Comparative Membrane Characterization of *Xanthomonas campestris* pv. *cassavae* and *X. campestris* pv. *manihotis*

R. M. D. B. dos Santos and J. C. Dianese

Former graduate student and associate professor, respectively, Departamento de Biologia Vegetal, Universidade de Brasília, 70910, Brasília, DF, Brazil.

Portion of an M.S. thesis submitted to the Universidade de Brasília by the senior author, with support of a graduate fellowship of the Brazilian National Research Council (CNPq).

The authors thank E. W. Kitajima for the electron microscopy, M. Haridasan for the phosphorus analysis, N. B. de Lima for the photographic work, and J. R. P. Frazão for typing the manuscript.

Accepted for publication 15 November 1984.

ABSTRACT

Santos, R. M. D. B., dos, and Dianese, J. C. 1985. Comparative membrane characterization of *Xanthomonas campestris* pv. *cassavae* and *X. campestris* pv. *manihotis*. Phytopathology 75:581-587.

Cell envelopes of *Xanthomonas campestris* pv. *manihotis* (*Xcm*) ENA-2648 and *X. campestris* pv. *cassavae* (*Xcc*) CIAT-1165 were obtained by sonication in the presence of lysozyme and fractionated into two bands by sucrose density gradient centrifugation. In both pathovars, the light band (density = 1.142 g/cm^3) showed high nicotinamide adenine dinucleotide oxidase and succinate dehydrogenase activities, low contents of 2-keto-3-deoxyoctonate, and larger numbers of peptides in SDS-PAGE than are usually found in inner membrane. The heavy band (density = 1.255 g/cm^3) showed a high level of 2-keto-3-deoxyoctonate, low enzymatic activity, and characteristic outer membrane pattern in SDS-PAGE. Changes in incubation temperature did not affect the SDS-PAGE patterns of the cell envelope of *Xcm* ENA-2648; however, a major 15-kdalton protein present in *Xcc* CIAT-1165 changed into a minor component when incubation was at 27.5 and 35 C. Four strains of *Xcm* and five of *Xcc* revealed major peptide bands at 114, 100, 41, 28.5, 26, and 22 kdaltons in SDS-PAGE. Membranes treated with 2% SDS at different temperatures showed heat-modifiable proteins in cell envelopes of both pathovars. A 41-kdalton, heat-modifiable peptide, shared in common by both pathovars, is apparently the OmpA protein of *X. campestris*. Major differences in peptide profiles between strains of *Xcm* and *Xcc* in SDS-PAGE were detected which suggested that the Colombian strains of *Xcc* are phylogenetically more related to *Xcm* than to the African strains of *Xcc*.

The cell envelope of Gram-negative bacteria is a complex structure containing three morphologically distinct layers: the cytoplasmic or inner membrane (IM), the peptidoglycan layer, and the outer membrane (OM) which is externally bound to the intermediate peptidoglycan (41). Both the IM and the OM contain proteins and phospholipids. Lipopolysaccharide (LPS) is also a major component of the OM (37). The OM contains the sites for interaction with bacteriophages and bacteriocins and possesses the porin proteins responsible for the transmembrane transport of larger molecules (5,12,16,21,27,37,42). The OM proteins and LPS are apparently involved in interactions between plants and pathogens (6,29,32,50). Volk (55–57) isolated and characterized the LPS from whole cells of *Xanthomonas campestris* pv. *campestris* and *X. sinensis*.

Sequeira (50) found that pathogenic strains of *Pseudomonas solanacearum* produce an extracellular polysaccharide which functions as a blocker of the agglutinative action of plant lectins against the LPS of the bacterial cells. Only the avirulent cells of *Erwinia amylovora* were agglutinated by the lectins extracted from apple seeds (44), whereas the cell surface of virulent *Agrobacterium tumefasciens* is chemically attached to plant cells before pathogenesis can begin (29). Research is needed to explain the involvement of bacterial OM in these interactions with plant host cell surface. The available data on membranes of bacterial plant pathogens (11,51,54) is very limited compared to the abundant literature dealing with other Gram-negative bacteria (8,17,19,33,35,42,45). Moreover, attention must be paid to bacteria

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

pathogenic to tropical plants. An example is the two pathovars of *X. campestris* causing "bacterial blight" and "bacterial necrosis" on cassava (*Manihotis esculenta* Krantz), namely *X. campestris* pv. *manihotis* (Berthet and Bondar, 1915), Dye 1978 (*Xcm*) and *X. campestris* pv. *cassavae* Wiehe and Dowson 1953 (Maraite and Weyns, 1953) (*Xcc*) (13), respectively.

Comparative studies of the pathovars are limited to physiological observations (18,31) and the isozymic analysis by Kimura and Dianese (26). Membrane comparisons have not been used to characterize the two pathovars.

The objective of this study was to compare the membrane profiles of the cassava pathovars of *X. campestris* obtained by SDS-polyacrylamide gel electrophoresis and to describe the relationships between membrane composition, geographical origin of the strains, and their phylogeny.

MATERIALS AND METHODS

Isolates and culture maintenance. The sources of the isolates of *Xcc* and *Xcm* are presented in Table 1. Cultures were stored temporarily at 5 C in solid 523 medium (22) and preserved for long-term storage by the slow drying technique of Takatsu (53).

Bacterial cultivation and membrane isolation. Bacteria were grown on liquid 523 (22) at 140 rpm in a New Brunswick incubator-shaker at 30 C, except for one experiment in which temperatures of 27.5, 30, 32.5, and 37 C were compared. Fifteen-milliliter seed cultures in 523 were used to start 2.8-L Fernback flasks containing 1.5 L of the same medium. At the logarithmic phase of culture growth (absorbance at 600 nm between 0.8 and 0.9) the cells were harvested by centrifugation at 13,200 *g* for 20 min at 4 C. Cells were washed twice in cold tris-(hydroxymethyl)aminomethane-HCl (tris-HCl) 3.3 mM and pH 7.4. The pellet was resuspended in tris-HCl 10 mM, pH 7.4, in 0.75 M sucrose containing 100 μg of lysozyme per milliliter. This cell suspension was incubated for 10 min on ice. Osmotic shock for spheroplast production was applied by addition of two volumes of cold tris-HCl 3.3 mM, pH 7.4. Six 30-sec sonication cycles were applied to cold spheroplast suspensions with a Biosonik sonicator (Bronwill Scientific Co., Rochester, NY) operating at maximum power. The suspension obtained was incubated for 30 min at 5 C before centrifugation at 4,080 *g* for 15 min at 4 C to separate the whole cells. The pellet was discarded and the supernatant was centrifuged at 36,900 *g* for 1 hr to pellet the total membranes (TM). Resuspension was in a minimum volume of tris-HCl 3.3 mM, pH 7.4, in 0.25 M sucrose. The TM sample was then layered on top of a 70% (w/w) sucrose cushion and centrifuged for 3 hr in a Beckman SW 25.1 rotor at 75,000 *g*. The pellet was discarded and the purified TM was extracted from the cushion with a 10-ml plastic syringe. TM samples were stored at −20 C after they had been washed in tris-HCl 10 mM, pH 7.4.

Density gradient fractionation. TM samples with 7 mg of protein per milliliter were fractioned in a step gradient containing tris-HCl 10 mM, pH 7.4, and 12.8, 12.8, and 10 ml of sucrose at the molar concentrations of 0.77, 1.44, and 2.02, respectively. Gradients were centrifuged in a Beckman SW 28 rotor for 18 hr at 130,000 *g*. Fractionation was done with an ISCO model 640 (Instrumentation Specialties Co., Lincoln, NE) gradient fractionator coupled to an ISCO UA-5 absorbance monitor. Peak samples were pooled before a fivefold dilution with tris-HCl 3.3 mM, pH 7.4, was performed. Membrane samples from each peak were pelleted by centrifugation at 90,000 *g* for 3 hr and resuspended in tris-HCl 3.3 mM, pH 7.4, in 0.25 M sucrose. Fractions were stored at −20 C before chemical characterization. Fresh membrane preparations were always used in enzymatic assays. The density of the fractions was measured with a Zeiss/Jena refractometer at 20 C from 1-ml gradient fractions.

Membrane extraction with Triton X-100. Outer membranes were obtained by treating the TM with Triton X-100 as described by Schnaitman (47). One to 10 mg of TM protein was suspended in 2% Triton X-100 at 20 C for 20 or 40 min before centrifugation at 90,000 *g* for 3 hr at 4 C to separate insoluble OM fraction from the dissolved IM proteins.

Chemical analysis of the membranes. Protein was measured by a modified Lowry test (30) in which bovine serum albumin was used as a control. LPS was estimated by measuring the content of 2-keto-3-deoxyoctonate (KDO) of the membranes by the thiobarbiturate method (59). The pink chromogen was taken up in *n*-butanol and the absorbances were read at 552 and 508 nm. A molar extinction coefficient of 19×10^{-3} was used to determine the KDO concentration (25). Phospholipids were extracted from lyophilized membranes as described (15) and their concentration was determined by following the procedures of Murphy and Riley (36) and using 700 as the average molecular weight for the phospholipids (52).

Enzymatic assays. The activity of succinate dehydrogenase (SDH) was determined according to Kasahara and Anraku (24). Nicotinamide adenine dinucleotide (NADH) oxidase was measured as described by Orndorff and Dworkin (40). β-mannanase concentration was estimated by following the procedures of Dekker and Candy (10), and the reducing sugars were measured by using Nelson's method (39).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE in 0.1% SDS was performed according to Laemmli (28). Stacking gel containing 5% acrylamide was layered on top of a 16% acrylamide analytical gel between 14.5 × 16.2-cm glass plates separated by 1.5-mm spacers in a slab gel apparatus. Membrane

TABLE 1. Description of the strains of *Xanthomonas campestris* pv. *cassavae* (*Xcc*) and *X. campestris* pv. *manihotis* (*Xcm*) used in a comparative study of their cell membranes

Pathovar and strain	Location	Source
Xcc		
CIAT-1164	Colômbia	O. Kimura[a]
CIAT-1165	Colômbia	O. Kimura
NCPPB-101	África	O. Kimura
CIAT-1195	África	O. Kimura
Xcm		
Xm-35	Goiânia, Brazil	A. Takatsu[b]
Xm-48	Taiwan	A. Takatsu
Xm-55	África	A. Takatsu
ENA-2648	Brasília, Brazil	A. Takatsu

[a] Departmento de Fitopatologia, Universidade Federal Rural do Rio de Janeiro, Km 47 da antiga Rio-São Paulo, 23560 Seropédica, RJ, Brazil.
[b] Departamento de Biologia Vegetal, Universidade de Brasília, 70910, Brasília, DF, Brazil.

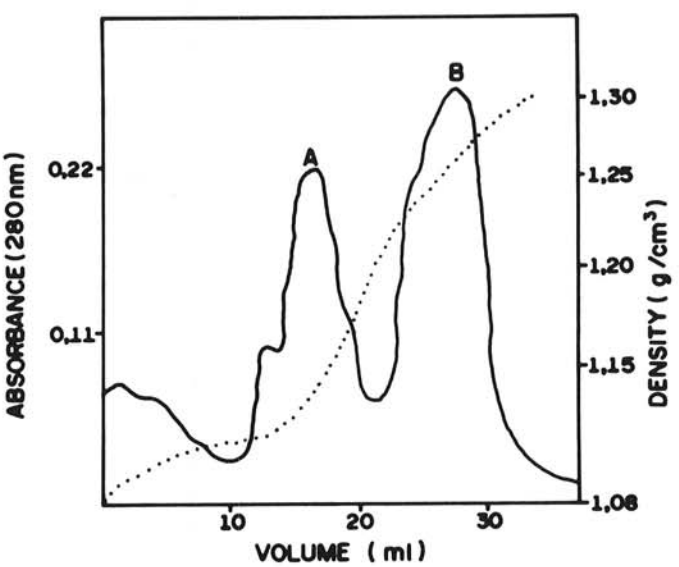

Fig. 1. Separation of inner (A) and outer (B) membrane fractions of *Xanthomonas campestris* pv. *manihotis* ENA-2648 in Schnaitman's sucrose density gradient centrifuged for 18 hr at 4 C and fractionated with an ISCO model 640 fractionator. The dotted line indicates density (grams per cubic centimeter) and the solid line indicates absorbance at 280 nm.

samples were solubilized in Laemmli sample buffer (28) at 100 C for 5 min, except for one experiment in which 25, 37, and 60 C were also used. Samples containing 30 μg of protein were placed in each sample well, and the electrophoresis was initiated with a 5-mA current which was increased to 12 mA constant current whenever the front reached the analytical gel. A Bio-Rad model 500 power supply (Bio-Rad Laboratories, Richmond, CA) was used to control the current. Gels were fixed for 12 hr in 5% trichloracetic acid and then stained with 0.65% Coomassie Blue G (Sigma Chemical Co., St. Louis, MO) in methanol-acetic acid-water (5:1:4, v/v). Destaining was performed by using 5% methanol in 7% acetic acid. Molecular weights were determined by using the method of Weber and Osborn (58) with a Bio-Rad low-molecular-weight standard.

Electron microscopy. IM and OM samples were suspended in tris-HCl 3.3 mM, pH 7.4, and centrifuged for 3 hr at 90,000 *g*. The pellets were fixed in 3% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, for 2 hr. A 0.2 M phosphate buffer, pH 7.2, was used to resuspend and wash the fixed pellet three times. Postfixation in 1% osmium tetroxide was performed in 0.15 M phosphate buffer, pH 7.2, for 1 hr. After three washings in the same buffer, the fixed membranes were dehydrated in an acetone-water series before being imbedded in EPON 812. Ultrathin sections were stained in 4% uranyl acetate and 1% lead citrate before they were observed in a JEOL-JEM-100 C electron microscope.

RESULTS

Isolation of membrane fractions in sucrose density gradient. After 18 hr of centrifugation of the gradient, TM samples of *Xcc* CIAT-1165 and *Xcm* ENA-2648 produced two distinct bands upon fractionation (Fig. 1). The lighter band of both pathovars included IM-rich fractions with a density of 1.142 g/cm^3 and higher activity of NADH oxidase and SDH when compared to the heavier band (1.255 g/cm^3). β-mannanase activity was not detected in either band. Chemical analysis revealed high phospholipid and lack of KDO in the lighter band, indicating a high IM content. Less phospholipid and higher concentrations of KDO detected in the heavier band are also characteristic OM features present in both pathovars (Table 2).

Electron microscopy. Both pathovars showed similar membrane morphology when IM and OM were viewed under the electron microscope (Fig. 2). IM sections showed vesicular appearance with characteristic bilaminar structure. OM obtained from gradient fractionation or from Triton X-100 extraction revealed a more rigid membrane structure in which *C*-shaped open sections were present.

TABLE 2. Density, chemical analysis, and enzymatic assays of total, inner, and outer membranes of *Xanthomonas campestris* pv. *manihotis* ENA-2648 and *X. campestris* pv. *cassavae* CIAT-1165

	Cell membranes of					
	ENA-2648			CIAT-1165		
	Total	Inner	Outer	Total	Inner	Outer
Density[a]	ND[b]	1.142	1.255	ND[b]	1.142	1.255
Protein[c]	126.0	50.0	73.0	150.0	36.0	83.0
Phospho-lipid[d]	71.2	122.2	6.3	124.2	180.0	47.0
KDO[e]	0.5	0	3.8	0.4	0.1	1.7
NADH oxidase[f]	273.3	369.8	64.3	257.2	289.4	64.3
Succinate dehydro-genase[g]	118.1	227.2	36.4	35.0	64.0	21.0

[a] Grams per cubic centimeter of inner or outer membrane fractions obtained from sucrose density gradient.
[b] ND = not determined.
[c] Micrograms of protein per milligram of freeze-dried membrane.
[d] Microgram of phospholipid per milligram of protein.
[e] Nanomoles of 2-keto-3-deoxyoctonate per milligram of protein.
[f] Micromoles of NADH oxidized per minute per milligram of protein.
[g] Nanomoles of succinate oxidized per minute per milligram of protein.

SDS-PAGE. SDS-PAGE was used to compare TM of both pathovars to their respective IM or OM fractions obtained by separation in a sucrose density gradient. IM fractions exhibited a larger number of peptides than did OM fractions. Bands containing low protein concentrations were shared in common in TM, IM, and OM samples of each pathovar, indicating some cross contamination, although characteristic major bands were shown by each fraction of both pathovars (Fig. 3).

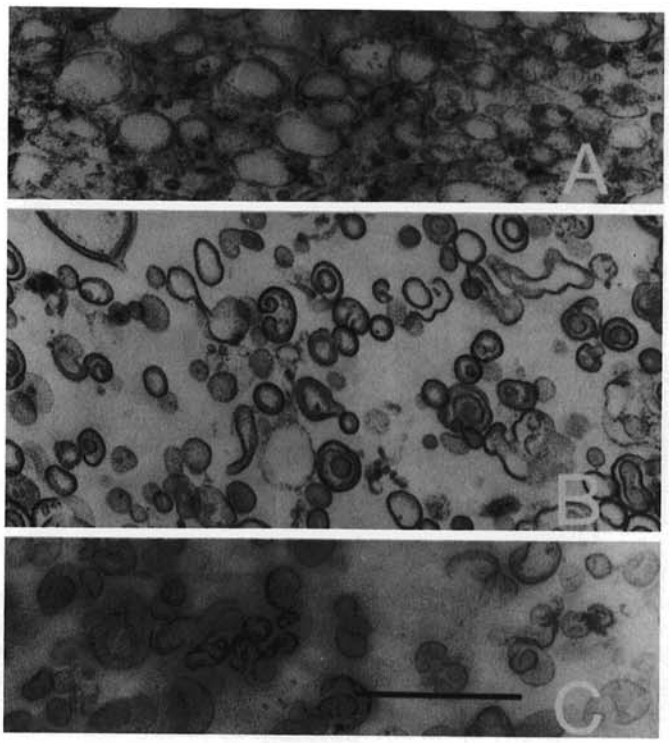

Fig. 2. Electron micrographs of purified membranes of *X. campestris* pv. *cassavae*. Inner (A) and outer (B) membranes obtained by sucrose density gradient fractionation versus outer membranes (C) obtained by treatment of the cell envelope with 2% Triton X-100 at 20 C for 20 min. Scale bar = 1 μm.

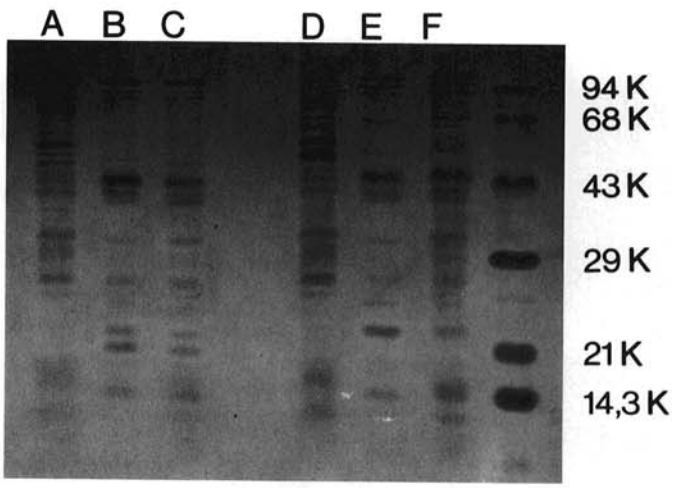

Fig. 3. Electrophoretic profiles of membranes of two pathovars of *Xanthomonas campestris* in 16% polyacrylamide gel with 0.1% sodium dodecyl sulfate, obtained after solubilization in Laemmli sample buffer at 10 C for 5 min. Letters indicate inner (A), outer (B), and total (C) membranes of *X. campestris* pv. *cassavae* CIAT-1165 and inner (D), outer (E), and total (F) membranes of *X. campestris* pv. *manihotis* ENA-2648, respectively. The right lane shows the molecular weight standards with their respective values in kdaltons (K).

TMs of *Xcm* and *Xcc* were compared on a single slab gel (Fig. 4). The main bands shared in common by both pathovars had approximate molecular weights (AMWs) of 114, 100, 41, 28.5, 26, and 22 kdaltons.

Strains of *Xcm* showed an average of 20 bands each, varying from 19 to 22 bands. A major difference detected among the four strains of *Xcm* occurred in proteins of AMWs above 94 kdaltons. The strain Xm-35, besides the two major peptides (114 and 100 kdaltons) also present in the other strains, had two major bands with 115 and 94 kdaltons, respectively. The largest peptide (115 kdaltons) was also present as a main band in strains Xm-55 and ENA-2648. A peptide of 68 kdaltons was detected only in ENA-2648.

Major differences in protein patterns were detected among the five strains of *Xcc* (Fig. 4). Besides the bands present in both pathovars, five strains of *Xcc* (CIAT-1164, CIAT-1165, NCPPB-101, CIAT-1195, and CIAT-1196) showed two major peptides (88 and 45 kdaltons) in common. Furthermore, CIAT-1164 had a minor band (26 kdaltons) also present in *Xcm*. The strain CIAT-1165 showed two unique peptides of low-molecular-weight (21 and 15 kdaltons) while NCPPB-101 had a characteristic band of 59 kdaltons. A 49-kdalton peptide was found in NCPPB-101 and CIAT-1195, whereas CIAT-1196 presented a unique major protein of 54.5 kdaltons. A major band of 23 kdaltons was detected in NCPPB-101, CIAT-1195, and CIAT-1196.

Temperature effect on SDS-PAGE patterns. Total membranes of the strains ENA-2648 (*Xcm*) and NCPPB-101 (*Xcc*) were solubilized in sample buffer at 25, 37, 60, and 100 C for 5 min. Temperature treatment of 37 C resulted in weak electrophoretic profiles (Fig. 5) with ENA-2648 showing major bands of 114, 100, 68, 29, 27.5, and 26.5 kdaltons. At 60 C, a protein with an AMW of 42 kdaltons appeared as a major band, while at 100 C three other major peptides were revealed (41, 26, and 22 kdaltons), and a fourth one (29 kdaltons) disappeared. Temperatures of 25 and 37 C had different effects upon NCPPB-101 which showed major bands with AMWs of 114, 100, 28, and 27.5 kdaltons. Protein at 33 kdaltons was minor at 25 and 37 C but major at 60 C. The 100-C treatment revealed three major bands with AMWs of 52.5, 45, and 41 kdaltons. A peptide of NCPPB-101 at 23 kdaltons appeared to be minor at 25, 37, and 60 C and major at 100 C.

Membrane treatment with Triton X-100. Outer membranes were produced by the treatment of TM samples with the non-ionic detergent Triton X-100. The OM profiles obtained (Figs. 6 and 7) showed a major protein at 41 kdaltons in all nine strains that were studied. Among all four strains of *Xcm* (Fig. 6) common bands appeared with AMWs of 94, 26, and 22 kdaltons. Another major protein (68 kdaltons) was detected in ENA-2648.

The five strains of *Xcc* had two major proteins (41 and 22 kdaltons) in common. CIAT-1164 also showed six other major proteins with AMWs of 115, 110, 88, 79, 42, and 25 kdaltons, while the major proteins of CIAT-1165 had AMWs of 115, 110, 88, 82, 25, and 21.1 kdaltons. A 23-kdalton peptide was present as a major band in strains NCPPB-101, CIAT-1195, and CIAT-1196. A major protein of 100 kdaltons was found in isolates CIAT-1195 and CIAT-1196.

The time of incubation (20 or 40 min) of the TM samples in 2% Triton X-100 did not affect the kind of peptides that were detected.

Effect of culture temperature on the electrophoretic profiles. The electrophoretic protein profile of *Xcc* CIAT-1165 was affected by changes in culture temperature. A major band was present at 15 kdaltons when the incubation temperature was 30 C, but it could not be detected at 27.5, 33, or 35 C. The electrophoretic pattern of *Xcm* ENA-2648 was not affected by the temperature (Fig. 8).

DISCUSSION

Fractionation of the bacterial cell envelope was originally done using ethylenediamine tetraacetic acid (EDTA) to produce spheroplasts prior to fraction separation in a density gradient (2). Osmotic shock in the presence of lysozyme followed by sonication as described by Hasin et al (17), was successfully used for spheroplast production and separation of the membranes of *Xcc* and *Xcm*.

Membrane fractionation was achieved and resulted in two distinct peaks in Schnaitman's density gradient (45). The characterization of both membrane fractions was based on chemical and biochemical markers previously utilized for other Gram-negative bacteria (11,17,23,33,35,41). Dianese and Schaad (11) used β-mannanase as a marker of the IM for *X. campestris* pv. *campestris* following extraction of the IM in a French press without prior production of spheroplasts. The lack of β-mannanase activity in the IM of *Xcc* and *Xcm* may be related to genetic variability of the bacteria or methodological differences.

Both pathovars had IM and OM densities of 1.142 and 1.255 g/cm^3, respectively. The value for IM is in the normal range of other Gram-negative bacteria (7,11,17,41). The density found for

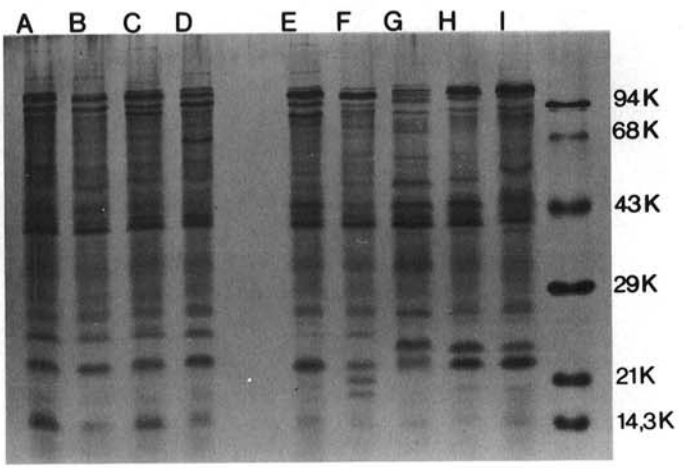

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of the total membranes of isolates Xm-35 (A), Xm-48 (B), Xm-55 (C), and ENA-2648 (D) of *Xanthomonas campestris* pv. *manihotis* and strains CIAT-1164 (E), CIAT-1165 (F), NCPPB-101 (G), CIAT-1195 (H), and CIAT-1196 (I) of *X. campestris* pv. *cassavae*. The right lane shows the molecular weight standards with their respective values indicated in kdaltons (K).

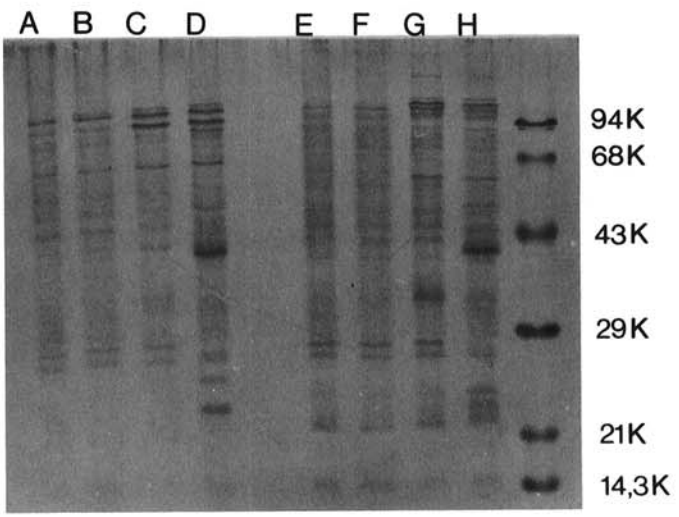

Fig. 5. Effect of sample buffer temperature on the sodium dodecyl sulfate-polyacrylamide gel electrophoretic profiles of total membranes extracted from two pathovars of *Xanthomonas campestris*. Lanes A, B, C, and D contained proteins of total membranes of *X. campestris* pv. *manihotis* ENA-2648 treated for 5 min at 25, 37, 60, and 100 C, respectively. Same temperatures applied to *X. campestris* pv. *cassavae* NCPPB-101 yielded profiles E, F, G, and H, respectively. The right lane shows the molecular weight standards with their respective values indicated in kdaltons (K).

the OM was higher than that of several other species (8,17,41). The plant pathogenic species *E. carotovora* (51) and *E. amylovora* (54) had similar values for OM density. Another pathovar of *X. campestris* presented lower OM densities when the cell envelope was separated in four fractions (11).

The electron micrographs confirmed the separation of IM and OM. The membrane structures observed were similar to those found in other species of Gram-negative bacteria (7,11,17,23,41,45). Schnaitman (45) detected *C*-shaped segments of the OM of *E. coli* when extracting membranes without lysozyme in a French press. The rigidity of those fragments was attributed to the presence of peptidoglycan (45). Similar structures obtained here with lysozyme-treated membranes indicated that peptidoglycan may not be the sole compound responsible for this morphological feature of the OM.

SDS-PAGE profiles of TM, OM, and IM of *Xcc* and *Xcm* were similar to those of other bacterial species (17,23,34,41,49) with the IM fraction showing a larger number of bands than the OM. The fractionation of the TM into two peaks leads to patterns of IM and OM different from those present in four-peak separation of membranes of *X. campestris* pv. *campestris* (11). Methodological differences related to different solubilization temperatures and time of exposures to SDS limited comparisons with the published literature. In general, however, the Gram-negative bacteria have shown a major OM protein with an AMW between 40 and 44 kdaltons (11,17,41,46,54). Both pathovars studied here showed a 41 kdalton peptide as a major OM protein. *Proteus mirabilis* (17), *E. amylovora* (54) and *Salmonella typhimurium* (41) had a 40-kdalton main protein, whereas the major OM protein of *X. campestris* pv. *campestris* was 42 kdaltons (11). Schnaitman (46) compared six Gram-negative species and detected a 44-kdalton peptide as the main one in all cases. As the 41-kdalton protein studied here was heat-modifiable (Fig. 5), according to Minsavage and Schaad (32), it can be considered the OM protein A or the OmpA protein of *Xcc* and *Xcm* by using the nomenclature of Osborn and Wu (42).

Braun's lipoprotein (3), a 7.2-kdalton protein, was not detected because the tracking dye migrated to the 14-kdalton position, and no other band which was stained had lower molecular weight than lysozyme (14.3 kdaltons).

The effects of culture conditions on SDS-PAGE profiles vary with the bacterial species (1,34). Mizuno and Kageyama (34) did not detect differences when *P. aeruginosa* was grown at 30, 37, and 42 C. Different profiles were obtained for *Yersinia pestis* growing at 26 and 37 C (9) and for *X. campestris* pv. *campestris* grown at 30 and 37 C (32). Temperatures of 27.5, 33 and 35 C caused no changes on electrophoretic patterns of the TM of *Xcc* CIAT-1165 or *Xcm* ENA-2648. At 30 C, the strain CIAT-1165 presented a unique major band (15 kdaltons) which was not found in ENA-2648. This indicates the need for standard methods and conditions when comparing bacterial membranes by SDS-PAGE. Temperature and duration of membrane solubilization in SDS can affect the release of OM peptides (48). The 41-kdalton OM protein in *Xcc* and *Xcm* appeared when membranes were boiled in SDS for 5 min at 100 C, but it was not detected when temperatures of 25, 37, and 60 C were used. *Xcc* has three peptides (52.5, 45, and 23 kdaltons) and *Xcm* two others (22 and 26 kdaltons) which are also heat modifiable. Conformation and molecular weight changes "in vitro" have been studied in heat-modifiable purified proteins from *E. coli* (38,45). Darveau et al (9) found nine proteins in membranes of *Y. pestis* that

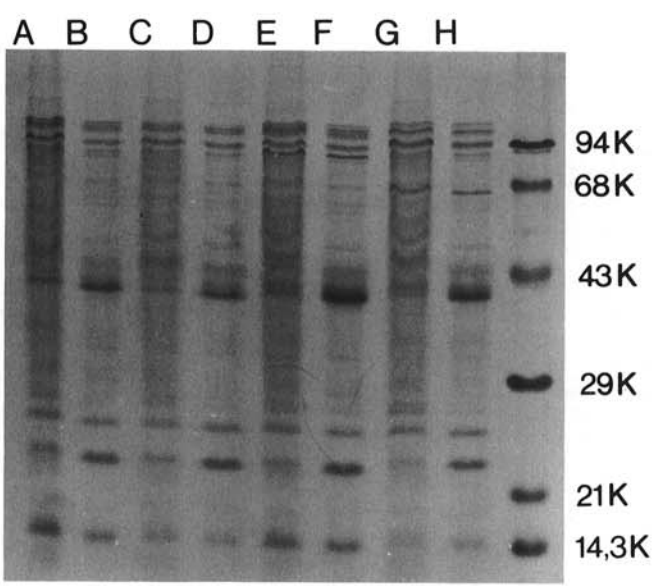

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic profiles of outer (B, D, F, and H) membranes of *Xanthomonas campestris* pv. *manihotis* obtained by 2% Triton X-100 extraction of the total membranes (A, C, E, and G) of the strains Xm-35, Xm-48, Xm-55, and ENA-2648, respectively. The right lane shows the molecular weight standards with their respective values in kdaltons (K).

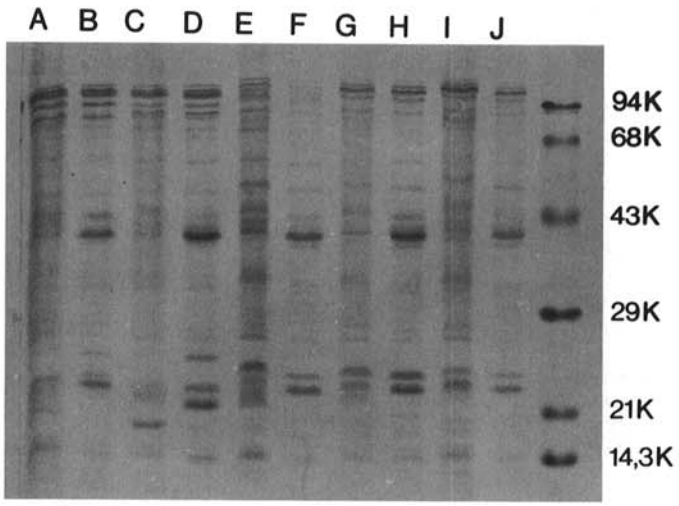

Fig. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of the outer (B, D, F, H, and J) membranes of *Xanthomonas campestris* pv. *manihotis* obtained by 2% Triton X-100 extraction of total membranes (A, C, E, G, and I) of the strains CIAT-1164, CIAT-1165, NCPPB-101, CIAT-1195, and CIAT-1196, respectively. The right lane shows the molecular weight standards with their respective values in kdaltons (K).

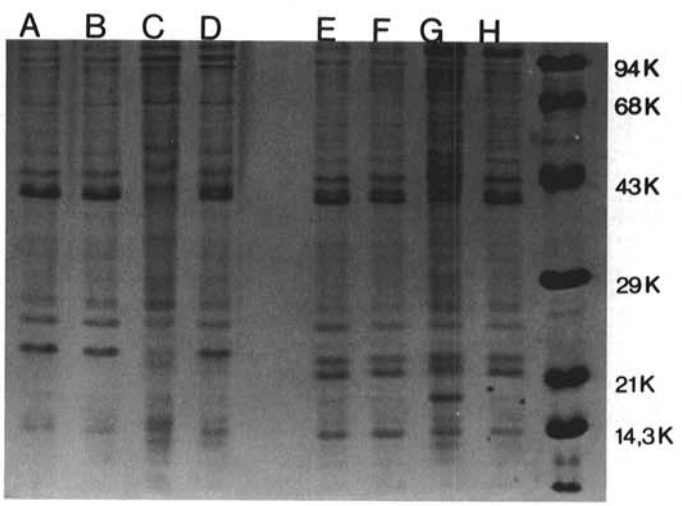

Fig. 8. Effect of temperatures of cultivation on two pathovars of *Xanthomonas campestris* as shown by their sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles. Patterns A, B, C, and D resulted from the growth of *X. campestris* pv. *manihotis* ENA-2648 at 35, 33, 30, and 27.5 C, respectively, for 15 hr. Profiles E, F, G, and H were produced from *X. campestris* pv. *cassavae* CIAT-1165, grown at 35, 33, 30, and 27.5 C, respectively, for 15 hr. The right lane shows the molecular weight standards with their respective values in kdaltons (K).

had been modified by heat. Seven of them were derived by modifications from a 24-kdalton peptide present in nonheated OM. The main OM proteins of *P. aeruginosa* (34) and *Vibrio cholerae* (20) are heat-unstable. *S. typhimurium* (41), *Erwinia* sp. (43) and *X. campestris* pv. *campestris* (39) also have heat-modifiable proteins. Although the presence of such peptides seems to be a feature of the Gram-negative OM (42), further studies are needed to explain the chemical mechanisms responsible for conformation and molecular weight changes due to heat treatment of membranes in SDS (38,48).

Strains of *Xcm* from three geographical areas were separated from the African isolates of *Xcc* by differences on the SDS-PAGE profiles of the TM. The two Colombian isolates of *Xcc* revealed profiles similar to those of *Xcm* except for two bands (15 and 21 kdaltons) characteristically present in isolate CIAT-1164 of *Xcc*. Based on the analysis of the soluble enzymes produced by the two pathovars, Dianese and Kimura (26) suggested that the Colombian isolates of *Xcc* could be yellow strains of *Xcm*. Furthermore, the African strains of *Xcc* exhibited slower growth (31,45) and were found only at altitudes near 1,600 m. The Colombian isolates grew at rates similar to *Xcm* (14) and were isolated at sea level in Colombia (4). The data presented here and the literature (4,15,26,31,45) indicate that the South American strains of *Xcc* are phenotypically less related to the African isolates of the same pathovar than to *Xcm*.

LITERATURE CITED

1. Ames, G. F. L. 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs: Membrane, soluble, and periplasmic fractions. J. Biol. Chem. 249:634-644.
2. Baumgardner, D., Deal, C., and Kaplan, S. 1980. Protein composition of *Rhodopseudomonas sphaeroides* outer membrane. J. Bacteriol. 143:265-273.
3. Braun, V., and Rehn, K. 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein lipoprotein) of the *E. coli* wall: The specific effects of trypsin on the membrane structure. Eur. J. Biochem. 10:426-438.
4. Centro Internacional de Agricultura Tropical. 1976. Añublo bacterial de la Yuca (ABY). Pages B29-32 in: Informe Anual 1976, CIAT. Cali, Colombia. 403 pp.
5. Chai, T. J., Wu, V., and Foulds, J. 1982. Colicin A Receptor: Role of two *Escherichia coli* outer membrane proteins (OmpF protein and btu B gene product) and lipopolysaccharide. J. Bacteriol. 151:983-988.
6. Chatterjee, A. K., and Starr, M. P. 1980. Genetics of *Erwinia* species. Annu. Rev. Microbiol. 34:645-676.
7. Chetina, E. V., Suzina, N. E., and Trotsenko, Y. A. 1981. Isolation and characterization of the membrane of *Methylomonas methanica*. Biokhimiya 46:2100-2109.
8. Collins, M. L., and Niederman, R. A. 1976. Membranes of *Rhodospirillum rubrum*: Isolation and physicochemical properties of membranes from aerobically grown cells. J. Bacteriol. 126:1316-1325.
9. Darveau, R. P., Charnetzky, W. T., and Hurlbert, R. E. 1980. Outer membrane protein composition of *Yersinia pestis* at different growth and incubation temperatures. J. Bacteriol. 143:942-949.
10. Dekker, R. F. H., and Candy, G. P. 1979. The β-mannanase elaborated by the phytopathogen *Xanthomonas campestris*. Arch. Microbiol. 122:297-299.
11. Dianese, J. C., and Schaad, N. W. 1982. Characterization of cell membranes of *Xanthomonas campestris*. Phytopathology 72:1284-1289.
12. Di Masi, D. R., White, J. C., Schnaitman, C. A., and Bradbeer, C. 1973. Transport of vitamin B12 in *E. coli*: Common receptor sites for vitamin B12 and E-colicins on the outer membrane of the cell envelope. J. Bacteriol. 115:506-513.
13. Dye, D. W., and Lelliott, R. A. 1974. Genus II: *Xanthomonas* Dowson. 1939. Pages 243-249 in: Bergey's Manual of Determinative Bacteriology. Eighth edition. R. E. Buckman and N. E. Gibbons, eds. Williams & Wilkins Co., Baltimore. 1268 pp.
14. Elango, F. N., Lozano, J. C., and Peterson, J. F. 1981. Relationships between *Xanthomonas campestris* pv. *manihotis*, *X. c.* pv. *cassavae* and Colombian yellowish isolates. Pages 96-104 in: Proc. Fifth Int. Conf. Plant Pathological Bacteria, Cali, Colombia.
15. Folch, J., Lees, M., and Sloane-Stanley, G. H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.
16. Hantke, K. 1976. Phage T7-colicin K receptor and nucleoside transport in *Escherichia coli*. FEBS (Fed. Eur. Biochem. Soc.) Lett. 70:109-112.
17. Hasin, M., Rottem, W., and Razin, S. 1975. The outer membrane of *Proteus mirabilis*. I. Isolation and characterization of the outer and cytoplasmic membrane fractions. Biochem. Biophys. Acta. 375:381-394.
18. Ikotun, T. 1981. Some characteristics that distinguish *Xanthomonas cassavae* from *Xanthomonas manihotis*. Fitopatol. Bras. 6:1-14.
19. Johnston, K. H., and Gotschlish, E. C. 1974. Isolation and characterization of the outer membrane of the *Neisseria gonorrhoeae*. J. Bacteriol. 119:250-257.
20. Kabir, S. 1980. Composition and immunological properties of the outer membrane proteins of *Vibrio cholerae*. J. Bacteriol. 144:382-389.
21. Kadner, R. J., Bassford, P. J., Jr., and Pugsley, A. P. 1979. Colicin receptors and the mechanisms of colicin uptake. Zentralbl. Bakteriol. Parasitenkd. Infektuibskr. Hyg., Erste Abt. Orig. Reihe A Med. Mikrobiol. Parasitol. 244:90-104.
22. Kado, C. I., and Heskett, M. G. 1970. Selective media for isolation of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas*, and *Xanthomonas*. Phytopathology 60:969-976.
23. Kamio, U., and Takahashi, H. 1980. Isolation and characterization of outer and inner membranes of *Selenomonas ruminantium* lipid composition. J. Bacteriol. 141:888-898.
24. Kasahara, M., and Anraku, Y. 1974. Succinate dehydrogenase of *Escherichia coli* membrane vesicles. Activation and properties of the enzyme. J. Biochem. 76:959-966.
25. Keleti, G., and Lederer, W. J. 1974. 2-keto-3-deoxyoctonate. Pages 74-75 in: Handbook of Micromethods for the Biological Sciences. G. Keleti and W. H. Lederer, eds. Van Nostrand-Reinhold, New York.
26. Kimura, O., and Dianese, J. C. 1983. Caracterização proteica e isoenzimática dos patovares de *X. campestris* (Pammel) Dowson que atacam a mandioca. Pesqui. Agropec. Bras. 18:1215-1228.
27. Konisky, J. 1979. Specific transport systems and receptors for colicins and phages. Pages 319-359 in: Bacterial Outer Membranes: Biogenesis and Functions. M. Inouye, ed. John Wiley & Sons, New York.
28. Laemmli, V. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227:680-685.
29. Lippincott, B. B., Whatley, M. H., and Lippincott, J. A. 1977. Tumor induction by *Agrobacterium* involves attachment of the bacterium to a site on the host plant cell wall. Plant Physiol. 59:388-390.
30. Lowry, O. J., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265-375.
31. Maraite, J., and Weyns, J. 1978. Distinctive physiological, biochemical and pathogenic characteristics of *Xanthomonas manihotis* and *X. cassava*. Pages 103-107 in: Diseases of Tropical Food Crops. Proc. Int. Symposium. J. Maraite and J. A. Meyer, eds. Université Catholique de Louvain, Louvain-la-Neuve, Belgium.
32. Minsavage, G. V., and Schaad, N. W. 1983. Characterization of membrane proteins of *Xanthomonas campestris* pv. *campestris*. Phytopathology 73:747-755.
33. Miura, F., and Mizushima, S. 1968. Separation by density gradient centrifugation of two types of membranes from spheroplast membranes of *Escherichia coli* K-12. Biochem. Biophys. Acta 150:159-161.
34. Mizuno, T., and Kageyama, M. 1978. Separation and characterization of the outer membrane of *Pseudomonas aeruginosa*. J. Biochem. 84:179-191.
35. Mizushima, S., and Yamada, H. 1975. Isolation and characterization of two outer membranes from *Escherichia coli*. Biochem. Biophys. Acta. 375:44-53.
36. Murphy, J., and Riley, J. P. 1962. A modified single solution method for the determination of phosphate in natural waters. Anal. Chim. Acta 27:31-36.
37. Nakae, T. 1976. Outer membrane of *Salmonella*. J. Biol. Chem. 251:2176-2178.
38. Nakamura, K., and Mizushima, S. 1976. Effects of heating in dodecyl sulfate solution on the conformation and electrophoretic mobility of isolated major outer membrane proteins from *Escherichia coli* K-12. J. Biochem. 80:1411-1422.
39. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:325-380.
40. Orndorff, P. E., and Dworkin, M. 1980. Separation and properties of the cytoplasmic and outer membranes of vegetative cells of *Myxococcus xanthus*. J. Bacteriol. 141:914-927.
41. Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. J. Biol. Chem. 247:3962-3972.
42. Osborn, M. J., and Wu, H. C. P. 1980. Proteins of the outer membrane of Gram-negative bacteria. Annu. Rev. Microbiol. 34:369-422.
43. Rice, T. W., and Grula, E. A. 1974. Effects of temperature on extraction and electrophoretic migration of envelope proteins *Erwinia* species. Biochim. Biophys. Acta 342:125-132.

44. Romeiro, R., Karr, A., and Goodman, R. 1981. Isolation of a factor from apple that agglutinates *Erwinia amylovora*. Plant Physiol. 68:772-777.
45. Schnaitman, C. A. 1970. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. 104:890-901.
46. Schnaitman, C. A. 1970. Comparison of the envelope protein compositions of several Gram-negative bacteria. J. Bacteriol. 104:1404-1405.
47. Schnaitman, C. A. 1971. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. J. Bacteriol. 108:545-552.
48. Schnaitman, C. A. 1973. Outer membrane of *Escherichia coli*. I. Effect of preparative conditions on the migration of protein in polyacrylamide gel. Arch. Biochem. Biophys. 157:541-552.
49. Scott, C. C. L., Makula, R. A., and Finnerty, W. R. 1976. Isolation and characterization of membranes from a hydrocarbon-oxidizing *Acinetobacter* sp. J. Bacteriol. 127:469-480.
50. Sequeira, L. 1978. Lectins and their role in host-pathogen specificity. Annu. Rev. Phytopathol. 16:453-481.
51. Shukla, S. D., and Turner, J. M. 1975. Preparation and properties of membranes from the Gram-negative bacterium *Erwinia carotovora*. Biochem. Soc. Trans. 3:756-758.
52. Smit, J., Kamio, Y., and Nikaido, H. 1975. Outer membrane of *Salmonella typhimurium*: Chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. J. Bacteriol. 124:942-958.
53. Takatsu, A. 1980. Preservação das bacterias fitopatogênicas pelo método de dessecação. Fitopatol. Bras. 5:461.
54. Thurn, K. K., and Chatterjee, A. D. 1981. Isolation and characterization of the outer membrane of *Erwinia amylovora*. Curr. Microbiol. 7:87-92.
55. Volk, W. A. 1968. Isolation of D-galacturonic acid 1-phosphate from hydrolysates of cell wall lipopolysaccharides extracted from *Xanthomonas campestris*. J. Bacteriol. 95:782-786.
56. Volk, W. A. 1968. Quantitative assay of polysaccharide components obtained from cell wall lipopolysaccharides of *Xanthomonas* species. J. Bacteriol. 95:980-982.
57. Volk, W. A., Salomonsky, N. L., and Hunt, D. 1972. *Xanthomonas sinensis* cell wall lipopolysaccharide. I. Isolation of 4,7-anhydro- and 4,8-anhydro-3-deoxyoctulosonic acid following acid hydrolysis of *Xanthomonas sinensis* lipopolysaccharide. J. Biol. Chem. 247:3881-3887.
58. Weber, K., and Osborn, M. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.
59. Weissbach, A., and Hurwitz, J. 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. J. Biol. Chem. 234:705-709.