Immunochemical Characterization of a Subspecies-Specific Antigenic Determinant of a Membrane Protein Extract of *Xanthomonas campestris* pv. *campestris*

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ABSTRACT

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A subspecies-specific antigenic determinant(s) of membrane proteins of *Xanthomonas campestris* pv. *campestris* was identified and characterized. Extracts of membrane proteins contained sugar, protein, and lipopolysaccharide (LPS) with a relative molecular mass >2,000 kilodaltons (kDas). Treatments of membrane proteins with protease Type VII and XIV, trypsin, lysozyme, beta-galactosidase nucleases, periodate, acid, alkali, detergents, or heat at 100 C for 30 min failed to destroy the immunogen responsible for the specific line of precipitin but those treatments did result in additional lines in Ouchterlony double diffusion and immunoelectrophoresis tests. On the other hand, protease Type I destroyed the immunogen. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of membrane proteins showed major peptide bands of 125, 100, 44, 34, 29, and 23.4 kDas. Crossed immuno-electrophoresis (two-dimensional) of SDS-PAGE gels revealed that the specific antigenic determinant was present in the first three bands, the uppermost part of stacking gel, and at the interface between the stacking and resolving gels. Antiserum to purified 125-, 100-, and 44-kDa peptides resulted in reactions of identity when tested against membrane proteins or purified homologous peptides of membrane proteins in Ouchterlony double diffusion tests. Those peptide bands (visualized with SDS-PAGE) with the specific antigenic determinant contained sugar and protein but no LPS. The specific antigenic moiety of membrane proteins was identified as a heat-stable inner membrane peptide.

Additional key words: bacteria, bacterial membranes, serology.

Immunochemistry of bacterial antigens of medically important Gram-positive and Gram-negative pathogens has received considerable attention. Early work focused on the immunodeterminants of capsular polysaccharide antigens (10), lipopolysaccharide (LPS) O and K antigens (20), murein lipoprotein antigens (2), and entero-bacterial common antigen or Kunin antigen (14,18). More recent investigations have resulted in immunochemical characterization and identification of membrane protein antigens, especially the outer membrane of Gram-negative and Gram-negative-like bacteria in medically important genera such as *Escherichia* (25), *Salmonella* (11), *Neisseria* (9), *Mycoplasma* (13), and *Chlamydia* (3,4,7,21).

Considerably less is known about membrane protein envelopes of phytopathogenic bacteria. Inner membrane and outer membrane envelopes of *Erwinia amylovora* (28) have been isolated and characterized. Total membrane envelopes of *Xanthomonas sinensis* have been characterized (24). The inner membrane and outer membrane fractions of *Xanthomonas campestris* pv. *campestris* have been characterized (8). A 44-kDa peptide of the outer membrane of *X. c.* pv. *campestris* was species specific and characterized as heat-modifiable and peptidoglycan-associated (19). A major 44-kDa peptide was also present in LiCl-extracted membrane proteins of *X. c.* pv. *campestris* (27). However, antigenicity of this prominent peptide band of *X. c.* pv. *campestris* has not been studied. Membrane proteins are useful for identification of several phytopathogenic bacteria because of their high specificity (5,22,30).

The specific serotype antigen of membrane proteins of *Neisseria meningitidis* was identified as a 41-kDa lipoprotein-LPS complex derived from outer membrane envelopes (9). However, no direct evidence was presented that this peptide was in fact the specific immunodeterminant.

Properties of membrane proteins of phytopathogenic bacteria are not well known. Membrane proteins of *E. chrysanthemi* contain one neutral and one acidic antigen in immunoelectrophoresis tests (30). No serovar-specific peptide bands are observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (30). Membrane proteins of *X. c.* pv. *campestris* contain one specific and two to four nonspecific antigens depending upon the strain (27). The specific antigen is neutral, whereas the nonspecific antigens are acidic (27).

This paper presents for the first time the immunochemical identity of the subspecies-specific immunogen of membrane proteins of *X. c.* pv. *campestris*. Purification of peptides, preparation of antisera to purified peptides, and their immunogenic specificities are presented. The specific immunogen of membrane proteins of *X. c.* pv. *campestris* is a high-molecular-weight (>2,000 kDa) sugar-protein complex. Its antigenic moiety is a physiochemically stable protein associated with the inner membrane. The subspecies-specific immunodeterminants of membrane proteins are located in several bands (SDS-PAGE) ranging in relative molecular mass from 125 to 44 kDa.

MATERIALS AND METHODS

Bacterial growth and extraction of membrane proteins. Culturing of *X. c.* pv. *campestris* strain BT-27 (23,27) and

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extraction of membrane proteins (30) were described previously. Cells were grown in liquid medium 523 (12) for 20–24 hr, harvested by centrifugation, and extracted with 0.2 M LiCl at 45 C for 3 hr. Cells and large membranous particles were removed from the extracted suspension by centrifugations at 12,000 *g* for 20 min and 30,000 *g* for 40 min, respectively, at 4C. The supernatant was centrifuged at 100,000 *g* for 70 min at 4 C to collect membrane proteins. The sample of membrane proteins was washed once with sterile distilled water, centrifuged as before, suspended in a solution of 0.01% aqueous thimerosal, and stored at 4–5 C.

Preparation of antisera to membrane proteins and peptides. Antisera to membrane proteins were obtained from the previous study (27). Peptides recovered from the 125-, 44-, and 23.5-kDa area of polyacrylamide gels, as described below, were used for immunization of female, New Zealand white rabbits. Three graded injections containing 0.1, 0.2, and 0.4 mg of protein were given 10 days apart. Specificity of antisera to each homologous peptide was tested by Ouchterlony double diffusion (27) against homologous peptides and membrane proteins. Preimmune sera served as controls. Antisera to peptides were concentrated about 6-fold by ultrafiltration with a PM-10 membrane (Amicon Corp., Danvers, MA).

Serology. Ouchterlony double diffusion tests and immunoelectrophoresis were performed as described (27). Purified peptides were tested by Ouchterlony double diffusion and immunoelectrophoresis against antiserum to membrane proteins and isolated peptides.

Analytical SDS-PAGE. Preparation of samples and electrophoresis on a discontinuous gel (0.7 × 140 × 160-mm) of Laemmli (15) were done as previously described (27). After electrophoresis, gels were removed and staining was performed as follows: 0.05% Coomassie brilliant blue R250 (19) for protein; periodic acid-Schiff's (PAS) for glycoprotein; and Alcian blue (29) for acidic carbohydrates. Commercial LPS from *Salmonella typhimurium* and glycoprotein bovine fraction VI (Sigma Chemical Co., St. Louis, MO 63178) were used as controls for carbohydrate and glycoprotein, respectively.

Purification of peptide by preparatory SDS-PAGE. Gel composition, apparatus, and conditions were the same as for analytical SDS-PAGE (27) except that a 1.4-mm-thick blank comb was used in place of a 0.7-mm-thick comb. A sample containing 2 mg of protein in 0.4–0.5 ml was applied to the sample trough. Two 15-cm-wide gels were run at the same time. After electrophoresis, gels were removed from the apparatus and a 1-cm-wide strip was cut longitudinally from each side of the gel and stained to determine the exact position of each peptide band to be isolated. The uppermost 2-mm of the stacking gel, the interface between the stacking gel and running gel, and areas of 5 mm (in height) containing peptide bands of 120.0, 100.0, 44.0, and 23.5 kDas were excised from the remaining 13-cm-wide gel, pooled, and crushed with a glass rod in a 16-mm test tube. Two-to-three volumes of sterile distilled water were added, and the tubes were incubated overnight at 4–5 C to allow diffusion of the peptides from the crushed gels. The suspension was then filtered through an 8.0-μm membrane (Millipore Corp., Bedford, MA). The filtrate containing the peptide was concentrated by ultrafiltration through a YM-10 membrane (Amicon Corp.). Protein concentrations were determined by the method of Lowry et al (17) and the samples were stored at 4–5 C until use.

Characterization of membrane protein and total membrane by sucrose density gradient fractionation. Cells were broken in a French pressure cell and total membrane envelopes were obtained as described (8). The final sample was resuspended in 2–4 ml of a 0.01% aqueous thimerosal solution and stored at 4–5 C.

The total membrane envelopes were fractionated into inner, intermediate, and outer membrane fractions by using 45–70% step density gradients (8) as modified below. One milliliter of total membrane envelopes (13.6 mg of protein) and 1 ml of membrane proteins (5.2 mg of protein) was layered onto each of four and two gradients, respectively. The six gradients were centrifuged at 120,000 *g* in a Beckman SW-27 rotor at 4 C for 18 hr. The membrane fractions were collected from the gradients with an Isco model 640 gradient fractionater and UA-5 monitor at 280 nm. Fractions under the same peak were pooled, dialyzed, and centrifuged at 190,000 *g* for 90 min at 4 C. Pellets were resuspended in 1 ml of tris buffer and kept at 4–5 C (8).

Polypeptide profiles of membrane proteins, total membrane, and the gradient fractions of total membrane were characterized by analytical SDS-PAGE as described above.

Location of immunodeterminants of membrane proteins within cell envelopes. Samples of cell envelope fractions such as total, inner, and outer membranes including LPS were used to determine the location of the immunodeterminants of membrane proteins. LPS was isolated by the aqueous butanol method of Leive and Morrison (16). Whole cells and total membrane were used for extraction of LPS. The isolated LPS samples were further purified by digesting protein with protease Type XIV (Sigma Chemical Co.), concentrated by Diaflo ultrafiltration with an XM-100A membrane (Amicon Corp.), and stored at 4–5 C until used.

Specific antigenic activities of membrane fractions and LPS were determined by comparative Ouchterlony double diffusion against antiserum to membrane proteins. Membrane fractions solubilized with different detergents were compared.

Identification of specific antigenic determinants of membrane proteins. Crossed (two-dimensional) immunoelectrophoresis of SDS-PAGE gels (CIE-PAGE) and Ouchterlony double diffusion tests of the purified peptides were used in this study. For CIE-PAGE, a modified procedure of Chua and Blomberg (6) was used. GelBond support films (0.2 × 100 × 150-mm; FMC Corp., Rockland, ME) were used instead of glass support plates. A 1% agarose gel was prepared by dissolving 1 *g* of agarose HGT (FMC Corp.) in 1 ml of 1% aqueous thimerosal and 99 ml of buffer A (80 mM tris, 40 mM sodium acetate, 1 mM Na_2 EDTA, pH 8.6). CIE-PAGE gels (2 × 80 × 150 mm) were formed on GelBond film of 100 × 150 mm in a glass plate mold. The cathodal gel (15 mm in height) was made first. After the cathodal gel solidified, the first dimensional PAGE gel of 5 × 120 mm containing the separated peptides was placed (immediately after electrophoresis) adjacent to and parallel to the cathodal gel. Next, gel containing antibody was poured to a height of 50 mm above and around the PAGE gel. After the antibody gel solidifed, anodal support gel was added to a height of 15 mm. The total height of the second-dimension gel was 80 mm. Antibody-containing gel consisted of 2% antiserum in the gel described above. Electrophoresis of the composite gel was conducted at a constant voltage of 2 V/cm (10V) across the gel for 16–18 hr at 10 C by using buffer A and a Buchler horizontal electrophoresis cell (Buchler Instruments, Fort Lee, NJ). Gels were pressed dry between several layers of Whatman No. 1 filter paper under a 1-kg weight for 20 min, removed and washed over night in saline, and then washed once in distilled water for 30 min. Gels were again pressed dry for 20 min and then dried with a hair dryer. Immunoprecipitates were visualized by staining the dried gels in 0.05% Coomassie brilliant blue R250 (27). After being stained for 3–5 min, the gels were washed three times with methanol-water-glacial acetic acid (4.5:4.5:1.0, v/v) until the background cleared. Gels were then air-dried.

Homology of the specific antigenic determinant was confirmed by Ouchterlony double diffusion tests with antisera to membrane proteins, purified proteins, and purified peptides.

Partial characterization of membrane proteins. Physiochemical properties of the immunodeterminants of membrane proteins were analyzed by using the procedure of Caldwell and Kuo (3). Fifty milliliters of membrane protein antigen (1 μg/μl) were evaporated under N_2 in test tubes (0.6 × 50 mm). The antigen was resuspended in 50 μl of the following solutions: proteases Type I (200 mg/ml), VII (200 mg/ml), and XIV (350 mg/ml); trypsin (2 mg/ml); lysozyme Grade I (2 mg/ml); beta-galactosidase (200 mg/ml); lipase Type VII (200 mg/ml); DNase I (2 mg/ml) and RNase Type I-AS (25 mg/ml); 100 mM glycine-HCl buffered at pH 2.2 (for acid treatment); 100 mM glycine-NaOH buffered at pH 10.6 (for alkaline treatment); and 50 mM sodium meta-periodate (Sigma Chemical Co.). Solutions of the enzymes were prepared in 0.01 M sodium phosphate-buffered saline (0.85% NaCl) (PBS), pH 7.0, and periodate in 50 mM sodium acetate buffer, pH 4.5. All enzymes

were obtained from Sigma Chemical Co. except trypsin which was obtained from Calbiochem-Behring, San Diego, CA. Antigens were treated with enzymes for 2 hr at 37 C, acid, alkaline, and periodate for 24 hr at 4–5 C, and heat for 30 min at 60 and 100 C. Control treatments consisted of evaporated antigen suspended in buffer alone and incubated at 37 C for 2 hr or 4–5 C for 24 hr.

Membrane proteins were solubilized with detergents by mixing 25 μl of membrane proteins (1 μg/μl) with an equal volume of the following detergent solutions: 3% SDS (Bio-Rad Laboratories, Richmond, CA), 3% sodium desoxycholate (Fisher Scientific Co., Fairlawn, NJ), 6% Triton X-100 (Eastman Kodak Co., Rochester, NY), 6% Tween-20 (Bio-Rad Laboratories), or 6% Tween-80 (Nutritional Biochemicals Corp., Cleveland, OH). All treatments were incubated at room temperature for 24 hr. Detergent solutions were prepared in 62.5 mM tris-HCl buffer, pH 6.8, containing 10% glycerol and 5% 2-mercaptoethanol. Control treatment consisted of incubating antigens with buffer at room temperature for 24 hr.

The effect of each treatment on antigenicity of membrane proteins was determined by Ouchterlony double diffusion and immunoelectrophoresis using antiserum to membrane proteins. Enzyme activity in all enzyme treatments was inactivated by heating at 100 C for 3 min before testing antigenic activity.

Relative molecular mass of membrane proteins was estimated based on calibration of Sephacryl S-200 column (Pharmacia Fine Chemicals, Piscataway, NJ) with Blue dextran (2,000 kDas) for void volume and cytochrome C for bed column. Purified membrane proteins from the column were compared by SDS-PAGE with membrane proteins, total membrane, and membrane fractions.

Chemical analyses of membrane proteins and peptides were determined as follows: protein content by the method of Lowry et al (17) with bovine albumin (Sigma Chemical Co.) as a standard, total hexoses by the anthrone reaction of Spiro (26) with glucose as a standard, and 2-*keto*-3-deoxyoctonic acid by the thiobarbituic acid method of Ashwell (1) with lipopolysaccharide (LPS) of *S. typhimurium* (Sigma Chemical Co.) as a standard. All absorbance readings were measured with a Gilford model 240 spectrophotometer.

RESULTS

Characterization of membrane proteins and total membrane by sucrose density gradient fractionation.

Membrane proteins and total membrane preparations of *X. c.* pv. *campestris* BT-27 were separated into one and four fractions, respectively. The total membrane was separated into four distinct bands which agreed with previous results (8,19). The membrane protein fraction appeared at the top of the gradient (upper band) at a location similar to the inner membrane fraction (8,19). SDS-PAGE profiles of membrane proteins (Fig. 1A, lanes 2 and 5), membrane proteins from the upper density gradient band (Fig. 1A, lane 4), and membrane proteins from the pooled bottom part of the density gradient (Fig. 1A, lane 3), were identical. Those peptides present in the upper density gradient band (Fig. 1A, lane 4) were also present in the lower, pooled gradient fraction (Fig. 1A, lane 3) but in reduced amount. Furthermore, the same peptide bands were present in membrane proteins purified by Sephacryl S-200 gel filtration (Fig. 1A, lane 1). The SDS-PAGE profiles of membrane proteins contained major peptide species of 23.5, 29, 34, and 44 kDas. The 23.4- and 44-kDa peptides predominated. Two minor bands of 100 and 125 kDas were also visible. (Fig. 1A). Profiles of membrane proteins (Fig. 1A, lanes 2 and 5) were similar to total membrane profiles (Fig. 1A, lane 6) but differed from inner membrane profiles (Fig. 1A, lane 10).

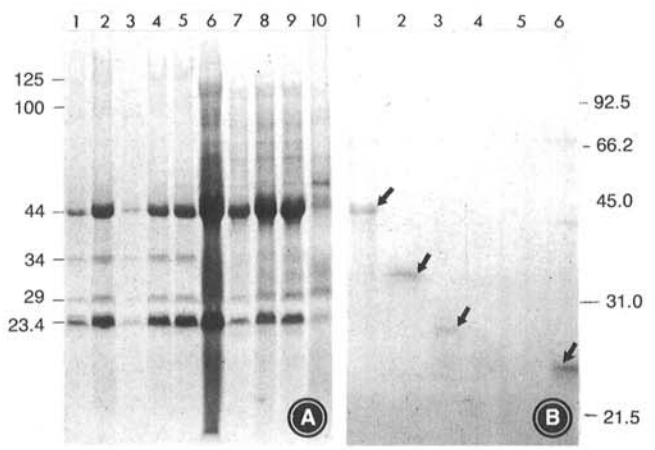

Fig. 1. A, Comparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of membrane proteins and membrane fractions of *Xanthomonas campestris* pv. *campestris* BT-27. Samples were boiled in Laemmli sample buffer 3 min before being applied to the sample wells. Sample lanes were: 1, purified membrane proteins from Sephacryl S-200 gel filtration; 2 and 5, membrane proteins; 3, pooled bottom of density gradient profile of membrane proteins; 4, upper band of density gradient fraction of membrane proteins; 6, total membrane; 7, fraction 2 of outer membrane; 8, fraction 1 of outer membrane; 9, intermediate membrane; and 10, inner membrane. Relative molecular masses (in kDas) of major peptides are indicated at the left of the figure. **B,** SDS-PAGE lanes of purified peptides were: 1, 44 KDa; 2, 34 kDa; 3, 29 kDa; 4 and 5, blank; and 6, 23.4 kDa. Relative molecular masses of standard proteins are indicated to the right of the figure.

Location of the specific immunogen of membrane proteins in cell envelopes. All membrane proteins (wells 1, 3, 5, and 7), inner membrane (well 2), and intermediate membrane (well 4) fractions reacted identically in Ouchterlony double diffusion tests with antiserum to membrane proteins (Table 1 and Fig. 2). In contrast, the total membrane (well 6) and outer membrane (well 8) fractions failed to react against antiserum to membrane proteins (Table 1 and Fig. 2A). However, the specific immunogen was present in the total membrane fraction but not in the outer membrane fraction after treating them with SDS and Triton X-100 (Table 1). LiCl extraction of total membrane, inner membrane, and outer membrane confirmed the presence of a specific antigenic determinant in the total membrane and inner membrane fractions but not in the outer membrane fraction (Fig. 2B, wells 2, 4, and 6, respectively).

Identification of specific antigenic determinant of membrane proteins. Crossed immunoelectrophoresis of membrane proteins of *X. c.* pv. *campestris* revealed specific activities (immunoprecipitin peaks) corresponding to at least five locations in the gel (Fig. 3, arrows). Three of the five immunoprecipitin peaks corresponded to peptides of 125, 100, and 44 kDas, whereas the other two immunoprecipitin peaks corresponded to the uppermost 2 mm of the stacking gel (Fig. 3, first arrow left to right) and the interface between the upper and lower PAGE gels (Fig. 3, second arrow). Membrane protein peptides were isolated from SDS-PAGE, and the purity of purified peptide preparations was demonstrated in SDS-PAGE showing no detectable contamination with other peptides (Fig. 1B). The 125-, 100-, and 44-kDa peptides and the gel area between 44 and 100 kDas (Fig. 1A, lane 1) were serologically identical to membrane proteins (Fig. 2C, wells 2, 4, 6, and 8. In

TABLE 1. Specificity of Ouchterlony double diffusion reactions relative to membrane proteins, total membrane, and membrane fractions of *Xanthomonas campestris* pv. *campestris* strain BT-27 treated with sodium dodecyl sulfate (SDS) and Trition X-100

	Antiserum to membrane protein		
Antigen	Nontreated	SDS	Triton X-100
Membrane proteins	++[a]	++	++
Total membrane	−	++	++
Inner membrane	++	+	+
Intermediate membrane	−	+	+
Outer membrane 1	−	+	+
Outer membrane 2	−	+	+

[a]Symbols: ++ = reaction of identify with specific immunoprecipitin, + = reaction of identity with nonspecific immunoprecipitin, and − = no precipitin reaction.

contrast, the 34-, 29-, and 23.4-kDa peptides did not react (Fig. 2D, wells 2, 4, and 6).

Antisera to purified 23.4-, 44-, and 125-kDa peptides reacted weakly with membrane proteins in Ouchterlony double diffusion tests. However, after the antisera were concentrated sixfold, precipitation increased. All antisera to purified peptides resulted in reactions of identity with membrane proteins of homologous strains (Fig. 2E and F, well 8) but not with membrane proteins of heterologous strains (Fig. 2E and F, wells 2, 4, and 6). Antiserum to purified 125-kDa peptide reacted by producing an additional precipitin line which was intense and similar to the nonspecific line (Fig. 2E, denoted by arrow). This precipitin line was removed by absorbtion with membrane proteins of a heterologous strain (BT-16). Reaction of antiserum to the 23.4-kDa peptide with membrane proteins was the weakest, whereas that of antiserum to the 125-kDa peptide was the strongest.

Staining SDS-PAGE gels with PAS and Alcian blue for the presence of glycoprotein failed to reveal any bands.

Partial characterization of membrane proteins. Several chemical and physical treatments of membrane proteins of *X. c.* pv. *campestris* BT-27 caused a separation of the specific line of precipitin into several additional lines and a reduction in intensity of the specific line, depending on the treatments (Fig. 4A, wells 2 and 5; 4B, wells 5 and 6; and 4C, wells 4, 6, and 8). None of the enzymatic treatments eliminated the specific antigenic determinant except protease Type I (Fig. 4B, C, and D, well 2). Treatments of membrane proteins with periodate, acid, base, or heat failed to eliminate the specific determinant (Table 2, Fig. 4A, wells 2, 3, 5, and 8; 4B, wells 3, 5, and 6; 4C, wells 4, 6, and 8). Detergents resolved the membrane proteins into several precipitin lines in immunoelectrophoresis (Fig. 5B and D). Samples of membrane proteins, total membranes, inner and outer membranes, and purified peptides contained protein and sugar (Table 3). On the

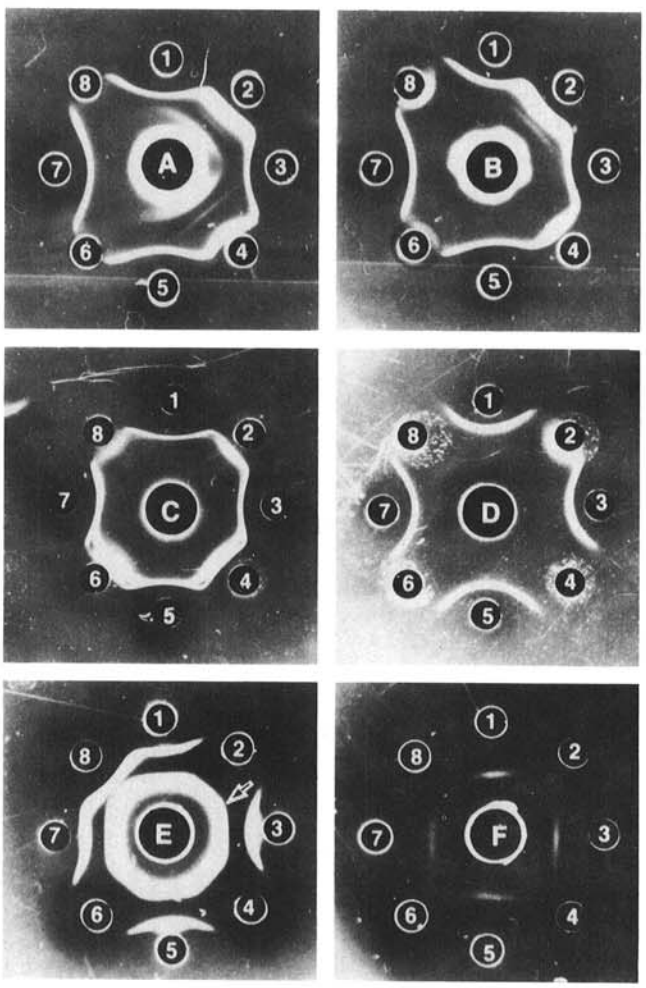

Fig. 2. Ouchterlony double diffusion reactions of density gradient fractions of bacterial membranes, LiCl-extracted total membrane, and purified peptides of *Xanthomonas campestris* pv. *campestris* strain BT-27 against antisera to membrane proteins and purified peptides. Center wells A to D contained 10 μl of antiserum to membrane proteins; center wells E and F contained 10 μl of sixfold-concentrated antisera to purified peptides with relative molecular masses of 125 and 44 kDas, respectively. Outer wells contained 5 μl of membrane proteins, membrane fractions, or purified peptides. Outer wells 1, 3, 5, and 7 of patterns A to D contained membrane proteins; those of pattern E contained the 125-kDa purified peptides; and those of pattern F the 44-kDa purified peptides. Pattern A outer wells contained: 2, inner membrane; 4, intermediate sample (M); 6, total membrane; and 8, outer membrane. Pattern B outer wells contained LiCl-extracted fractions: 2, total membrane; 4, inner membrane; 6, outer membrane fraction 1; and 8, outer membrane fraction 2. Pattern C outer wells contained purified peptides: 2, from the area between 44 and 100 kDas; 4, 125 kDas, 6, 100 kDas; and 8, 44 kDas. Pattern D outer wells contained purified peptides: 2, 34 kDas; 4, 29 kDas; 6, 23.4 kDas; and 8, dye front. Pattern E and F outer wells contained membrane proteins: 2, BT-1; 4, BT-16; 6, BT-4; and 8, BT-27. The arrow in pattern E indicates the nonspecific immunoprecipitin nearest the antiserum well.

TABLE 2. Specificity of Ouchterlony double diffusion reactions relative to physiochemical treatments of membrane proteins of *Xanthomonas campestris* pv. *campestris* BT-27

Membrane proteins treated with:	Type of precipitin lines formed by by reaction of antiserum to membrane proteins of BT-27[a]	
	Specific	Nonspecific
Phosphate-buffered saline	+[b]	−
Acetate buffer	+	+
Acid, Base	+	+
Heat (37, 60, or 100 C)	+	+
Periodate	+	+
RNase, DNase, lipase, beta-galactosidase, trypsin, lysozyme, protease (VII, XIV)	+	+
Protease (I)	−	+

[a]Specific refers to immunoprecipitin line nearest the antigen well and nonspecific to immunoprecipitin line nearest the antiserum well.
[b]Symbols: + = presence of precipitin line, and − = absence of pecipitin line.

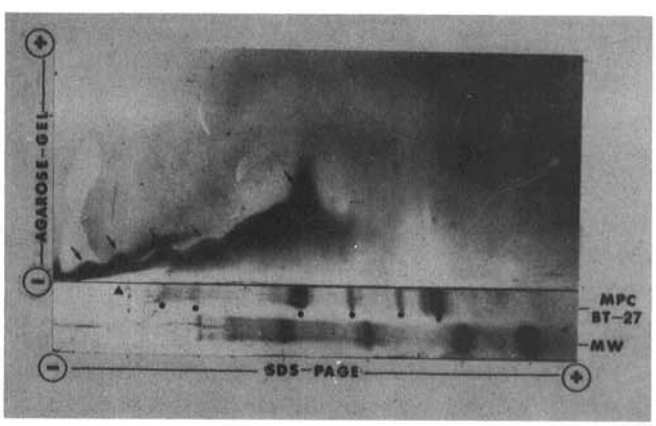

Fig. 3. Cross immunoelectrophoresis after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of membrane proteins of *Xanthomonas campestris* pv. *campestris* strain BT-27. The sample of membrane proteins containing 10 μg of protein was resolved in 10% discontinuous polyacrylamide gel of Laemmli (15) in the first dimension. In the second dimension, the resolved polypeptide in the SDS-PAGE gel was electrophoresed, as described (6), into 2% (v/v) agarose containing antiserum to membrane proteins. Gels were stained with 0.05% Coomassie brilliant blue R-250 to visualize immunoprecipitates. Arrows denote specific peptide peaks. The dark triangle indicates the interface of stacking and running SDS-PAGE gels. Dark dots from left to right denote peptides with relative molecular masses of 125, 100, 44, 34, 29, and 23.4 kDas, respectively.

other hand, LPS was found only in membrane proteins and total membranes (Table 3). Estimation of the relative molecular masses of intact membrane proteins by gel filtration revealed values greater than 2,000 kDas. When tested against antiserum to membrane proteins, LPS of *X. c.* pv. *campestris* BT-27 isolated from both whole cells or total membrane reacted identically to membrane proteins (Fig. 4D, wells 4, 6, and 8). However, specific antigenic properties of the LPS fraction were not inactivated by beta-galactosidase or lipase (Fig. 4D and E, wells 4 and 6) but were inactivated by protease Type I (Fig. 4D and E, well 2).

Immunoelectrophoresis of membrane proteins following treatment with different enzymes (Fig. 5C), chemicals (Fig. 5B and D), and heat (Fig. 5A and D) resulted in a partially modified specific antigenic molecule. Some treatments resulted in additional lines of precipitin (Fig. 5B and D, the first, third, and fourth wells from the top), whereas other treatments did not yield additional lines (Fig. 5A, the third and fourth wells from the top).

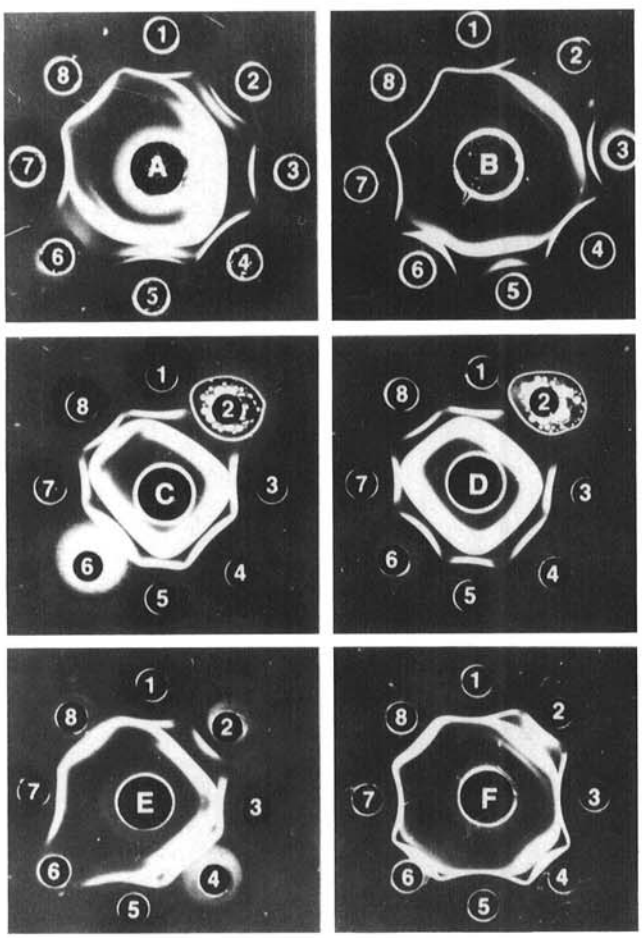

Fig. 4. Ouchterlony double diffusion reactions of membrane proteins and lipopolysaccharide (LPS) of *Xanthomonas campestris* pv. *campestris* strain BT-27 treated with various chemicals, enzymes, and heat and tested against antiserum to membrane proteins. Center wells A to E contained 10 μl of antiserum to membrane proteins. All outer wells contained 5 μl of membrane proteins or LPS antigens. Outer wells of pattern A: 1, 4, and 7, nontreated membrane proteins; 2 and 5, heat treated at 100 C; 3, sodium periodate; 6, protease Type I; and 8, trypsin. Outer wells of pattern B: 1, 4, and 7, nontreated membrane proteins; 2, protease Type I; 3, and 6, protease Type XIV; 5, protease Type VII; and 8, sodium acetate buffer. Outer wells of pattern C: 1, 3, 5, and 7, nontreated membrane proteins; 2, protease Type I; 4, beta-galactosidase; 6, lipase; and 8, phosphate-buffered saline (PBS). Outer wells of pattern E: 1, 3, 5, and 7, untreated LPS from total membrane; 2, lipase; 4, beta-galactosidase; 6, PBS alone; and 8, PBS. Outer wells of pattern F: 1, 3, 5, and 7, untreated membrane proteins; and inactivated enzymes—2, protease Type I; 4, protease Type VII; 6, protease Type XIV; and 8, trypsin.

DISCUSSION

The immunogen responsible for the subspecies specificity of membrane proteins of *X. c.* pv. *campestris* has been identified. As in previous studies on membranes, SDS-PAGE and crossed immunoelectrophoresis were useful techniques for characterization of the specific immunodeterminant (4,6). When reacted with homologous antiserum, membrane proteins of strain BT-27, resulted in a single, specific precipitin line. Formation of a new nonspecific line following treatments that failed to inactivate the specific line was unexpected. One possible explanation is that the antigenic part involved in the nonspecific line is loosely bound to

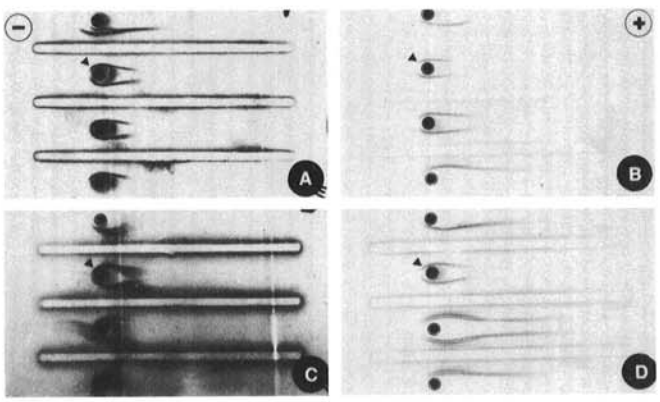

Fig. 5. Immunoelectrophoresis of membrane proteins of *Xanthomonas campestris* pv. *campestris* strain BT-27 treated with various detergents, chemicals, and heat. **A to D,** all antiserum troughs contain antiserum to membrane proteins. Sample wells from top to bottom of gel: **A,** 100 C, nontreated, 60 C, and 37 C; **B,** Tween-80 (3%), nontreated, Tween-80 (0.6%), and SDS (3%); **C,** protease Type I, nontreated, protease Type VII, and protease Type XIV; and **D,** SDS (0.6%), nontreated, SDS (3%, boiled 3 min), and SDS (0.6%, boiled 3 min). Dark triangles indicate specific immunoprecipitates.

TABLE 3. Chemical analyses of membrane proteins, membrane fractions, and purified peptides of *Xanthomonas campestris* pv. *campestris* strain BT-27.

	Chemical composition[a]			
Sample	Dry weight	Protein	Hexoses	LPS[b]
Membrane proteins	10.88	5.20	5.90	1.50
Total membrane	16.60	13.60	5.30	7.60
Gradient fractions				
Inner membrane	4.19	1.53	0.43	0.00
Intermediate	3.47	1.43	1.00	0.00
Outer membrane 1	3.12	1.09	0.50	0.00
Outer membrane 2	1.55	0.58	0.28	0.00
Purified peptides of SDS-PAGE profile				
Stacking gel				
Uppermost 2mm	ND	0.30	2.50	0.00
Interface area	ND	0.30	3.30	0.00
Running gel				
120.0 kDa	ND	0.30	1.90	0.00
100.0 kDa	ND	0.40	3.10	0.00
44.0 kDa	ND	2.70	3.55	0.00
23.4 kDa	ND	2.20	6.55	0.00
LPS from:				
Cells	ND	ND	6.00	10.40
Total membrane	ND	ND	4.90	4.00
S. typhimurium[c]	1.00	ND	0.51	1.00
Glycoprotein[c]	1.00	ND	0.56	ND

[a] Chemical compositions were expressed in milligrams per milliliter and LPS was calculated from 2-*keto*-3-deoxyoctonic acid reaction, ND = not determined.
[b] LPS = lipopolysaccharide.
[c] Obtained from Sigma Chemical Co., St. Louis, MO.

the specific part of the molecule and is easily released from the specific determinant. The evidence suggests that the specific immunodeterminant is part of a complex molecule containing either one molecule with multispecific determinants or several molecules with the same antigenic determinant.

Results from experiments reported here demonstrate that the specific antigenic determinant of of *X. c.* pv. *campestris* is associated with at least three peptides having relative molecular masses of 44, 100, and 125 kDas. This antigenic determinant responsible for the specific immunoprecipitin in Ouchterlony double diffusion tests is a heat-stable protein and is resistant to several chemical and physical treatments. The specific antigenic determinant is associated with the inner membrane but not the outer membrane. These results contrast with the report that the specific immunodeterminant of membrane proteins of *N. meningitidis* is an outer membrane protein (9). Furthermore, we found that the total membrane fraction did not demonstrate specific antigenic properties until after treatment with Laemmli sample buffer (15), Triton X-100, or LiCl. These results indicate that the specific part of the antigen is buried in the inner membrane or possibly is a hydrophobic protein moiety.

The inner membrane lacked the predominant 44-kDa and 23.4-kDa peptides in SDS-PAGE profiles, but still possessed the specific immunogenic activity. In addition, the gel contained no major protein band between 44 to 100-kDas even though antigens were detected in that area of the gel. This evidence supports the hypothesis that the major 44- and 23.4-kDa peptides are less involved or responsible for the specific antigenic determinant than are the high-molecular-mass peptides. Chemical analyses of membrane proteins, total membrane, and their fractions revealed that membrane proteins and total membrane fractions contained protein, carbohydrate, and a small amount of LPS. On the other hand, no LPS was detected in the purified 125-, 100-, and 44-kDa peptides from SDS-PAGE gels. Antisera to peptides of purified membrane proteins resulted in a homologous reaction between peptides and membrane proteins of different strains of the same organism but failed to react with peptides and membrane proteins of different organisms. Antiserum to the 125-kDa peptide resulted in a specific and nonspecific precipitin when tested in Ouchterlony double diffusion tests against homologous antigen. However, the nonspecific precipitin was absorbed out with membrane proteins of a heterologous strain (BT-16). We suggest that antiserum to the 125-kDa peptide contains multispecific antibodies. Furthermore, the absorption of antibodies to 44-kDa peptide by membrane proteins of BT-16 suggests that the 44-kDa peptide is common to heterologous strains and may play a role in nonspecific reactions.

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