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Characterization of a Flagellar Sheath Protein of *Vibrio cholerae*

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A flagellar sheath protein of *Vibrio cholerae* CA401 (Inaba) was characterized. Purity of the preparation was indicated by a single band on polyacrylamide gel electrophoresis gels and on Ouchterlony plates prepared with antibody against crude sheath material. The sheath protein was composed of three polypeptides with minimal molecular weights of 61,500, 60,000, and 56,500. The presence of sheath protein on the flagellum as well as on the outer membrane of the cell was demonstrated by ferritin labeling experiments with antiserum. Sheath protein antibody reacted similarly in labeling experiments and agglutination tests with a classical Ogawa strain and two nonagglutinating *V. cholerae* isolates, indicating that the sheath protein may represent the common *Vibrio* H antigen. Antibody specific for lipopolysaccharide labeled the cell but not the sehathed flagellum, which demonstrated that the sheath is not a simple extension of the outer membrane of the cell.

Most bacterial flagella are composed of the basal body, the flagellar hook, and the flagellar filament (15). The flagellum of *Vibrio* has in addition to the three components a flagellar sheath which encloses the flagellar filament (8, 17). The sheathed flagellum of *Vibrio cholerae* has a diameter of about 30.0 nm, and the sheath has a thickness of 7.5 nm (5, 9). Electron microscopy of thin sections through the cell wall and the flagellum of *Bdellovibrio bacteriovorus* indicated a layered sheath structure, and it has been suggested that the outer membrane of the cell and the flagellar sheath of *Bdellovibrio* and *Vibrio* is a continuum (8, 9, 14). The flagellar sheath does not seem to be firmly attached to the flagellar core. Washing cells of *Vibrio* with water (9) or treating them with acid or urea (8; J. Gordon and E. A. C. Follett, Proc. 5th Int. Congr. for Electron Microscopy, 2:M-5, 1962) separated the sheath from the flagellar filament.

If the flagellar sheath were indeed a continuation of the outer membrane, it should contain lipopolysaccharide (LPS) and protein as antigenic components. The evidence presented in this report indicates that the flagellar sheath of *V. cholerae* does not contain LPS but does possess a protein which is also associated with the cellular surface.

MATERIALS AND METHODS

Bacteria. *V. cholerae* (CA401, Inaba and CA411, Ogawa) were the same strains used in earlier studies (7). The nonagglutinating *V. cholerae* (NAG) strains, recent isolates from Louisiana waters and seafood, were provided by Henry Bradford, Louisiana Department of Health and Human Resources. Cultures were maintained in the lyophilized state and restored as needed. Brain heart infusion agar slants inoculated from lyophilized cultures were employed to grow seed cultures. For growing larger quantities of cells, 100 ml of brain heart infusion agar in 1-liter Erlenmeyer flasks was surface inoculated with a saline suspension of cells prepared from slants. The flasks were incubated for 14 to 18 h at 34°C. Four to 16 flasks were employed, depending on the quantity of cells desired.

Isolation of sheath material. Isolation and purification of antigens were done using strain CA401 (Inaba). The cells from Erlenmeyer flasks were recovered by washing cells from the agar surface with 0.85% saline. The cells were sedimented by centrifugation at 5,000 × *g* for 15 min. The sedimented cells were suspended in saline and standardized such that a 1/50 dilution gave an absorbance of 0.37 ± 0.03 at 600 nm on a Bausch and Lomb Spectronic 20 spectrophotometer. One hundred units, in a 5-ml volume of saline, of heat-stable hemolysin (10) produced by *Pseudomonas aeruginosa* was added to 95 ml of cell suspension, and the mixture was incubated at ambient temperature for 40 min. The cells were sedimented by centrifugation at 17,300 × *g* for 30 min. The supernatant fluid was recovered, dialyzed against distilled water, and lyophilized. The dried material will be referred to as crude sheath.

A sample of crude sheath containing 30 mg of protein was dissolved in 2.0 ml of 0.01 M phosphate buffer (pH 6.5) and applied to a column (1.5 by 15 cm) of diethylaminoethyl (DEAE)-Sephacel (Pharmacia, Inc., Piscataway, N.J.). Phosphate buffer (0.01 M, pH 6.5) was used to wash unadsorbed material through the column, and the protein was eluted with a 100-ml linear gradient of 0 to 0.7 M KCl in 0.01 M phosphate buffer. Twenty-five-drop fractions were collected and

monitored for protein at 280 nm. Carbohydrate in the fractions was quantitated by the phenol-sulfuric acid method (1). All the fractions except for those in peak I were pooled, dialyzed against deionized water, and lyophilized. The lyophilized protein was employed for further purification and immunization of rabbits.

A 50-mg protein sample from the DEAE-Sephacel column was dissolved in 2.0 ml of 0.01 M tris(hydroxymethyl)aminomethane buffer (pH 8.0) and applied to a Sepharose 6B column (0.9 by 15 cm) (Pharmacia Inc., Piscataway, N.J.). Elution was accomplished with tris(hydroxymethyl)aminomethane buffer, and 30-drop fractions were collected. Fractions in peak II formed precipitin lines on gel immunodiffusion plates with DEAE-Sephacel sheath antiserum. These fractions were pooled and analyzed by polyacrylamide gel electrophoresis (PAGE) (6). The gels were stained with amido black for protein or were stained for glycoprotein (18).

The sheath protein was separated from excised gel slices by a reverse electrophoretic procedure (4). The eluted protein was dialyzed against water and lyophilized. The minimum molecular weights of the sheath protein were determined by sodium dodecyl sulfate (SDS) gel electrophoresis (19).

Ferritin labeling. Ferritin labeling was performed by a modification of a method previously described (21). A suspension of young cells washed once in 0.5 mM phosphate-buffered saline was adjusted to an absorbance of 0.34 at 600 nm with a Bausch and Lomb Spectronic 20 spectrophotometer. A sample (0.25 ml) of this suspension was treated with an equal volume of antiserum or a dilution of antiserum, and the mixture was incubated at 37°C for 30 min. The cell-antibody mixture was washed with 10 ml of phosphate-buffered saline, and the pellet was gently suspended in 0.5 ml of phosphate-buffered saline. An aliquot (0.25 ml) of the mixture was reacted with an equal volume of a 1/4 dilution of ferritin-conjugated goat anti-rabbit immunoglobulin G (Cappell Laboratories, Cochranville, Pa.) at 37°C for 30 min. The resulting complex was washed twice in 10 ml phosphate-buffered saline and suspended in 0.1 ml of phosphate-buffered saline. The complex was analyzed for ferritin labeling by electron microscopy using Formvar-coated grids coated with carbon.

Preparation of antiserum. Whole-cell antiserum was prepared by a series of intravenous and subcutaneous injections of a standardized suspension of live cells of CA401 (Inaba) (100 Klett units, blue filter) into rabbits over a period of 25 days. If high agglutination titers were obtained on sera from test bleedings, the rabbits were exsanguinated.

Antiserum against crude sheath and DEAE-sheath (crude sheath with the LPS removed by a DEAE-Sephacel column) were prepared in rabbits by mixing 50 mg of protein of each preparation with an equal volume of Freund incomplete adjuvant. The antigen preparations were injected intramuscularly. Six weeks postimmunization, test bleedings were performed. The titers of the antisera were determined by agglutination. At the time agglutination titers reached 1/4,000 or greater, the rabbits were exsanguinated.

Antibodies specific for LPS were prepared by affinity chromatography. LPS was prepared by the phenol-water method (20). The LPS was bonded to Sepharose 4B with a diaminohexane sidearm. The LPS (10 mg), dissolved in 2 ml of 0.15 M borate buffer (pH 8.6) containing 0.5 M NaCl (BNC buffer), was activated with 6.4 mg of CNBr and maintained at pH 11.0 until the reaction ceased. The activated LPS was added to 8 ml of Sepharose with the sidearm and maintained with stirring at 4°C for 18 h. The Sepharose-LPS was added to a 7-mm column and washed with BNC buffer. Heat-inactivated whole-cell antiserum was dialyzed against BNC buffer. Three milliliters of antiserum was loaded onto the column and washed with BNC buffer until the eluate showed no absorbance at 280 nm. The LPS-specific antibody was dissociated from the column with 0.2 M glycine–0.5 M NaCl buffer (pH 2.8) until all the antibody was eluted, as determined by zero absorbance of fractions at 280 nm. The eluate containing antibody was adjusted to pH 7.6 with dilute NaOH and dialyzed against phosphate-buffered saline. The antibody in dialysis tubing was concentrated with solid polyvinylpyrrolidone and was titered by the passive hemagglutination technique of Auzins (2). We found it necessary to treat the LPS with base for adsorption of LPS to erythrocytes to occur (2). A passive hemagglutination titer of 1/800 was obtained with the LPS antibody preparation.

Antiserum against proteins in peak II from Sepharose 6B was prepared from proteins separated by PAGE (6). The three most prominent proteins (bands 1, 2, and 3) were located by staining a reference gel with amido black. Gel slices from unstained gels containing the respective bands were minced with a scalpel and suspended in saline. The suspended minced gel slices were inoculated into the axillary and lingual lymph nodes of rabbits. Three weeks later the rabbits were boosted with the minced gel containing the separated protein bands, and they were exsanguinated 3 to 5 weeks after this when sera from test bleedings indicated sufficient agglutination titers for experimentation.

All protein measurements were determined by the method of Lowry et al. (12).

RESULTS

The LPS prepared by the phenol-water procedure formed a single band on immunogel diffusion plates when reacted with whole-cell antiserum. In addition, LPS antibody prepared by affinity chromatography forms a single line with crude sheath on gel immunodiffusion plates (21). This line formed a line of identity with pure LPS. The LPS antibody reacted with the cell surface but did not react with the flagellar sheath (Fig. 1). A number of labeling experiments with LPS antibody were done, but in no case were flagella labeled.

A number of different methods were tested for removal of sheath in a specific manner. The assay we employed for determining sheath removal was measurement of the diameters of flagella after staining with phosphotungstic acid and viewing by electron microscopy. Subjective

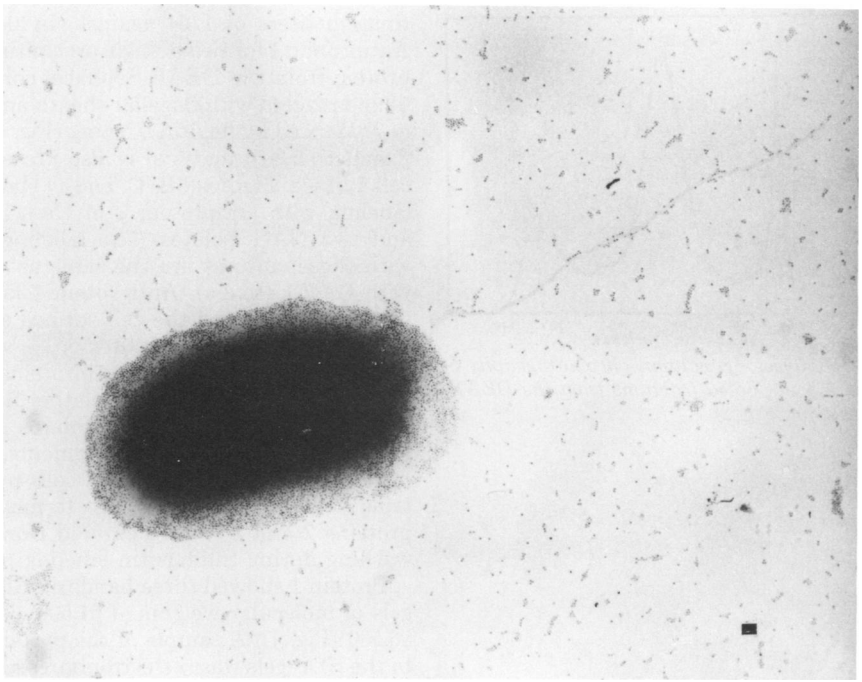

FIG. 1. *Electron micrograph showing the distribution of antibody against LPS on a cell of V. cholerae CA401 (Inaba) as seen by ferritin labeling. Note lack of labeling on the flagellum, but heavy labeling of the cell. Bar, 0.1 μm.*

evaluation of electron micrographs favored removal with low concentrations of hemolysin. Washing with distilled water was inefficient, and treatment with 3% NaCl or low concentrations of SDS of Triton X-100 appeared to affect either sheath or sheath, flagella, and somatic material. We chose the hemolysin for solubilization of sheath for purification procedures.

DEAE-Sephacel chromatography did not provide for clean separation of components of the crude sheath (Fig. 2), although peak I, which was eluted by starting buffer, contained all the LPS. In addition, peak I contained a protein which formed a line of identity on immunogel diffusion plates with a protein in the later-eluting fractions. We attempted to improve the performance of the DEAE-Sephacel column by making runs at pH values of 7.0, 7.5, 8.0, and 8.5. In these cases the separation of protein peaks was no better, and the LPS material was smeared throughout most of the fractions. At pH 6.5 we were successful in separating the LPS from the proteins in the later peaks which contained no carbohydrates as determined by the phenol-sulfuric acid method.

The elution pattern of protein from the Sepharose 6B column is shown in Fig. 3. Only the material in peak II formed a line of precipitation on gel immunodiffusion plates employing

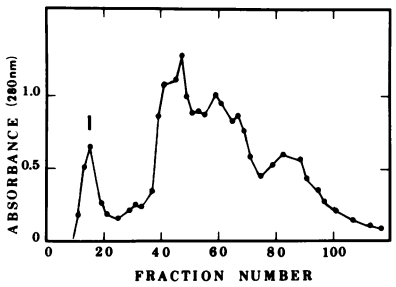

FIG. 2. *DEAE-Sephacel column chromatography of crude sheath material (30 mg of protein). Fractions in peak I contained all of the LPS. The remaining peaks contained protein, forming precipitin lines on gel immunodiffusion plates with crude sheath antiserum, and they were pooled and applied to a Sepharose 6B column.*

DEAE-sheath antiserum. The protein in peak II was analyzed by PAGE (Fig. 4). Three major and six minor protein bands were apparent. None of the bands was glycoprotein, as determined by staining of gels for carbohydrate. The major protein bands were designated 1, 2, and 3 (Fig. 4), with the protein of band 1 being near the top of the gel. Antisera against proteins 1, 2, and 3 (antiproteins) were employed in ferritin-labeling experiments. Antiprotein 1 had agglu-

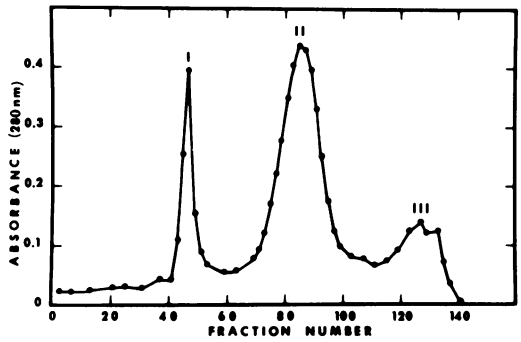

FIG. 3. *Sepharose 6B column chromatography of protein (50 mg) in pooled fractions from the DEAE-Sephacel column (Fig. 2).*

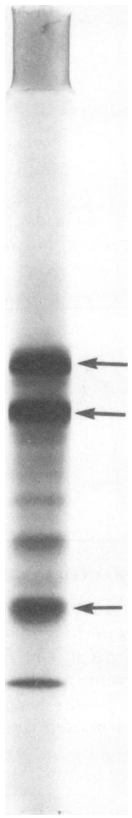

FIG. 4. *Polyacrylamide gel electrophoresis of pooled fractions from peak II of the Sepharose 6B column. Protein (200 μg) was applied to a 7% polyacrylamide gel. The three major protein bands are indicated by the arrows. Protein band 1 is nearest to the top of the gel, followed by protein bands 2 and 3.*

tination titers of 1/4,096 against live cells of *V. cholerae* CA401 (Inaba) and CA411 (Ogawa) and two NAG cultures.

Antiprotein 2 and antiprotein 3 had low agglutination titers of 1/64 against CA401 (Inaba). Antiprotein 1 formed a single precipitin line with protein from the DEAE-Sephacel column (Fig. 5) and reacted with flagellar sheath and the cell, as evidenced by ferritin labeling (Fig. 6A). Thus flagellar sheath material is also present on the cell surface. Figures 6B, C, and D show ferritin labeling with antiprotein 1 of CA411 (Ogawa) and two NAG isolates. The labeling patterns with these cultures are the same as were seen with CA401 (Inaba). Antiprotein 1 labeled the flagellar sheath and the cell surface even if diluted to an agglutination titer of 1/64, which was the agglutination titer of antiproteins 2 and 3. Antiproteins 2 and 3 labeled neither flagella nor cells. The cellular origin of proteins 2 and 3 is not apparent from our experiments, and the mechanism of agglutination of cells by antiproteins 2 and 3 is not known. It may be that proteins 2 and 3 were separated from cells by washing during the ferritin labeling procedure.

Protein 1 showed three bands on SDS-PAGE gels of molecular weights of 61,500, 60,000, and 56,500 (Fig. 7). A sample of the protein applied to the SDS gels was reelectrophoresed (4). Only a single band was observed. From our results it appears that protein 1 is a multimeric protein composed of three different subunits.

DISCUSSION

An interesting, although not a unique feature of *V. cholerae* is the presence of a flagellar sheath which appears in electron micrographs to be continuous with the outer membrane (8, 9,

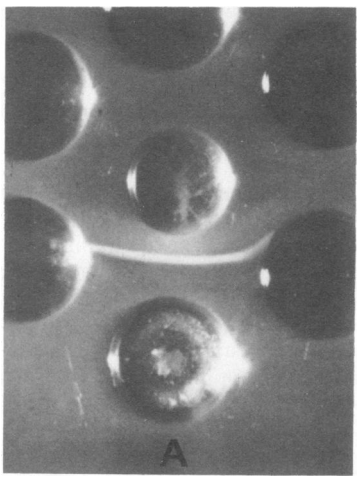

FIG. 5. *Gel immunodiffusion plate showing a single line of precipitation with antibody to band 1 protein (Fig. 4) and pooled fractions (all fractions except those in peak I) from the DEAE-Sephacel column (Fig. 2). The center well contained 30 μl of antiserum, and 50 μl of pooled fractions was added to the bottom well (A).*

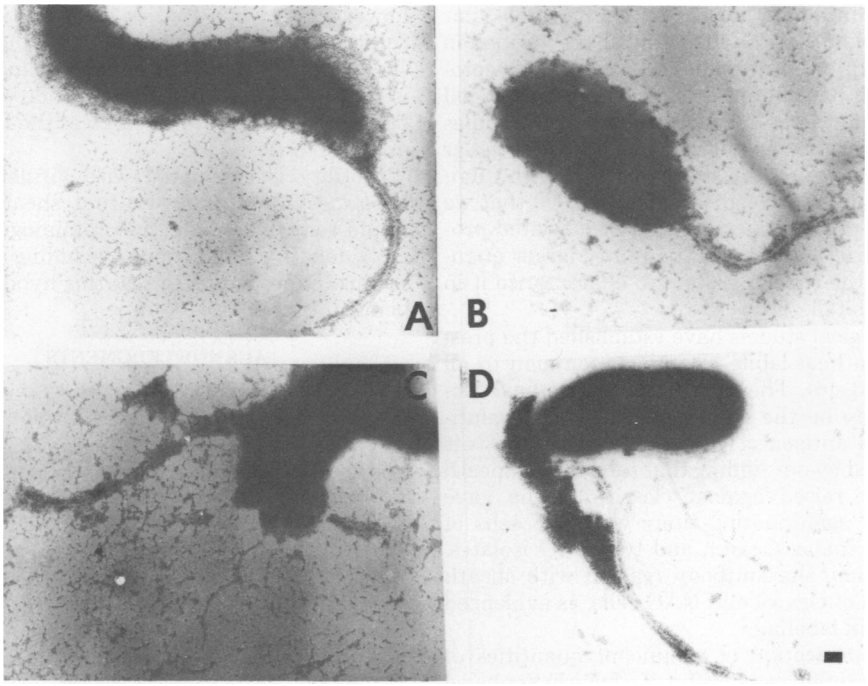

FIG. 6. *Electron micrographs showing the distribution of antibody against protein from band 1 of PAGE (Fig. 4) as demonstrated by ferritin labeling of cells of V. cholerae. (A) CA401 (Inaba). (B) CA411 (Ogawa). (C) A NAG isolate. (D) A NAG isolate. Both flagellar sheaths and cells of all strains are ferritin labeled. Bar, 0.1 μm.*

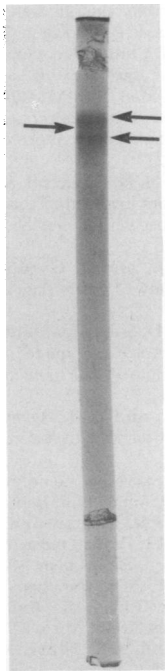

FIG. 7. *SDS-PAGE of protein from band 1 of PAGE. The protein was treated with 1% SDS and 1% mercaptoethanol and was electrophoresed on a gel containing 0.1% SDS and 10% polyacrylamide. Molecular weight standards employed were bovine serum albumin, ovalbumin, aldolase, chymotrypsinogen, and ribonuclease. Beginning with top arrow, molecular weights are 61,500, 60,000, and 56,500.*

15). If the sheath is continuous and identical with the outer membrane, identical components (LPS and proteins) should be present on the cell and in the sheath. Our data indicate that LPS is not a component because LPS-specific antibody (Fig. 1) did not react with flagellar sheath but reacted strongly with the outer surface of the cell. It is possible that LPS is present in the sheath but is masked and not available to antibody. We think this is improbable because LPS is a surface component of outer membrane of whole cells and reacts readily with antibody. The sheath does not appear to be a simple extension of the outer membrane.

From our experiments it appears that a protein is common to both the flagellar sheath and the cell surface. The protein formed a single band on PAGE, and SDS gels showed the protein to be composed of three protomers of a molecular weight of about 60,000. In the native state the protein could have a very high molecular weight, depending upon how many protomers are present in the molecule. If the sheath protein were a trimer, the molecular weight would be a minimum of about 180,000.

Flagellin of *V. cholerae* has a molecular weight of 45,000 (21), which we have verified in our laboratory. Antibodies against flagellin combine with flagella but do not react with the cell (21). In addition (data not presented), antibodies for sheath protein do not react with flagellar cores. Therefore, the sheath protein with minimum molecular weight of about 60,000 that we have described is not flagellin or a related protein. It will be necessary to purify larger quantities of the sheath protein to characterize it in greater detail.

Serological studies have established the presence of a heat-labile H antigen common to all vibrios (3, 16). The sheath protein we have isolated may be the common heat-labile agglutinating H antigen of vibrios. This hypothesis is supported by our finding that the specific sheath antibody raised against classical Inaba gave identical agglutinating titers with live cells of classical Inaba, Ogawa, and two NAG isolates. In addition, the antibody reacted with sheath and cells of Ogawa and NAG cells, as evidenced by ferritin labeling.

The purification of significant quantities of sheath protein was difficult. DEAE-Sephacel served only to separate the LPS and other carbohydrate-containing materials from proteins in the crude sheath material. The Sepharose 6B column was more efficacious in separating the proteins from the DEAE-Sephacel than was a Sepharose 4B column. These results indicated the high molecular weights of the proteins. One of the obvious difficulties in the purification of the sheath protein is the lack of a rapid assay method for sheath material. Thus, we were limited in the development of a purification procedure to immunizing rabbits with the fractions from columns and determining whether or not the antibodies reacted with flagellar sheath—a tedious process as compared to enzyme purification, for which simple and rapid assays for specific enzyme activities are usually available.

The success we had in identifying the sheath protein described in this report was based on PAGE separation and the immunization of rabbits with the minced gel slices containing the separated protein. Part of our success may be attributable to the strong antigenicity of the protein in band 1 of the gel, because bands 2 and 3 did not induce good antibody titers although the same methodology was employed. Only antibody to band 1 reacted with flagellar sheath even if diluted to the same titers as for antiprotein 2 and antiprotein 3. Thus, we believe that only protein 1 of the proteins separated by PAGE is present as a significant component of the flagellar sheath. It must be emphasized, however, that our studies have partially characterized only one component of the flagellar sheath. Our research does not preclude the existence of other sheath components, which may be identified in future research as the H antigens of *V. cholerae*.

Motility is associated with virulence in *V. cholerae* (10). It may be that sheath protein would serve as an effective immunogen against all *V. cholerae* infections, including the NAG strains. Experiments to test this hypothesis are being initiated.

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