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Composition and Immunochemical Properties of Outer Membrane Proteins of *Vibrio cholerae*

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The protein compositions of the outer membranes of various *Vibrio cholerae* strains, belonging to both biotypes (El Tor and classical) and both serotypes (Ogawa and Inaba), were analyzed by electrophoresis on polyacrylamide slab gels in the presence of sodium dodecyl sulfate. All these strains contained a major protein band of molecular weight 48,000. But they differed in the composition and proportions of minor proteins. The outer membrane protein profile was influenced by the growth medium. A protein band of molecular weight 15,000 was observed in the outer membrane when *V. cholerae* Ogawa 395 (classical) was grown in peptone-water, whereas a protein of molecular weight 68,000 appeared when it was grown in the synthetic medium. Under anaerobic growth conditions the proportion of the 13,000-molecular-weight protein was greatly enhanced. The outer membrane contained heat-modifiable proteins, as the protein bands with molecular weights 41,000 and 37,000 disappeared when membrane proteins were disaggregated in sodium dodecyl sulfate at or above 60°C. The antisera to the outer membrane proteins of *V. cholerae* Ogawa 395 (classical) produced immunoprecipitation to the outer membrane proteins of both biotypes and both serotypes. Also, the antisera agglutinated bacteria of both biotypes and both serotypes, suggesting the presence of a common protein antigen in the outer membrane of *V. cholerae*.

Vibrio cholerae, the etiological agent of cholera, is a noninvasive pathogen which colonizes the small intestine. It produces an enterotoxin which binds to an epithelial ganglioside receptor (19) and causes an outpouring of fluid into the intestinal lumen. Both antibacterial and antitoxic immunities are induced in the host after an infection by *V. cholerae*. Recently, Levine et al. (12) have studied the relative roles of antibacterial immunity and antitoxic immunity in protection against cholera in humans and have observed that the predominant operative immune mechanism is antibacterial rather than antitoxic in nature.

Although considerable work has been done in the field of cholera, antigens associated with antibacterial immunity have not been characterized in detail. *V. cholerae*, like other gram-negative bacteria, possesses three distinct layers: the outer membrane, the inner or cytoplasmic membrane, and the peptidoglycan (9). The outer membrane contains surface components, such as proteins and lipopolysaccharides (LPS), and it is most likely that these components would interact with the host to elicit antibacterial immune response.

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There are several studies related to the composition of the outer membrane proteins of such gram-negative bacteria as *Escherichia coli* and *Salmonella* (4). It appears that the outer membrane proteins of *E. coli* and *Salmonella* can act as surface antigens (1, 8). Also, antisera raised against the outer membrane proteins of *Salmonella* have been found to render protection against experimental salmonellosis (11). However, little is known about the composition and immunochemical properties of the outer membrane proteins of *V. cholerae*.

There are two biotypes of *V. cholerae*: classical and El Tor. Within each biotype are found two major serotypes: Ogawa and Inaba. This paper describes the compositions of the outer membrane proteins of these strains under the influence of a number of cultural conditions. Immunochemical studies demonstrated that the strains of *V. cholerae* belonging to both serotypes and both biotypes have a cross-reacting protein antigen located in the outer membrane.

MATERIALS AND METHODS

Bacterial strains. The following strains of *V. cholerae* were used in this investigation: Ogawa strain 395 (classical), Ogawa strain 10255 (El Tor), Inaba strain 3661 (El Tor), and Inaba strain 10732 (classical). Ogawa strain 395 was a gift from N. F. Pierce, The

Johns Hopkins University, Baltimore, Md., whereas the other strains were supplied by the National Collection of Type Cultures, London, United Kingdom.

Media. Three types of media were used in this investigation.

(i) Peptone-water. For peptone-water, a 3% solution of peptone, pH 7.4, was prepared as directed by Burrows et al. (2).

(ii) Semisynthetic or Syncase medium. Semisynthetic or Syncase medium was prepared according to the procedure described by Finkelstein et al. (5).

(iii) Synthetic medium. For synthetic medium the ingredients were the same as those described in the semisynthetic medium except that Casamino Acids had been replaced by 2.5 g of each of the following acids per liter: glutamine, serine, aspartic acid, and arginine.

Growth conditions. (i) Aerobic growth. For aerobic growth, cultures were incubated at 37°C with continuous shaking and harvested at the stationary phase of growth.

(ii) Anaerobic growth. For anaerobic growth, medium containing bile salts was prepared by adding 1 g (2.2 mM) of sodium deoxycholate (Fisher Scientific Co., Philadelphia, Pa.) to 1 liter of Syncase. The medium was prereduced for 24 h in an atmosphere of H_2 and CO_2, and the growth was maintained in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) in a similar atmosphere at 37°C for 16 h.

Preparation of the outer membrane. *V. cholerae* cultures (250 ml) were grown in 1-liter Erlenmeyer flasks at 37°C with shaking to an absorbance at 600 nm of 0.8 to 1.0 and harvested by centrifugation at 12,000 × *g* for 15 min at 0 to 4°C. The cell pellet was suspended in 25 ml of 0.1 M Tris-hydrochloride, pH 7.8, and sheared at 4°C in a Waring blender (19,500 rpm, 45 s) to remove flagella as described for *E. coli* (3). The suspension was diluted sixfold in the Tris buffer and subjected to centrifugation at 12,000 × *g* for 10 min. The cell pellet was rapidly suspended in cold 0.75 M sucrose–10 mM Tris-hydrochloride, pH 7.8 (0.7 ml of sucrose solution per 10 units of absorbance at 600 nm of original culture). The suspension was transferred to an Erlenmeyer flask containing lysozyme (2 mg/ml of water, 0.05 ml/ml of cell suspension), and the mixture was incubated in ice for 2 min. The suspension was diluted with 2 volumes of cold 1.5 mM EDTA and allowed to stay at 4°C for 3 h. The spheroplast was lysed by osmotic shock by slowly pouring the suspension into 4 volumes of cold water with magnetic stirring and was stirred for 10 min in the cold. The total membrane fraction was recovered from the osmotic lysate by centrifugation at 12,000 × *g* for 15 min at 2 to 4°C. Unlysed cells were removed, and the supernatant was centrifuged at 48,000 × *g* for 30 min. The pellet was suspended in 0.01 M Tris-hydrochloride (pH 7.8) containing 10 mM EDTA by a syringe and was washed once with 0.01 M Tris-hydrochloride (pH 7.8) containing 5 mM $MgCl_2$. The inner membrane was removed from the total membrane by extraction at 23°C for 20 min with 2% Triton X-100 according to the procedure of Schnaitman (17). The outer membrane was recovered as a pellet.

Enzyme assay. NADH oxidase activity in the outer membrane was assayed as described by Osborn et al. (15). The incubation mixture for the measurement of NADH oxidase activity contained 50 mM Tris-hydrochloride, (pH 7.5), 0.12 mM NADH, 0.2 mM dithiothreitol, and the membrane preparation (0.1 to 0.2 mg of protein) in a volume of 1.0 ml. The rate of decrease in absorbance was measured at 340 nm at 25°C.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to the method of King and Laemelli (10). The slab gels (10% acrylamide) were cast between glass plates (15 by 15 cm) to a height of 10 cm, using spacers 1.5 mm thick. The gel solution was overlaid with water until polymerization was completed. A stacking gel (3% acrylamide) 1 cm high was applied on top of the analytical gel. Electrophoresis was performed at 30 mA until the tracking dye reached the end of the gel. Immunoglobulin G (molecular weight, 150,000 [150K]), bovine serum albumin (68K), ovalbumin (43K), α-chymotrypsinogen (25.7K), myoglobin (17.2K), and cytochrome *c* (11.7K) were used as marker proteins.

The slabs were fixed by treating them with a solution of 45.4% methanol and 9.2% acetic acid in water. The gels were stained with gentle shaking for 2 h in 0.025% Coomassie brilliant blue containing 25% isopropanol and 10% acetic acid. Gels were destained by shaking in a solution containing 10% methanol and 10% acetic acid in water.

Unless otherwise stated, membrane proteins were disaggregated by mixing an equal volume of sample buffer (0.125 M Tris-hydrochloride [pH 6.9], 4% SDS, 10% 2-mercaptoethanol, and 0.002% bromophenol blue) with a sample containing 10 to 100 μg of protein. The mixture was heated at 100°C for 3 min.

Recovery of proteins from SDS-polyacrylamide gels. The polyacrylamide gel section containing proteins was cut into pieces and macerated by passing the pieces through a syringe. Proteins were eluted by shaking at 37°C into 10 volumes of 0.05 M NH_4HCO_3 containing 1% SDS. After 6 h the polyacrylamide fragments were removed by centrifugation and then washed once with a small volume of elution buffer. The combined supernatants were dialyzed against 1% SDS in order to remove the buffer originally present in the gel. Upon lyophilization, NH_4HCO_3 was removed, leaving a protein-SDS mixture which was subjected to amino acid analysis.

Amino acid analysis. The protein samples, containing 0.8 to 0.9 mg, were hydrolyzed in 6 M HCl at 110°C for 24 h and analyzed in a Durram D-500 amino acid analyzer.

Electron microscopy. Both the bacteria and the total membrane were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate (pH 7) buffer containing 0.1 M sucrose for 1 week. The samples were then centrifuged, and the pellets were suspended in distilled water. One drop from each sample was applied to a Formvar-coated grid onto which a thin carbon layer had been placed, and the grids were rinsed with several drops of 1% sodium silicotungstate in water. The excess sodium silicotungstate was removed after 1 min, and the grids were allowed to dry. The specimens were examined in a JEOL 100-C transmission electron microscope at 60 kV.

Antisera. Antisera against *V. cholerae* membrane proteins were raised in rabbits by immunizing each rabbit with 1 mg of *V. cholerae* Ogawa 395 total membrane suspended in 0.5 ml of saline and an equal volume of Freund incomplete adjuvant. Antigens were administered on days 0, 14, 28, and 42. Blood was collected 1 week after the last injection. The antisera were stored at −70°C until use.

Absorption with LPS. Antibody against LPS was removed from the serum by absorption with LPS (5 mg/ml of serum), incubation at 4°C overnight, and centrifugation at 105,000 × *g* for 5 h at 4°C. The LPS-absorbed antiserum was subjected to immunoprecipitation reaction with *V. cholerae* LPS.

Immunodiffusion. The outer membrane proteins were solubilized in the nonionic detergent Tween 20. Briefly, 1 volume of a 0.10 M Tris-hydrochloride (pH 8.0) containing 5% Tween 20 was added to 1 volume of the membrane suspension. The mixture was shaken for a period of 2 h at room temperature and was centrifuged at 10,000 × *g*. The supernatant was subjected to immunodiffusion in 2% agarose.

Immunoelectrophoresis. The outer membrane proteins, solubilized in Tween 20 as described above, were subjected to immunoelectrophoresis in a barbital buffer, pH 8.2, in 2% agarose for 1 h and allowed to diffuse against the LPS-absorbed antiserum to *V. cholerae* total membrane.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed on glass plates (5 by 5 cm), using agarose gel (Sea Plaque) and barbital buffer, pH 8.6, as described by Weeke (20). Membrane proteins (5 μl) were electrophoresed in the first dimension at 10 V/cm for 1 h at 4°C and against the LPS-absorbed antiserum in the second dimension at 2 V/cm for 16 h.

Bacterial agglutination. Cultures were adjusted to 10^{10} cells per ml in 0.9% (wt/vol) NaCl. Twofold-diluted serum was added to an equal volume of the bacteria, and the agglutination pattern was recorded after an incubation of 2 h at 37°C.

RESULTS

Composition of the outer membrane proteins of *V. cholerae*. The outer membrane proteins of *V. cholerae* were isolated from living cells. The flagella were removed from living bacteria by shearing in a Waring blender. The morphology of the total membrane of *V. cholerae* was examined by electron microscopy. The cytoplasmic membrane proteins were solubilized from the total membrane by 2% Triton X-100 as described by Schnaitman (17). The purity of the outer membrane protein (Triton X-100 insoluble) was tested for the presence of NADH oxidase, an enzyme characteristic of the cytoplasmic membrane of a gram-negative bacterium (15). Very little specific activity of this enzyme was detected in the outer membrane (0.013 μmol/min per mg of protein). This value was of the order of 1.4% of that obtained for the total membrane (0.920 μmol/min per mg of protein).

In this investigation the compositions of the outer membrane proteins were studied with the following strains of *V. cholerae*: Ogawa 395 (classical), Ogawa 10255 (El Tor), Inaba 10732 (classical), and Inaba 3661 (El Tor). All these strains possessed the 48K polypeptide as the major outer membrane protein band (Fig. 1). Several minor protein bands were also present. Although in Inaba 3661 (El Tor) the low-molecular-weight polypeptide 13K was greatly reduced, this strain contained two other polypeptides, 16K and 28K, which were not present in other strains. The 13K protein was present in all other strains; however, the relative amount of this protein was more pronounced in Ogawa 10255 (El Tor) and Inaba 10732 (classical).

Effect of heating. Figure 2 shows the effect of temperature of solubilization on the polypeptide composition of *V. cholerae* Ogawa 395 membrane proteins disaggregated for 3 min at 25, 37, 60, and 100°C. At 25°C protein bands 37K and 41K were observed. On heating at 37°C the intensities of these bands decreased. At 60°C these bands disappeared, whereas at 100°C the 48K band became very prominent. These results demonstrated the presence of heat-modifiable proteins in the outer membrane of *V. cholerae*.

Effect of growth medium. To determine the effect of the growth medium on the composition of the outer membrane proteins of *V. cholerae*, the bacteria were grown aerobically in (i) peptone-water, (ii) Syncase medium, and (iii)

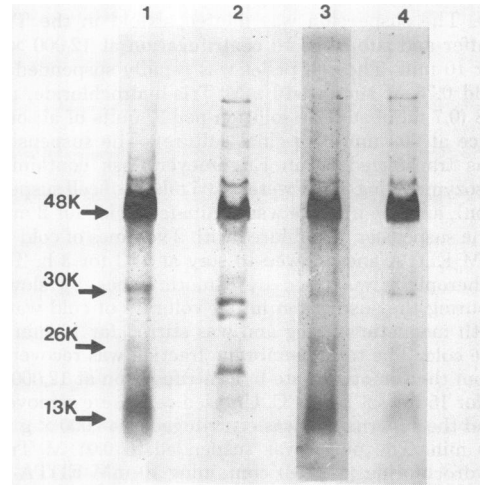

FIG. 1. *Protein composition of the outer membrane of V. cholerae strains Inaba 10732 (classical) (1), Inaba 3661 (El Tor) (2), Ogawa 10255 (El Tor) (3), and Ogawa 395 (classical) (4). Electrophoresis was carried out on a slab polyacrylamide gel (10%) in the presence of SDS according to the method of King and Laemelli (10).*

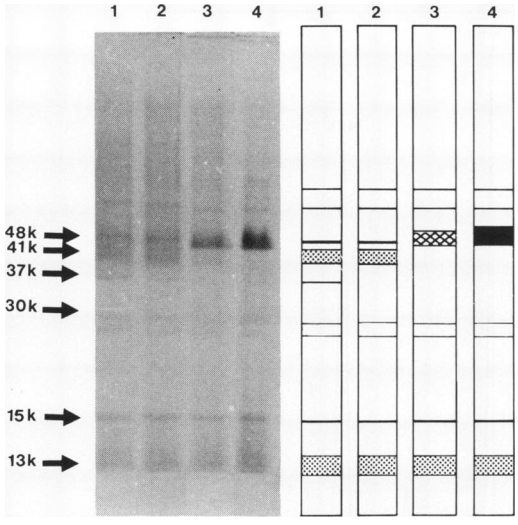

FIG. 2. *Protein composition of the outer membrane of V. cholerae Ogawa 395 (classical) grown in peptone-water and disaggregated in Tris-hydrochloride buffer containing SDS at 25°C (1), 37°C (2), 60°C (3), and 100°C (4). Line drawings of the electrophoretic patterns are also shown.*

synthetic medium. *V. cholerae* was grown anaerobically in Syncase medium containing bile salts. The composition of the growth medium had an influence on the outer membrane protein profile. *V. cholerae* grown in all these media contained the major protein band 48K. However, the following differences were observed: (i) *V. cholerae* grown in peptone-water contained a 15K polypeptide which was absent in others (Fig. 3), (ii) a 68K polypeptide was observed when the bacteria were grown in the synthetic medium (Fig. 3), (iii) the low-molecular-weight polypeptide 13K was more pronounced in synthetic medium and peptone-water than in Syncase (Fig. 3), and (iv) when grown anaerobically in the presence of bile salts, the relative amount of the low-molecular-weight protein 13K was increased (Fig. 4).

Amino acid analysis. Membrane proteins 13K and 48K were isolated from slab polyacrylamide gels and were analyzed for amino acid contents. The results are shown in Table 1. The amino acids are grouped as basic, acidic, neutral, and hydrophobic. The following features are noteworthy: (i) both proteins were rich in neutral amino acids, representing about 36% of the total amino acids; (ii) the acidic residues outnumbered the basic residues; (iii) protein 13K was richer in the basic amino acids lysine, histidine, and arginine (16.36% of the total amino acids) than was protein 48K (12.76%), and (iv) protein 48K was richer in hydrophobic amino acid residues (27.12%), especially in tyrosine, as compared with protein 13K (23.39%).

Immunoelectrophoretic studies. To determine cross-reactivity among the outer membrane proteins of various strains of *V. cholerae*, these proteins were subjected to immunoelectrophoresis. The LPS-absorbed antiserum to *V. cholerae* Ogawa 395 total membrane produced immunoprecipitation reactions to the outer membrane preparations of (i) Ogawa 395 (classical), (ii) Ogawa 10255 (El Tor), (iii) Inaba 10732 (classical), and Inaba 3661 (El Tor) (Fig. 5). These results demonstrated the antigenic cross-reactivity among the biotypes and serotypes of *V. cholerae*.

Agglutination tests. The LPS-absorbed antiserum to the total membrane of *V. cholerae* Ogawa 395 did not produce any immunoprecipitation reaction with *V. cholerae* Ogawa LPS, suggesting that anti-LPS antibody molecules were removed by the absorption technique. The absorbed antiserum showed positive agglutination reactions against all the strains of *V. cholerae* (both biotypes and serotypes) tested, i.e., Ogawa 395, Ogawa 10255, Inaba 10732, and Inaba 3661. The agglutination titer was 256 with Ogawa 395, Ogawa 10732, and Inaba 10732, whereas with Inaba 3661 it was 128.

Crossed immunoelectrophoretic analysis. When the outer membrane proteins of Ogawa 395 were analyzed by crossed immunoe-

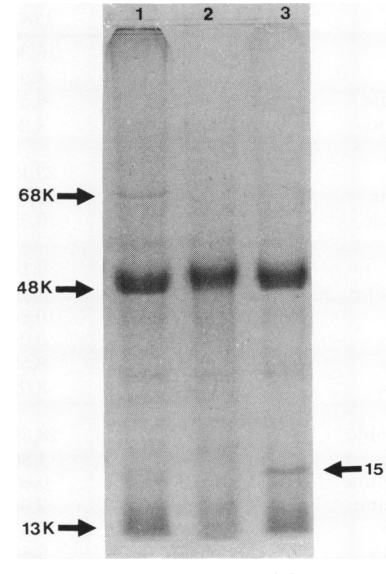

FIG. 3. *Protein composition of the outer membrane of V. cholerae Ogawa 395 grown in synthetic medium (1), Syncase or semisynthetic medium (2), and (3) peptone-water. Electrophoresis was performed on slab polycrylamide gels (10%) in the presence of SDS.*

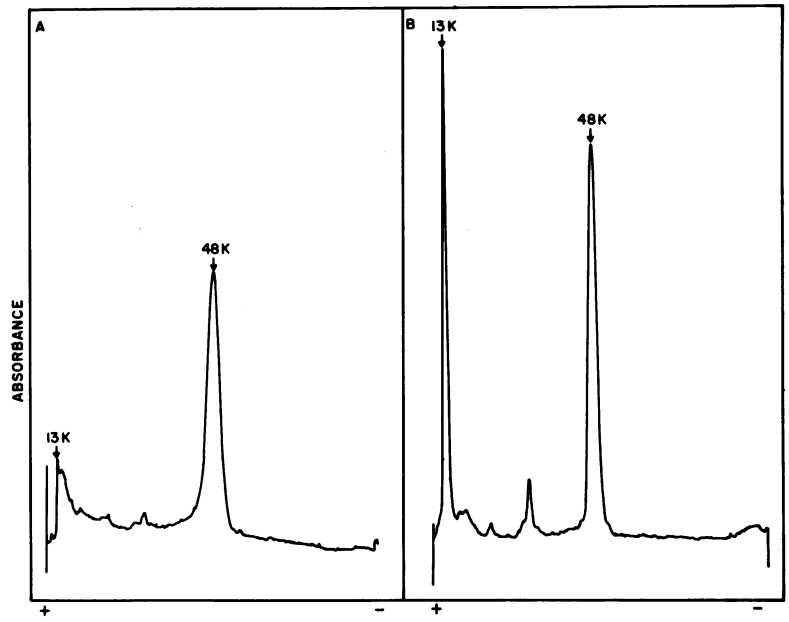

FIG. 4. *Densitometric scans of Coomassie brilliant blue-stained gels at 600 nm of V. cholerae grown aerobically (A) and anaerobically in the presence of bile salts (B). Electrophoresis was performed on slab polyacrylamide gels (10%) in the presence of SDS.*

TABLE 1. *Amino acid analysis of purified outer membrane proteins from V. cholerae Ogawa 395 (classical)*

Amino acids	Concn (%) of amino acid in protein:	
	48K	13K
Basic	12.76	16.36
Lysine	5.00	4.91
Histidine	4.21	6.51
Arginine	3.55	4.94
Acidic	23.37	23.19
Aspartic	14.90	12.84
Glutamic	8.47	10.35
Neutral	36.97	36.14
Threonine	6.30	5.10
Serine	7.77	10.86
Proline	2.00	3.04
Glycine	9.09	8.52
Alanine	10.81	8.62
Hydrophobic	27.12	23.39
Valine	5.24	4.86
Methionine	1.10	0.85
Isoleucine	3.48	3.98
Leucine	6.88	6.95
Tyrosine	6.30	3.64
Phenylalanine	4.22	3.11

lectrophoresis, one major precipitation band and one minor precipitation band were observed (Fig. 6). This indicated the presence of two antigens, one strong and one weak, among the outer membrane proteins of *V. cholerae* Ogawa 395.

DISCUSSION

V. cholerae can be killed by either physical treatment, heat, or chemical treatment, formaldehyde or acetone, but such treatments were avoided because they might denature the outer membrane proteins and alter their immunogenicity. Hence, the outer membrane proteins were prepared from living cells of *V. cholerae*.

In the present investigation four strains of *V. cholerae*, belonging to both biotypes (classical and El Tor) and both serotypes (Inaba and Ogawa), were used. It was found that the major outer membrane protein, 48K, as obtained by SDS-polyacrylamide gel electrophoresis, was similar in all these strains. Various investigators have studied the outer membrane proteins of such enteric bacteria as *E. coli* and *Salmonella* and have found that the major outer membrane proteins have an apparent molecular weight range of 32,000 to 37,000 (4).

The outer membrane protein profile depended on the medium of cultivation. When grown in the presence of peptone, a 15K protein band appeared, whereas a 68K protein was observed when Ogawa 395 was grown in the synthetic medium. Also, when this strain was grown in an anaerobic medium, the relative proportion of

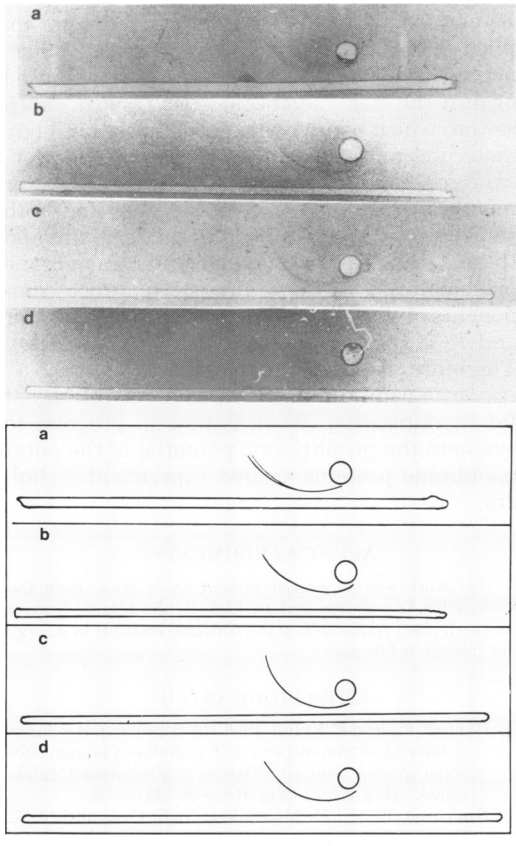

FIG. 5. *Immunoelectrophoretic patterns of the outer membrane proteins of V. cholerae strains Ogawa 395 (classical) (a), Ogawa 10255 (El Tor) (b), Inaba 10732 (classical) (c), and Inaba 3661 (El Tor) (d). Membrane proteins were solubilized in a nonionic detergent (Tween 20) and electrophoresed in barbital buffer as described in the text. Immunodiffusion was performed with the antiserum (LPS adsorbed) raised against the total membrane of V. cholerae Ogawa 395 (classical). Line drawings of the immunoelectrophoretic patterns are also shown.*

polypeptide 13K was increased. The precise mechanism behind the effect of the cultivation medium on the outer membrane protein profile should be studied. A similar phenomenon has also been observed with other gram-negative bacteria, such as *E. coli* (18), and might be common to all gram-negative bacteria.

The migration of the outer membrane proteins in SDS-polyacrylamide gel electrophoresis depends on the temperature of solubilization in SDS. When membrane proteins were solubilized at room temperature or at 37°C, 37K and 41K protein bands were observed which disappeared on heating at or above 60°C. The 48K band became the only major protein band when membranes were digested at 100°C. Similar findings regarding the presence of heat-modifiable proteins have been observed with other gram-negative bacteria, such as *E. coli* (16) and *Pseudomonas aeruginosa* (14). In the case of *E. coli* the apparent molecular weight increased from 28,500 to 33,000 (16) when membrane proteins

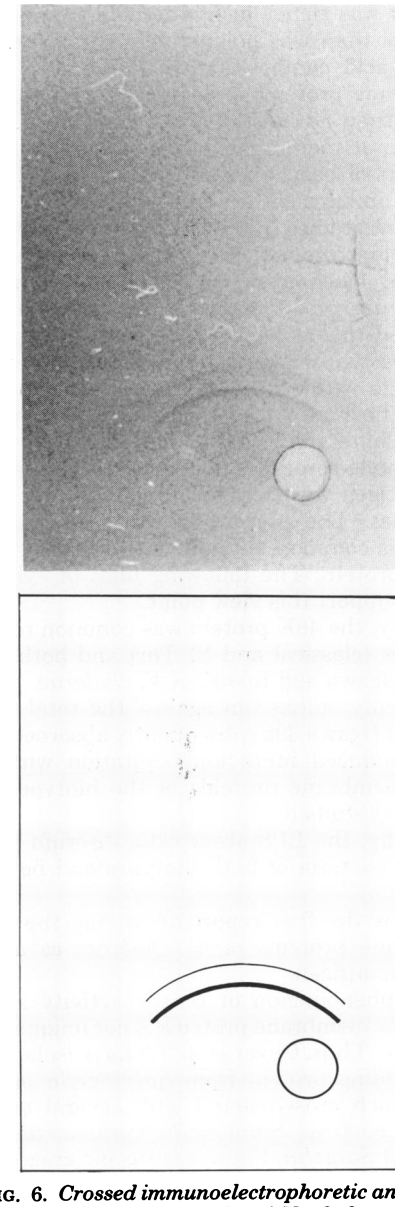

FIG. 6. *Crossed immunoelectrophoretic analysis of the outer membrane protein of V. cholerae Ogawa 395 dissolved in 5% Tween 20. Electrophoresis was performed in barbital buffer (i) for 90 min in the first dimension and (ii) for 16 h in the second dimension in gels containing LPS-adsorbed antimembrane immunoglobulins. A line drawing of the crossed-immunoelectrophoretic pattern is also shown.*

were solubilized at temperatures higher than 60°C. Heat-modifiable proteins might be present in all gram-negative bacteria, and, as suggested by Reithmeir and Bragg (16), some native structures present in the unmodified membrane might be lost upon heating.

Amino acid analyses indicate that the 48K protein was richer in hydrophobic amino acid residues than was polypeptide 13K. When the amino acid composition of *V. cholerae* outer membrane proteins is compared with that obtained from *E. coli* (6), it is found that *V. cholerae* outer membrane proteins contain higher amounts of basic and lower amounts of hydrophobic amino acid residues.

Recent studies by Levine et al. (12) with human volunteers indicated that the predominant immune mechanism was antibacterial rather than antitoxic in nature. Also, these authors observed that clinical infection with one serotype (Ogawa) conferred immunity to subsequent challenge with the heterologous serotype (Inaba). The reason behind this mechanism was not explained. The phenomenon of cross-protection among serotypes suggests that the protective antigen was common to Ogawa and Inaba serotypes. The present investigation suggests that this common antigen was the outer membrane protein. The following lines of evidence would support this view point.

Firstly, the 48K protein was common to both biotypes (classical and El Tor) and both serotypes (Ogawa and Inaba) of *V. cholerae*.

Secondly, antiserum against the total membrane of Ogawa 395 subsequently absorbed with LPS produced immunoprecipitation with the outer membrane proteins of the biotypes and serotypes studied.

Thirdly, the LPS-absorbed antiserum agglutinated bacteria of both biotypes and both serotypes.

This is the first report describing the outer membrane proteins of *V. cholerae* as a new common antigen.

The phenomenon of cross-reactivity among the outer membrane proteins is not unique to *V. cholerae*. Thus, Geyer et al. (7) have isolated an LPS-binding protein from *Salmonella minnesota* which cross-reacted with several enteric strains, such as *Salmonella typhimurium*, *E. coli*, and *Shigella*. Similar antigenic cross-reactivities of major outer membrane proteins among several serotypes of *E. coli* and *Salmonella typhimurium* have been demonstrated (8).

At present there is a great need to develop a vaccine which would provide protection against challenges with both biotypes and both serotypes of *V. cholerae*. Also, this vaccine should be nontoxic. The LPS absorption technique applied in this investigation removed all LPS-reacting antibody from the antiserum raised against the total membrane. The resulting antiserum, which contains predominantly antibody molecules to the outer membrane proteins of *V. cholerae*, not only cross-reacted with the outer membrane proteins of both biotypes and both serotypes of *V. cholerae* but also agglutinated these bacteria. Besides, the outer membrane proteins of *V. cholerae* are the nontoxic components of the *V. cholerae* cell surface (S. Kabir and X. X. Mann, J. Gen. Microbiol., in press). Therefore, the outer membrane proteins of *V. cholerae* can be considered as a suitable agent for immunization. Work is now in progress to evaluate the prophylactic potential of the outer membrane proteins against experimental cholera.

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