Corp., New York, NY 10001) at 520 nm.

Identification of heat-modifiable polypeptides in the membranes of *X. campestris*. Freshly thawed total, inner, intermediate, and outer membrane samples were solubilized in Laemmli sample buffer and treated at different temperatures to identify heat-modifiable proteins as described (12,24). Total membrane fractions were treated at 37 C for 30 min or at 100 C for 3 min before electrophoresis.

To investigate the presence of peptiodoglycan-associated protein in the membranes, samples were prepared according to Mizumo and Kageyma (23). Briefly, total membranes of strain B-24 were solubilized in 2% SDS, 10%, glycerol in tris buffer at 37 C or 60 C for 60 min. The samples were then centrifuged at 130,000 *g* for 60 min at 4 C. The pellets, containing peptidoglycan and peptidoglycan-associated proteins, were washed once in tris buffer and prepared for electrophoresis as usual.

Solubilization of membrane polypeptides by different detergents. Solubilization of membrane proteins by different detergents was determined using 2.75 mg samples of membranes of strain B-24 in 1 ml of tris buffer. The samples were diluted 1:1 (v/v) in each of the following detergent solutions, all in tris buffer (w/v): 6% SDS, 3% sodium desoxycholate (DOC; Fisher Scientific Co., Fair Lawn, NJ 07410), 6% octyl phenoxypolyethoxyethanol (Triton X-100; Sigma Chemical Co., St. Louis, MO 63178), 4% hexadecyltrimethylammonium bromide (CTAB; J. T. Baker Chemical Co., Phillipsburg, NJ 08865), 6% polyoxyethylene [20] cetylether (Brij-58; Sigma Chemical Co.), 6% sodium monoheptadecyl sulfate (Teritol 7; J. T. Baker Chemical Co.), 0.6% polyoxyethylene sorbitan monolaurate (Tween-20; Sigma Chemical Co.), 6% polyoxyethylene [20] sorbitan monoolate (Tween-80; J. T. Baker Chemical Co.). The preparations were incubated at 37 C for 3 hr and then centrifuged at 190,000 *g* for 90 min at 4 C. The pellets were washed once in tris buffer and resuspended in 0.5 ml of the same buffer. The supernatant fractions were dialyzed at 20 C against three changes of 0.1% SDS in 3 L of tris buffer during 72 hr. Dialyzed samples were concentrated by freeze-drying and resuspending the proteins in 0.5 ml of tris buffer. Protein concentrations of the soluble and insoluble fractions were determined by using a modification (20) of the Lowry method. Electrophoresis was performed as described above.

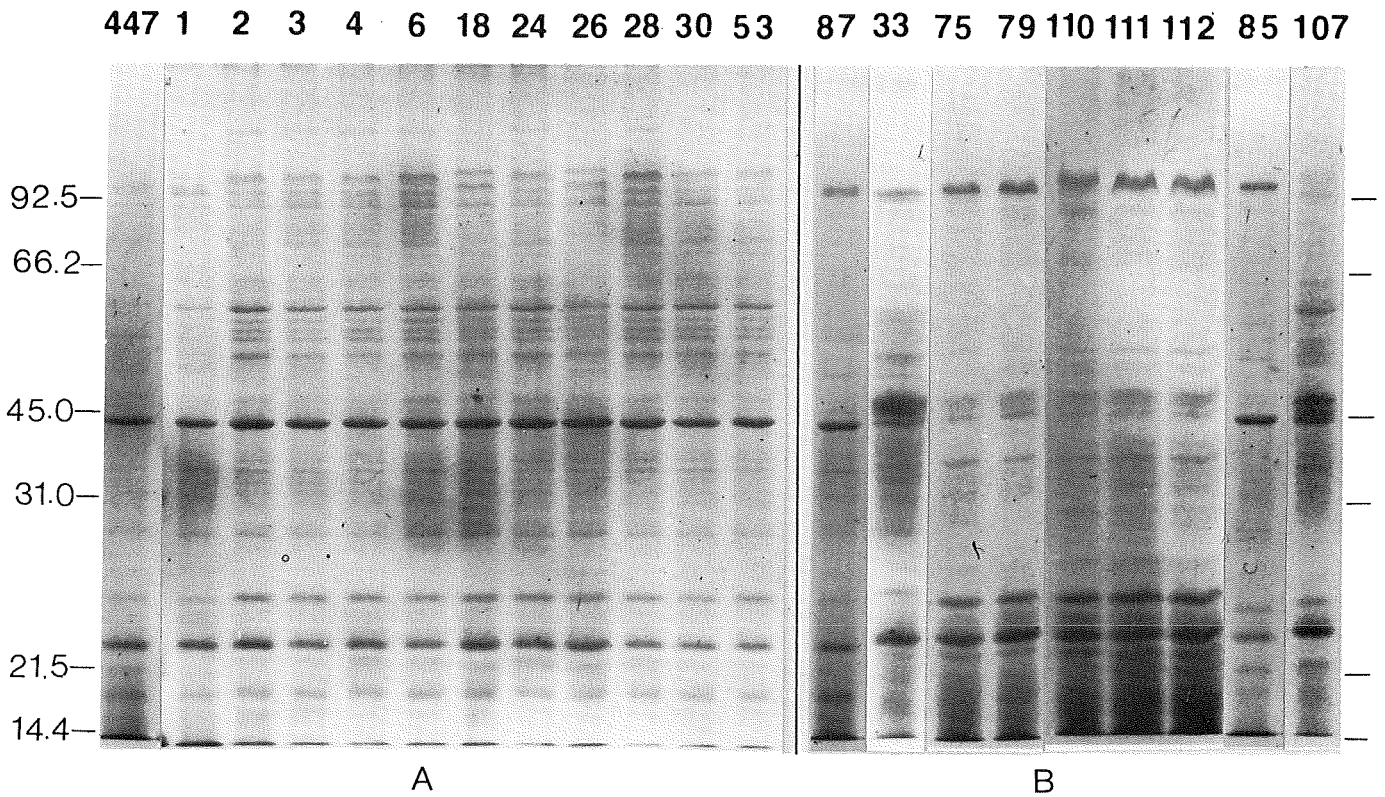

Fig. 1. SDS-PAGE profiles of total membrane proteins of 21 strains of *Xanthomonas campestris* pv. *campestris*. Strains are grouped according to virulence. **A,** Strains that resulted in typical black rot symptoms. **B,** Strains that were avirulent or that resulted in reduced virulence or vein blackening without chlorosis. Strain numbers are given at the top of each lane. Samples, containing 10 μg of protein, were boiled in Laemmli sample buffer for 3 min before being applied to the sample well. Molecular weights (kdaltons) of standard proteins are indicated to the left of the figure.

RESULTS

Authenticity of strains of *X. campestris*. All strains used in this study produced typical yellow mucoid colonies on YDC agar, hydrolyzed starch on SX agar, and were protease positive. Strains B-32, B-33, B-70, B-79, B-82, B-87, B-89, B-111, and B-114 produced colonies with a somewhat darker yellow pigmentation on YDC agar whereas colonies of strains B-30 and R-26 were lighter yellow. All strains, except B-33 and B-75, were pathogenic to cabbage. Strains B-85, B-87, and B-107 were less virulent, producing typical black rot symptoms, but only after 4–6 days of additional incubation. Strains B-70, B-79, B-110, B-111, B-112, B-113, and B-114 produced a general blackening of the veins, but no yellow chlorosis. In addition, the blackening was less pronounced. These 12 strains were designated as atypical in reference to virulence.

Comparison of polypeptide profiles of total envelopes of strains of *X. campestris* and other bacteria. The total membrane profile of *X. campestris* contained approximately 26 distinct polypeptide species of which the 62-, 44-, 26-, and 23-kdalton species (Fig. 1A) (hereafter referred to as "major" polypeptides) predominated. All 26 strains of *X. campestris*, which produced typical black rot symptoms on cabbage, possessed the four major polypeptides. Most variations in the profiles of the typical strains occurred only in the minor polypeptides of 86 to 112 kdalton and, less frequently, in the minor polypeptides between 54 kdalton and 60 kdalton and the major 62 kdalton polypeptide.

All atypical vein-blackening strains and avirulent strains B-33 and B-75 possessed the major 23- and 26-kdalton polypeptides (Fig. 1B). However, either the major 44 or 62-kdalton polypeptides and minor polypeptides from 54 to 60 kdaltons were greatly reduced or absent. These atypical strains had an increase in the quantity of polypeptides in the 86- to 112-kdalton range, often present in the form of a single, predominant polypeptide of approximately 93 kdaltons. Two of the strains (B-85 and B-107) with reduced virulence exhibited shifts in the 23- and 26-kdalton polypeptides to apparent molecular weights of 24 and 25 kdaltons, respectively (Fig. 1B).

Polypeptide profiles for the eight pathovars of *X. campestris* exhibited an overall similarity, but were clearly distinguishable from one another (Fig. 2, lanes A to H). The 62-kdalton polypeptide was shared in common by strains of all pathovars. Polypeptide profiles of the six species and pathovars of *Pseudomonas* (Fig. 2, lanes I to N) appeared less homologous with each other than did profiles of pathovars of *X. campestris* and were easily distinguishable from the xanthomonads (Fig. 2, lanes A to H). No single common major peptide was shared by all the pseudomonads. Several polypeptides of various apparent molecular weights were common between different strains of *Xanthomonas* and *Pseudomonas*. Polypeptide profiles of *Agrobacterium tumefaciens*, *Erwinia chrysanthemi*, and *Escherichia coli* (Fig. 2, lanes O to Q) were easily distinguishable from one another and from the xanthomonads and pseudomonads (Fig. 2, lanes A to N).

Coefficients of similarity among the typically virulent strains of *X. campestris* were 0.5 and 0.7 or greater for total (Fig. 3) and major (Fig. 4) polypeptides, respectively. On the other hand, coefficients of similarity among the atypical strains ranged from 0.2 to 0.5 and 0.2 to 0.7 for total and major polypeptides, respectively. All other bacteria, except *X. malvacearum*, had coefficients of similarity to *X. campestris* of 0.2 or less (Figs. 3 and 4). Coefficients of similarity of *X. malvacearum* (B-412) were 0.4 and 0.5 for total and major polypeptides, respectively.

Effect of growth conditions on membrane protein profiles. Variation in the time of incubation from 12 to 72 hr had no effect on the protein profiles of strain B-1 or B-24, whereas variation in temperature of incubation from 30 and 37 C did. When strain B-1 was grown at 37 C for 24 hr (Fig. 5, lane B), the minor 36-kdalton polypeptide present at 30 C (Fig. 5, lane A) was absent. In addition, growth at 37 C decreased the amount of 18-kdalton polypeptide. Growth of strain B-24 at 37 C (Fig. 5, lane D) resulted in a similar reduction and increase, respectively, in the relative amounts of the

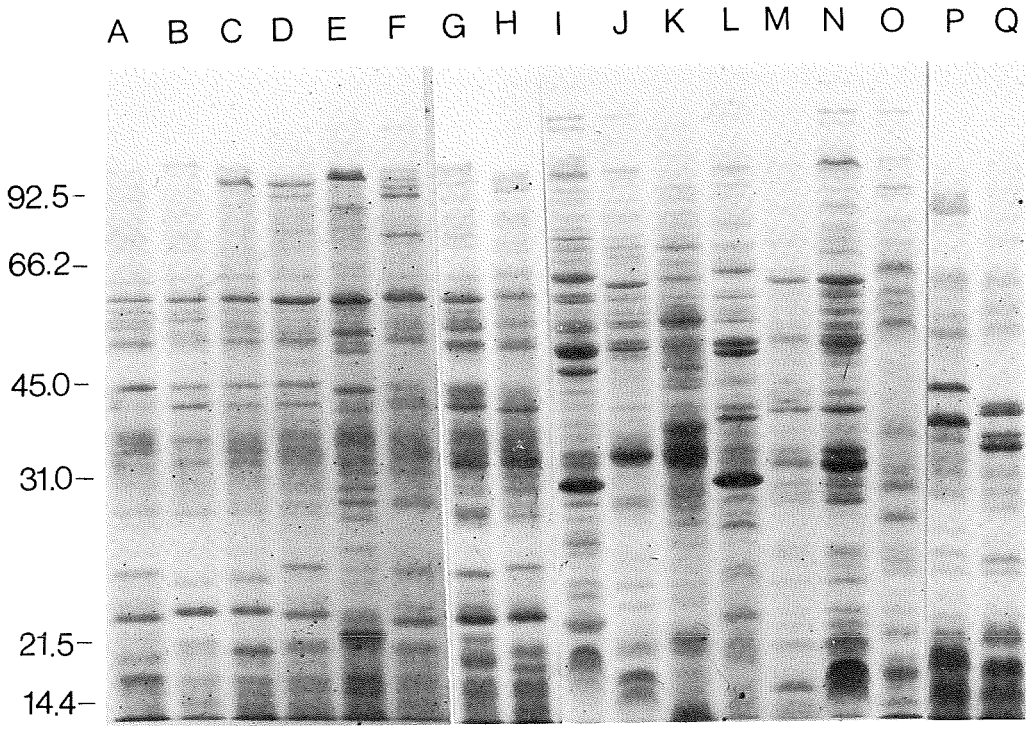

Fig. 2. SDS-PAGE profiles of total membrane proteins of several xanthomonads **(A–H)** and pseudomonads **(I–O)** and three other bacteria **(P–Q). A,** *Xanthomonas campestris* pv. *campestris* strain B-24; **B,** *X. campestris* pv. *pruni*; **C,** *X. campestris* pv. *vesicatoria*; **D,** *X. campestris* pv. *malvacearum*; **E,** *X. campestris* pv. *translucens*; **F,** *X. campestris* pv. *oryzae*; **G,** *X. campestris* pv. *phaseoli* (fuscans); **H,** *X. campestris* pv. *manihotis*; **I,** *Pseudomonas syringae* pv. *syringae*; **J,** *P. syringae* pv. *tomato*; **K,** *P. caryophylli*; **L,** *P. fluorescens*; **M,** *P. syringae* pv. *coronafaciens*; **N,** *P. solanacearum*; **O,** *Agrobacterium tumefaciens*; **P,** *Erwinia chrysanthemi*; and **Q,** *Escherichia coli*. Samples, containing 10 μg protein, were boiled for 3 min before electrophoresis. Molecular weights (kdaltons) of standard proteins are indicated to the left of the figure.

19- and 22-kdalton polypeptides (arrows). Furthermore, minor polypeptides of apparent molecular weights of 37 kdaltons and 28 kdaltons were absent (arrows). Finally, the 60-, 59-, 56-, and 54-kdalton minor polypeptides of strain B-24 were absent, while the relative amount of the major 62-kdalton polypeptides increased at 37 C (Fig. 4, lane D, arrow).

Identification of heat-modifiable polypeptides in the membranes of *X. campestris*. Treatment of total membranes at 37 (Fig. 6, lane A) or 60 C (Fig. 6, lane B) for 30 min gave identical polypeptide profiles, but these differed from those that received our standard treatment of 100 C for 3 min (Fig. 6, lane C). Testing different membrane fractions showed that the differences in the polypeptide profiles due to temperature variation were present in the OM (Fig. 6, lanes H and I), but not in the IM (Fig. 6, lanes D and E). Two sets of OM heat-modifiable proteins were noted. Treatment of OM at 37 C for 3 min resulted in the appearance of major bands at 38 kdaltons and 25 kdaltons and a minor polypeptide with an apparent molecular weight of 29 kdalton (Fig. 6, lane H). Upon treatment at 100 C for 3 min, these bands exhibited changed mobilities and shifted to different positions in the profile. The 38-kdalton band shifted to form a minor 37-kdalton polypeptide, and the major OM protein of 44 kdaltons (Fig. 6, lane I). This shift was confirmed by extraction of the 38-kdalton protein from a sample profile treated at 37 C in a gel, and rerunning the protein from the band with no additional treatment and after treating at 100 C for 3 min (*unpublished*). Treatment of OM at 100 C for 3 min resulted in a shift of the 25 kdalton and 29-kdalton polypeptides to apparent molecular weights of 26, 23, and 22 kdaltons (Fig. 6, lane A).

SDS-PAGE of peptidoglycan samples treated at 60 and 37 C indicated that the 44-kdalton major OM protein and the 107-kdalton OM protein were peptidoglycan-associated at 37 C (Fig. 6, lane J). No peptidoglycan-associated proteins were found in the 60 C sample.

Solubilization of membrane proteins by different detergents. Treatment of membranes with different detergents resulted in different polypeptide profiles and degrees of solubility (Table 2 and Fig. 7A and B). SDS-PAGE profiles of DOC- and CTAB-soluble proteins were identical (Fig. 7A, lanes 1 and 2), Most polypeptides present in the SDS-solubilized membrane sample (Fig. 7 A, lane 8) were present along with a new polypeptide with an apparent molecular weight of 48 kdaltons (Fig. 7 A, lanes 1 and 2). In addition, the amounts of the 62-, 44-, and 23-kdalton polypeptides were reduced. Some of the 23-kdalton protein missing in the CTAB soluble fraction was found in the CTAB insoluble (pellet) fraction (Fig. 7 B, lane 2). Profiles of the Tergitol 7 and Triton X-100 soluble fractions (Fig. 7 A, lanes 3 and 4) were nearly identical to the SDS profile, but the insoluble fractions differed significantly. The

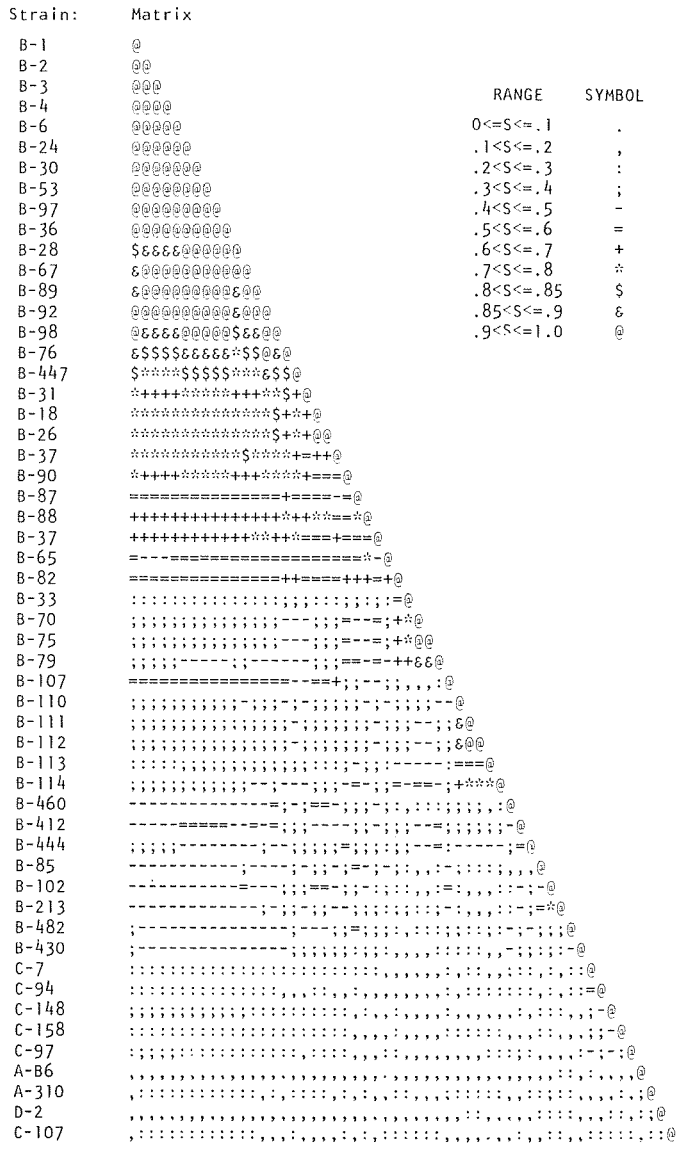

Fig. 3. Sorted and differentially shaded matrix based on the comparison of electrophoretograms of total membrane envelope proteins.

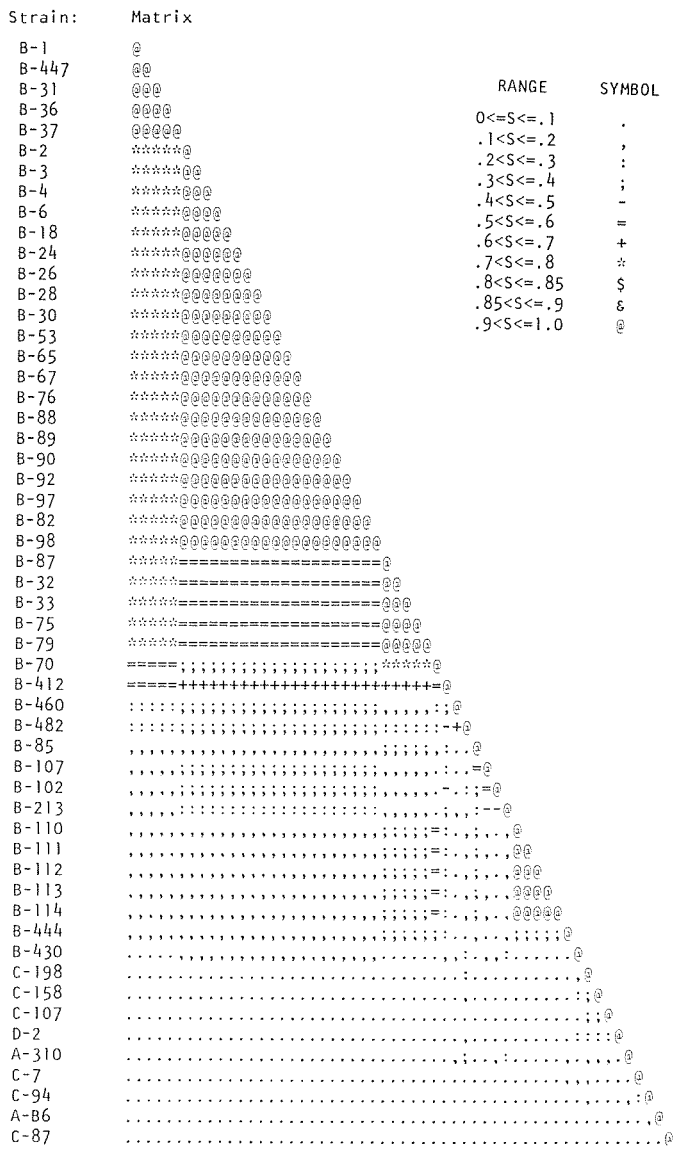

Fig. 4. Sorted and differentially shaded matrix based on the comparison of electrophoretograms of major membrane envelope proteins.

Tergitol-7 insoluble profile lacked the 105-, 93-, 89-, 86-, and 60-kdalton polypeptides, indicating a selective solubilization of these species (Fig. 7 B, lane 3). No polypeptides were visible in the Tween-80 and Brij-58 soluble fractions (Fig. 7 A, lanes 5 and 7). The pellet fractions from these detergents, as well as for the Tween-20 and Triton X-100 pellets, were similar to total membranes solubilized in SDS (Fig. 7 B, lanes 4–7). SDS-PAGE of the Tween-20 soluble fraction revealed a single major polypeptide band (arrow) with an apparent molecular weight of 65 kdaltons (Fig. 7 A, lane 6). This polypeptide was unique to the Tween-solubilized preparation.

DISCUSSION

The genus *Xanthomonas* has few distinguishing morphological and biochemical properties (36). Unequivocal laboratory identification of all xanthomonads is difficult without pathogenicity tests (8). The problem has been partially alleviated by including all but four species of *Xanthomonas* as pathovars of *X. campestris* (9). Differentiation at the genus level between *Xanthomonas* and *Pseudomonas* often must be based on differences in pigmentation.

SDS-PAGE, of membrane proteins of the 55 organisms, distinguished all 26 typical strains of *X. campestris* from the other bacteria based on visible differences in gel profiles and on computer determined similarity coefficients of R_f values. Similar results have been reported with membrane proteins of *Mycoplasma* species (27). Similarities in protein profiles of *X. campestris* were correlated with differences in virulence but not in colony morphology, as with *Salmonella typhimurium* (3), or pigmentation, as with *Neisseria gonorrhoeae* (41).

All strains of *X. campestris* that produced typical symptoms formed a tight group based on either total or major protein profiles. On the other hand, variations occurred between similarity coefficients of total and major protein strains of *X. campestris* that produced atypical symptoms. Five atypical strains had major protein similarity coefficients similar to typical strains, but only one such strain was similar with respect to total proteins. Also, whereas no other pathovar of *X. campestris* had a similar total membrane similarity coefficient to *X. campestris* of 0.3 or greater, one, *X. malvacearum*, had a major membrane similarity coefficient to *X. campestris* of 0.5.

Based on membrane profile similarity coefficients and virulence reactions, strains B-70, B-79, B-85, B-87, and B-107 should be considered as pathovars of *X. campestris*. These strains infect cabbage and have similar membrane protein profiles, but produce altered symptoms when compared to typical strains of *X. campestris*. Strains B-85 and B-107 are included because the minor shift of 1 kdalton of two major proteins was counted as negative by the computer yet perhaps should have been counted as positive. Because strains B-110, B-111, B-112, B-113, and B-114 differ significantly in protein profiles and virulence, they should probably be considered as subspecies of *X. campestris*. However, the term pathovar is already used for such organisms as *X. manihotis* and *X. translucens*. It is clear from results of membrane protein analysis and nutritional differences that these other xanthomonads such as *X. manihotis* and *X. translucens* are not simply pathovars of *X. campestris*. We conclude that true pathovars of *X. campestris* do exist and that the current list of pathovars of *X. campestris* is untenable. Only one pathovar, *X. malvacearum*, could be considered closely related to *X. campestris* based upon membrane proteins. Since most atypical strains of *X. campestris* originated from crucifer seed or weeds, such strains should be designated differently for etiological reasons. Strains B-79 and B-82 were obtained as atypical, low temperature, black-flecking strains. Strain B-82 should be considered as *X. campestris*. On the other

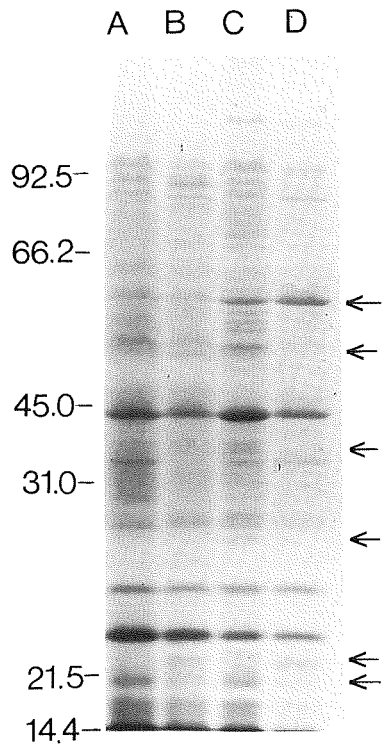

Fig. 5. Effect of temperature of growth of *Xanthomonas campestris* pv. *campestris* on SDS-PAGE membrane protein profiles. Strain B-1 at **A**, 30 C and **B**, 37 C for 24 hr and strain B-24 at **C**, 30 C and **D**, 37 C for 24 hr. Figures to left are standard protein molecular weights (kdaltons). Arrows point to polypeptides that varied with temperature of growth.

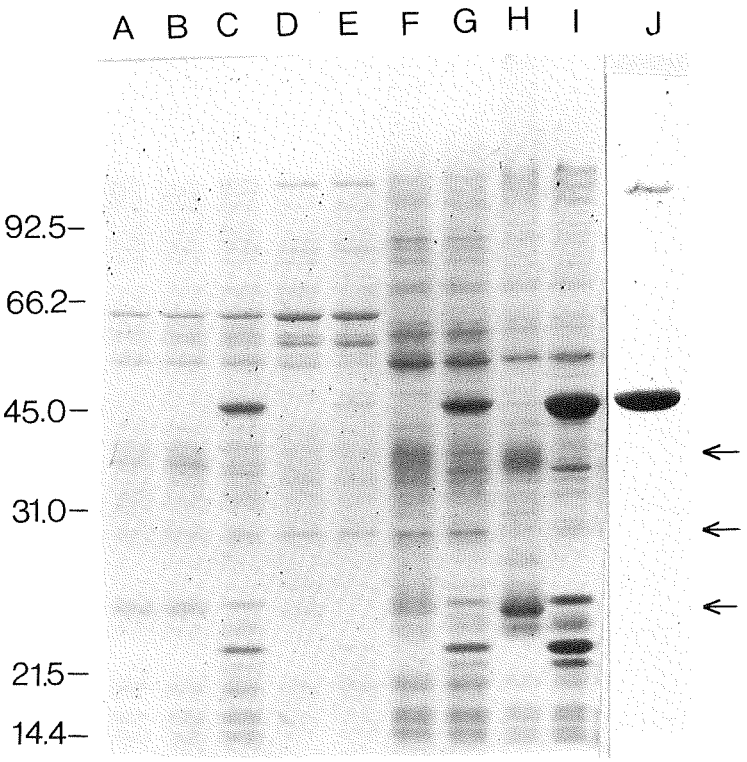

Fig. 6. Effect of temperature of solubilization of SDS-PAGE profiles of total envelope and membrane fractions of *Xanthomonas campestris* pv. *campestris* strain B-24. Total membrane incubated 30 min at **A**, 37 C, **B**, 60 C, and **C**, 100 C. Inner membrane incubated 30 min at **D**, 37 C, and **E**, 100 C, for 3 min. Intermediate membrane fraction incubated 30 min at **F**, 37 C, and **G**, 100 C, for 3 min. Outer membrane incubated 30 min at **H**, 37 C, and **I**, 100 C, for 3 min. **J**, Peptidoglycan-associated proteins at 37 C (sample was boiled for 3 min before being placed in the well). A total of 10 μg of protein was added per well. Molecular weights (kdaltons) of standard proteins are indicated to the left of the figure. Arrows to the right of the figure: upper arrow shows the 38-kdalton polypeptide in lanes F and H, middle arrow shows the 29-kdalton polypeptide in lane H, and the lower arrow shows the 25-kdalton polypeptide in lane H.

hand, B-79 should be considered as a pathovar of *X. campestris* because it produced atypical symptoms even though it had a similar membrane protein profile.

Although all 26 typically virulent strains of *X. campestris* have nearly identical protein profiles that are distinguishable from the other xanthomonads, these results can be discussed only in terms of serving as a preliminary indication of their actual relationships. Additional study of other xanthomonad and pseudomonad strains will help establish the value of total- and/or major-membrane profiles in bacterial taxonomy. Whereas the total polypeptide system represents a simple +/− comparison, the use of major polypeptides entails the arbitrary distinction of major versus minor polypeptides. Differentiation between major or minor polypeptides is further complicated by possible changes in the relative amounts of specific proteins in different culture growth conditions.

For most Gram-negative bacteria, structural OM proteins remain unchanged over a wide range of growth conditions (2,22). Variations in age of culture (39) and temperature of incubation (5), however, cause changes in the profiles of several major and minor polypeptides. Changes in single proteins and covariant increases or decreases in two or more proteins is reported for *N. meningitidis* (10) and *S. typhimurium* (2) grown under different culture conditions. The major OM proteins of *P. aeruginosa* are unaffected by changes in the temperature, but vary with culture media (22). Membrane profiles of *X. campestris* are unaffected by different-aged cultures. However, single and covariant changes in polypeptides species occur with a temperature shift from 30 to 37 C. Alterations noted in both IM and OM polypeptides of *X. campestris* caused by a shift to a higher temperature could be a result of decrease in growth since optimal growth is at 28–30 C.

The 44-kdalton polypeptide of *X. campestris*, like the 33.5-kdalton protein of *E. coli* (4,25), is heat-modifiable and appears to be peptidoglycan-associated at 37 C. The 44-kdalton polypeptide is therefore considered to be the outer membrane protein A (25) (OmpA) protein for *X. campestris*. An OmpA-like protein has been observed in membranes of most Gram-negative bacteria, including *E. amylovora* (37) and *E. carotovora* (26), and is an excellent taxonomic marker.

All typical strains of *X. campestris* had the 44-kdalton polypeptide, but it was reduced or absent in all strains with atypical virulence. The 44-kdalton polypeptide may play a role in determining the virulence of *X. campestris*. A mutation at the OmpA locus was associated with altered virulence in *N. gonorrhoeae* (43). LPS defects in mutants of *S. typhimurium* with altered virulence is associated with OM proteins (3). Genetic and biochemical studies are needed to confirm the true role of the

TABLE 2. Solubilization of total membrane proteins of *Xanthomonas campestris* pv. *campestris* strain B-24 by different detergents

Detergent	Type	Final solubilization concentration (%)	Protein solubilized[a] at 37 C for 3 hr (%)
SDS[b]	Anionic	3.0	95
DOC	Anionic	1.5	90
CTAB	Cationic	2.0	88
Tergitol 7	Anionic	3.0	41
Triton X-100	Non-ionic	3.0	25
Brij-58	Non-ionic	3.0	2
Tween-20	Non-ionic	3.0	2
Tween-80	Non-ionic	3.0	2

[a] Percentage, by weight, of initial total membrane sample (2.75 mg) present in the soluble fraction of each detergent extraction, after dialysis against 0.1% SDS in tris buffer, determined by a modification (19) of the Lowry method.

[b] SDS (sodium dodecyl sulfate), DOC (sodium desoxycholate), CTAB (hexadecyltrimethylammonium bromide), Tergitol 7 (sodium monoheptadecyl sulfate), Triton X-100 (octyl phenoxypolyethoxyethanol), Brij-58 (polyoxyethylene cetylether), Tween-20 (polyoxyethylene sorbitan monolaurate) and Tween-80 (polyoxyethylene sorbitan monooleate).

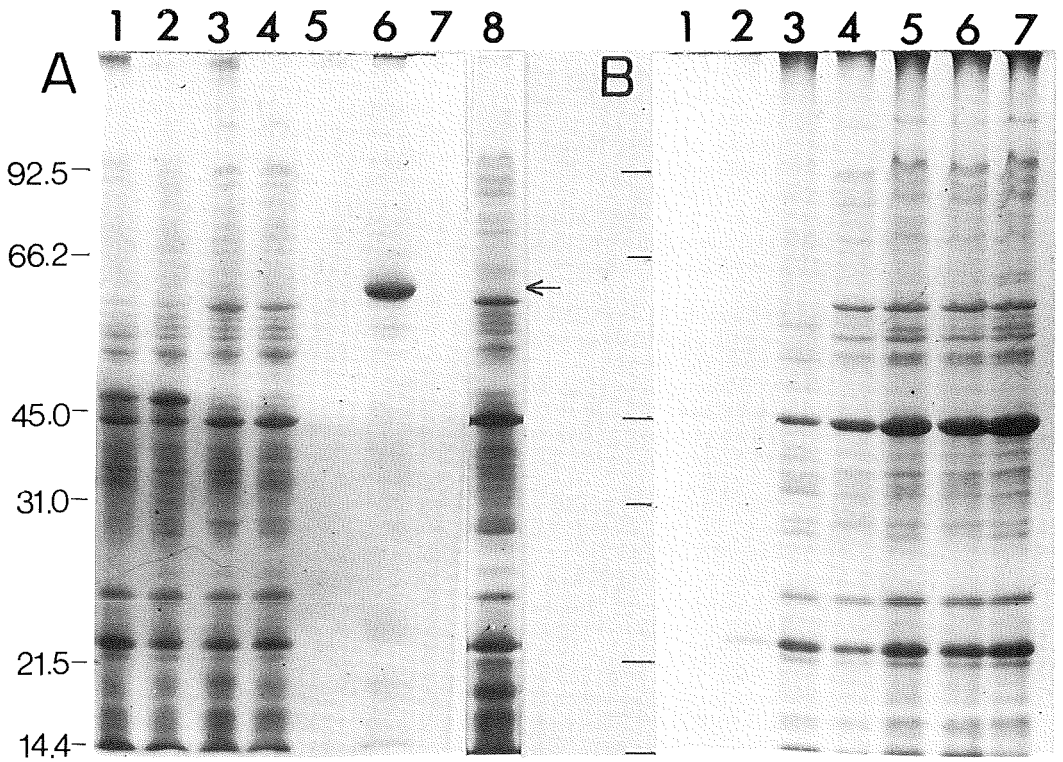

Fig. 7. Solubilization of proteins of total membranes of *Xanthomonas campestris* pv. *campestris* strain B-24 by different detergents at 37 C. **A,** Soluble fractions of 1) DOC, 2) CTAB, 3) Tergitol 7, 4) Triton X-100, 5) Tween-80, 6) Tween-20, 7) Brij-58, and 8) SDS. **B,** Insoluble (pellet) fractions as above. Samples containing 10 μg of protein were treated at 100 C for 3 min in Laemmli sample buffer before electrophoresis. The arrow indicates the position of the 65 kdaltons polypeptide in the Tween-20 soluble fraction (lane 6). See Table 2 for abbreviations.

OmpA-like protein in the virulence of *X. campestris*.

Proteins associated with membranes are termed peripheral (extrinsic) and integral (intrinsic [15]). Peripheral proteins can be released by chelating agents and by lowering or increasing the ionic strength of pH of the extraction buffer. Tightly bound integral proteins can only be solubilized by disrupting the membrane with organic solvents or detergents. Triton X-100 treatment of unfractionated cell envelopes of *E. coli*, in the presence of small amounts of Mg^{++}, results in the specific solubilization of only IM proteins (29). No major specific solubilization of IM proteins of *X. campestris* was found. Nonspecific Triton X-100 solubilization, in the absence of Mg^{++}, of 25% of the total protein of the membranes of *X. campestris* was comparable to previous reports of 15–28% for *E. coli* (29), and 58% of *P. aeruginosa* at 35 C (12). The apparent specific solubilization of a single major polypeptide of 65 kdaltons by Tween-20 suggests the possible use of differential solubilization to obtain single antigens for serological identification. However, the presence of a single major band following solubilization with such weak non-ionic detergents can result from aggregation of polypeptides into mixed micelles (38) or from the hydrophobic interactions between two proteins being greater than the interaction between the detergent and the protein (14). One protein band does not necessarily imply homogeneity of the protein. The major 40 kdalton polypeptide of *E. coli* has been differentiated into two, three, and four distinct bands of different molecular weights by varying the electrophoresis systems and conditions (24). The existence of multiple protein components with similar properties and molecular masses is not unique to *E. coli* and could occur in membranes of other organisms as well (30). Major proteins (eg, the 44-kdalton polypeptide) offer more promise for obtaining specific antiserum for serological identification of bacteria than do preparations of whole cells or total envelopes. Characterization of membrane proteins of plant pathogenic bacteria should improve serological techniques and increase understanding of bacterial taxonomy, serology, and host-pathogen interactions.

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