JOURNAL OF BACTERIOLOGY, Apr. 1988, p. 1488–1494
0021-9193/88/041488-07$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 170, No. 4

Demonstration of Lipopolysaccharide on Sheathed Flagella of *Vibrio cholerae* O:1 by Protein A-Gold Immunoelectron Microscopy

J. A. FUERST* AND J. W. PERRY
Department of Microbiology, University of Queensland, St. Lucia, 4067, Queensland, Australia

Received 27 October 1987/Accepted 30 December 1987

Monoclonal antibodies with group and type specificity for lipopolysaccharide antigens were used in combination with protein A-colloidal gold labeling and transmission electron microscopy to demonstrate the presence of lipopolysaccharide antigens on both the sheathed flagellum and the cell surface of Inaba and Ogawa strains of *Vibrio cholerae* O:1. Labeling was associated with the sheath of the flagellum rather than the core, and flagellar cores were not labeled. Flagellum and cell shared a common set of lipopolysaccharide antigens characteristic of the strain serotype.

Recent research on the pathogenesis of cholera has concentrated on the factors responsible for adhesion of *Vibrio cholerae* cells to the intestinal mucosa and the nature of the antigens responsible for stimulating an immune response to infection (1, 4–6, 20). Although the important protective antigens have yet to be unequivocally defined, purified lipopolysaccharide (LPS) has been reported to induce significant protection against cholera in humans and experimental animals (16). The flagellum of *V. cholerae* has been implicated in pathogenesis of cholera in two ways: with respect to its role as a motility organelle (12, 18, 25) and with respect to its possible role in adherence of *V. cholerae* to the host intestinal surface (1). The role of the flagellum as a carrier of the adhesins responsible for attachment to intestinal tissue may be of greater significance than its role in conferring motility (1).

While the flagellum of *V. cholerae* is of possible significance in pathogenesis, its chemical and antigenic composition are poorly understood. The flagellum possesses a membranous sheath covering a core which is similar in structure to naked flagella of other bacteria (7, 8). In *Vibrio metchnikovii*, continuity between the sheath and the outer membrane has been demonstrated (10). The question of whether the flagellar sheath of *V. cholerae* shares the macromolecular composition of the cell wall outer membrane has not been resolved, however. It has been proposed, on the basis of immunoferritin electron microscopy using polyclonal antibodies to LPS, that the sheath does not contain LPS and that the sheath is thus not a simple extension of the outer membrane (17). However, reports that polyclonal antibodies against somatic O antigen and monoclonal antibodies against LPS determinants immobilize motile *V. cholerae* cells are consistent with the occurrence of LPS in the flagellar sheath (1, 14). One way to resolve this problem is to localize LPS directly, utilizing highly specific monoclonal antibodies against LPS antigenic determinants and an electron-dense protein A-gold marker for immunoelectron microscopy. LPS determinants distinguish two major serotypes of *V. cholerae* O:1: Ogawa, with determinants A and B, and Inaba, with determinants A and C (13). The establishment of hybrid cell lines producing monoclonal antibodies against such group- and type-specific O antigens of *V. cholerae* O:1 (13, 15) has made such an approach possible. We report here the localization of LPS on the sheathed flagellum of *V. cholerae* by employing such a technique.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *V. cholerae* NCTC 8021 (UQM 2928) and NCTC 7254 (UQM 2929) were obtained from the National Type Culture Collection (United Kingdom) and entered into the University of Queensland Department of Microbiology Culture Collection. *V. cholerae* NIH 41 (UQM 2931), NIH 35A3 (UQM 2932), and NCTC 4711 (UQM 2934) were kindly donated by Riichi Sakazaki and Toshio Shimada, National Institute of Health, Tokyo. Strains UQM 2928 and UQM 2931 are O:1 Ogawa serotype, while UQM 2929 and UQM 2932 are O:1 Inaba serotype. Serotypes were checked routinely by slide agglutination with polytypic (anti-O:1) antiserum and anti-Inaba and anti-Ogawa typing sera (all from Difco Laboratories, Detroit, Mich.). Strains were routinely maintained in semisolid heart infusion–0.3% agar Craigie tubes at room temperature. For immunolabeling experiments, 20 ml of heart infusion broth in a 250-ml Erlenmeyer flask was inoculated with 1 ml of an overnight culture in heart infusion broth and incubated with shaking on a New Brunswick Gyrotory water bath shaker (model G76) at 288 rpm, at 37°C, to achieve a logarithmic-phase culture of sufficient density for preparative purposes.

Immunological reagents. Monoclonal antibodies against LPS antigens of *V. cholerae* were generously donated by Bjorn Gustafsson of the Karolinska Institutet, Stockholm, Sweden. Production and characterization of the hybridomas producing the antibodies and enzyme-linked immunosorbent assay characterization of the antibodies are described by Gustafsson and Holme (13). Monoclonal antibody H4 possesses specificity for the A determinant of *V. cholerae* LPS and is immunoglobulin M (IgM); H8 and C6 are specific for B and C determinants, respectively, and are both IgG3 antibodies (13). These antibodies were all received as preparations of mouse ascites fluid. The negative control used in experiments involving these monoclonal antibodies was FNBA4, a mouse monoclonal antibody (IgG2a kappa) against *Fusobacterium nucleatum* 263, supplied by Phil Bird of University of Queensland Dental School. Rabbit anti-mouse immunoglobulins used as a second antibody in indi-

rect immunolabeling experiments were purchased from Dakopatts, Glostrup, Denmark (product Z109).

Protein A-colloidal gold. Colloidal gold particles (9 nm) were prepared by the method of Slot and Geuze (21). Protein A (Sigma Chemical Co., St. Louis, Mo.) was conjugated to the gold particles, and the conjugate was purified by the methods of Slot and Geuze (21).

Immunolabeling of whole cells. Cells were grown in heart infusion broth (Difco) for 3 h, shaken at 37°C, after inoculation from an overnight heart infusion broth culture. Cells were fixed by centrifuging the broth culture and suspending the pellet in 1% glutaraldehyde in Sorensen's 0.1 M phosphate buffer (pH 7.2). Cells were fixed for 5 min at room temperature and were then centrifuged, washed once in phosphate-buffered saline (PBS), and resuspended in PBS to an absorbance of 0.7 at 540 nm (ca. 5×10^9 cells ml^{-1}). A drop (15 to 35 μl) of this suspension was placed on a sheet of Parafilm. A carbon-stabilized nitrocellulose-filmed copper specimen support grid was placed on the drop of cell suspension, carbon side down, for 1 min and then onto drops (15 to 35 μl) of the following reagents on the same parafilm sheet: (i) for direct protein A-gold labeling, PBS–0.02 M glycine (10 min), PBS–2% bovine serum albumin (PBS-BSA) (5 min), monoclonal antibody diluted in PBS-BSA (30 min), four washes in PBS-BSA (1 min each), protein A-gold (1:20 dilution in PBS-BSA) (30 min), and three washes in distilled water (5 min each); (ii) for indirect labeling, PBS–0.02 M glycine (10 min), PBS-BSA (5 min), monoclonal antibody diluted in PBS-BSA (30 min), four washes in PBS-BSA (1 min each), rabbit anti-mouse immunoglobulin diluted 1:100 in PBS-BSA (30 min), four washes in PBS-BSA (1 min each), protein A-gold (1:20 dilution in PBS-BSA) (30 min), and three washes in distilled water (5 min each). After a completed treatment protocol, excess fluid was removed from grids with filter paper and the grid was either air dried without staining (unstained preparations) or negatively stained by applying a drop of 1% ammonium molybdate containing 0.05% bovine serum albumin, removing the excess fluid with filter paper, and allowing to air dry. H4 and H8 monoclonal antibodies were used at a dilution from ascites fluid of 1:100; C6 was diluted at 1:400. In some experiments, LPS from *V. cholerae* Inaba 569B (product no. L-5262, Sigma) at 1 mg ml^{-1} was incubated for 30 min with diluted monoclonal antibody preparations, and the mixture was used in indirect labeling experiments in place of the normal primary monoclonal antibody.

Immunolabeling of thin sections. Cells were grown for 2 h in heart infusion broth, shaken at 37°C, and harvested by centrifugation, and the pellet was fixed with 5% glutaraldehyde in 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 8.0) for 1 h at room temperature. Cells were then washed with buffer, suspended in 3% agarose, fixed in 1% osmium tetroxide in HEPES buffer for 1 h at room temperature, treated with 2% aqueous uranyl acetate for 30 min, dehydrated through two changes of 70% ethanol for 30 min, and infiltrated overnight at 4°C with LR White resin (London Resin Company Ltd., Basingstoke, U.K.) before being embedded in LR White resin by polymerization at 50°C for 24 h.

Thin sections were cut on a Sorvall MT-5000 ultramicrotome, collected on grids, and then immunolabeled by the indirect protocol outlined above, with the omission of the PBS-glycine treatment. Sections were then stained with uranyl acetate (5 min) and Reynolds lead citrate (2 min).

Electron microscopy. Specimens were examined using a Hitachi H-800 transmission electron microscope operated at 100 kV accelerating voltage.

RESULTS

Use of the protein A-gold immunolabeling technique with mouse monoclonal antibodies must take into account the limited reactivity of protein A with IgM mouse antibodies (11), so that a direct protein A-gold labeling protocol using mouse monoclonal antibodies is limited to monoclonals of an appropriate class and subclass. Thus, direct protein A-gold labeling of *V. cholerae* O:1 Inaba cells and flagella was achieved by using a method employing only monoclonal antibody H8 directed against the B determinant of the *V. cholerae* LPS with protein A-gold (Fig. 1). However, the full spectrum of antibodies against A, B, and C LPS determinants in *V. cholerae* (including the IgM anti-A monoclonal antibody) could not be tested by this direct protein A-gold method, and an indirect method employing anti-LPS monoclonal antibody as the primary antibody and rabbit anti-mouse immunoglobulins as the secondary antibody before protein A-gold treatment was used for most experiments.

Indirect immunolabeling demonstrated that both cells and flagella of all *V. cholerae* strains tested showed protein A-gold labeling when the group-specific antibody H4, directed against the A determinant of *V. cholerae* LPS, was used as the primary antibody (Table 1; Fig. 2A and B; Fig. 3A). Both Ogawa strains tested (UQM 2928 and UQM 2931) displayed label on both cells and flagella when an anti-B-determinant antibody (H8) was the primary antibody, but were not labeled when an anti-C-determinant antibody (C6) was used (Table 1; Fig. 3C and D). The Inaba strains (UQM 2929 and UQM 2932) displayed label of both cells and flagella

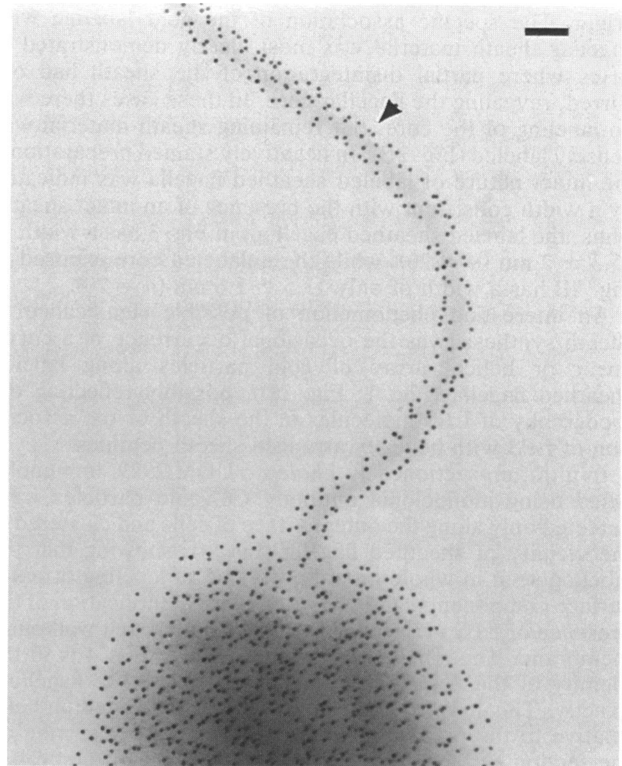

FIG. 1. *V. cholerae* UQM 2928, immunolabeled with anti-B-determinant monoclonal antibody H8 and protein A-colloidal gold by the direct protein A-gold labeling protocol. Arrows indicate helical banding of gold particles around flagellum. Unstained. Bar, 100 nm.

TABLE 1. Results of protein A-gold immunolabeling of *V. cholerae* O:1 Ogawa and Inaba strains, using monoclonal antibodies as a primary antibody with the indirect protocol

Antibody	Labeling[a] of strain:			
	Ogawa		Inaba	
	UQM 2928	UQM 2931	UQM 2929	UQM 2932
H4 (anti-A)	+	+	+	+
H4 + LPS	−	−	−	−
H8 (anti-B)	+	+	−	−
C6 (anti-C)	−	−	+	+
FNBA4 (negative control)	−	−	−	−

[a] +, Dense labeling of cells and flagella; −, no labeling of cells and flagella. Twenty cells were scanned on each grid, using an instrument magnification of ×12,000 and fluorescent screen-scanning binoculars; a negative result was recorded if fewer than 10 gold particles were observed on cell and flagellum surface.

when anti-C-determinant antibody (C6) was the primary antibody, but not when anti-B-determinant antibody (H8) was used. The pattern of labeling of intact flagella of the *V. cholerae* strains with type- and group-specific antibodies against LPS determinants therefore does not differ from the pattern of labeling of the cell soma surface and exactly reflects the LPS determinant pattern of the strain serotype. Consistent with this evidence for occurrence of LPS antigenic determinants on both cell soma and flagella was the inhibition of labeling with anti-A (H4) antibody by purified LPS from *V. cholerae* Inaba strain 569B (Table 1; Fig. 3B). A similar inhibition reaction attempted with anti-C antibody did result in some residual patchy labeling of unknown origin. The specific association of the gold labeling with flagellar sheath material was most clearly demonstrated in cases where partial disintegration of the sheath had occurred, revealing the flagellar core. In these cases there was no labeling of the core, but remaining sheath material was densely labeled (Fig. 2B). In negatively stained preparations, the intact nature of labeled sheathed flagella was indicated by a width consistent with the presence of an intact sheath. Thus, the labeled sheathed flagellum in Fig. 3 has a width of 25.3 ± 2 nm (n = 20), while the unlabeled core exposed in Fig. 2B has a width of only 13.5 ± 1.6 nm (n = 20).

An interesting phenomenon of possible significance to sheath synthesis was the occasional occurrence of a curvilinear or helical array of gold particles along labeled sheathed flagella (Fig. 1; Fig. 2D), possibly reflecting the topography of LPS molecules in the sheath or the association of LPS with helically arranged sheath peptides.

In ultrathin-sectioned *V. cholerae* UQM 2929, immunolabeled using monoclonal antibody C6, gold particles were detected only along the outer surface of cells and clustered in the vicinity of sheathed flagella (Fig. 4), showing that the labeling seen in whole mounts was due to labeling of outer surface components, and consistent with identification of the presence of LPS in flagellar sheath as well as cell wall outer membrane. The labeling of the flagellum in Fig. 4 is in the vicinity of the flagellum but does not follow the flagellum exactly. This may be due to the angle of the flagellum helix relative to the plane of section, labeling only occurring on the section surface, combined with the effect of the projection of LPS molecules away from the flagellum surface. Extension of LPS molecules away from the surface of gram-negative bacteria has been reported in *Escherichia coli* and *Pseudomonas aeruginosa* with the aid of immunoelectron microscopy techniques (2, 19).

DISCUSSION

This study was initiated to resolve the question of whether the sheathed flagella of *V. cholerae* possess LPS antigenic determinants. The use of immunolabeling involving group- and type-specific monoclonal antibodies developed by Gustafsson et al. (15) against LPS antigens of *V. cholerae* O:1 and protein A-gold particles as markers has enabled demonstration of such LPS determinants on sheathed flagella, and these are specifically associated with the flagellar sheath. The LPS antigenic pattern of the sheathed flagellum corresponds to that of the cell surface, that is, of the outer membrane of the cell wall. Thus, on Ogawa strains flagella possess A and B determinants, while on Inaba strains they possess determinants A and C. The lack of any C determinants on either Ogawa cells or flagella is consistent with results of enzyme-linked immunosorbent assay-inhibition experiments of Gustafsson and Holme (13) which demonstrated specificity of the C antigenic determinant to Inaba strains and of B antigen to Ogawa strains. There is no evidence from the present study for any discontinuity in composition of sheath membrane relative to the composition of the outer membrane of the wall, with respect either to the presence of LPS or to the distribution of the LPS determinants. The protein A-gold immunocytochemical technique enabled examination of flagella at high resolution so that both antigen distribution and flagellum ultrastructure could be determined in the same preparation.

Previous studies of *V. cholerae* flagella have conflicted with respect to the question of whether sheathed flagella of this species possess LPS. Benenson et al. (3) had observed that somatic antibody (presumably anti-LPS) inhibited vibrio motility in wet mounts of stools from cholera patients, and motility inhibition experiments with polyclonal anti-LPS antibodies (1) or monoclonal anti-LPS antibodies identical to those used in the present study (14) have confirmed this for cultured *V. cholerae* strains. Zykin (26) reported that fluorescent anti-O antiserum stained flagella as well as cell bodies of cholera vibrios, a result similar to that obtained by Williams et al. (24) for flagellated cells in infected rabbit ileal loops. However, from experiments using affinity-purified anti-LPS antibody and an indirect ferritin immunolabeling technique, it has been reported that LPS is not a component of the flagellar sheath of *V. cholerae* (17). In those experiments, anti-LPS antibody reacted with the cell surface, as indicated by labeling with ferritin-conjugated anti-rabbit immunoglobulin, but did not react with flagellar sheath. Figure 1 of the report by Hranitsky et al. (17) indicates some labeling of a flagellum. However, it is not clear that the flagellum is sheathed, and the labeling is difficult to distinguish against a high background.

Results obtained here are consistent with those found in motility inhibition experiments with *V. cholerae* strains by employing the same monoclonal antibodies (14). In the latter study, it was suggested that inhibition of motility by antibodies specifically directed against LPS determinants might be due to steric hindrance of flagellar movement or to alteration of bacterial metabolism resulting in depletion of energy required for motility. The immunolabeling results presented here support the former hypothesis. However, at least two alternative mechanisms for the mode of action of sheathed flagella have been proposed, one involving the rotation of the whole flagellum in a fluid membrane and the other involving helical wave propagation along a flagellum with a rigid sheath and outer cell wall membrane (9). Rotation of the flagellum in a fluid portion of cell wall outer

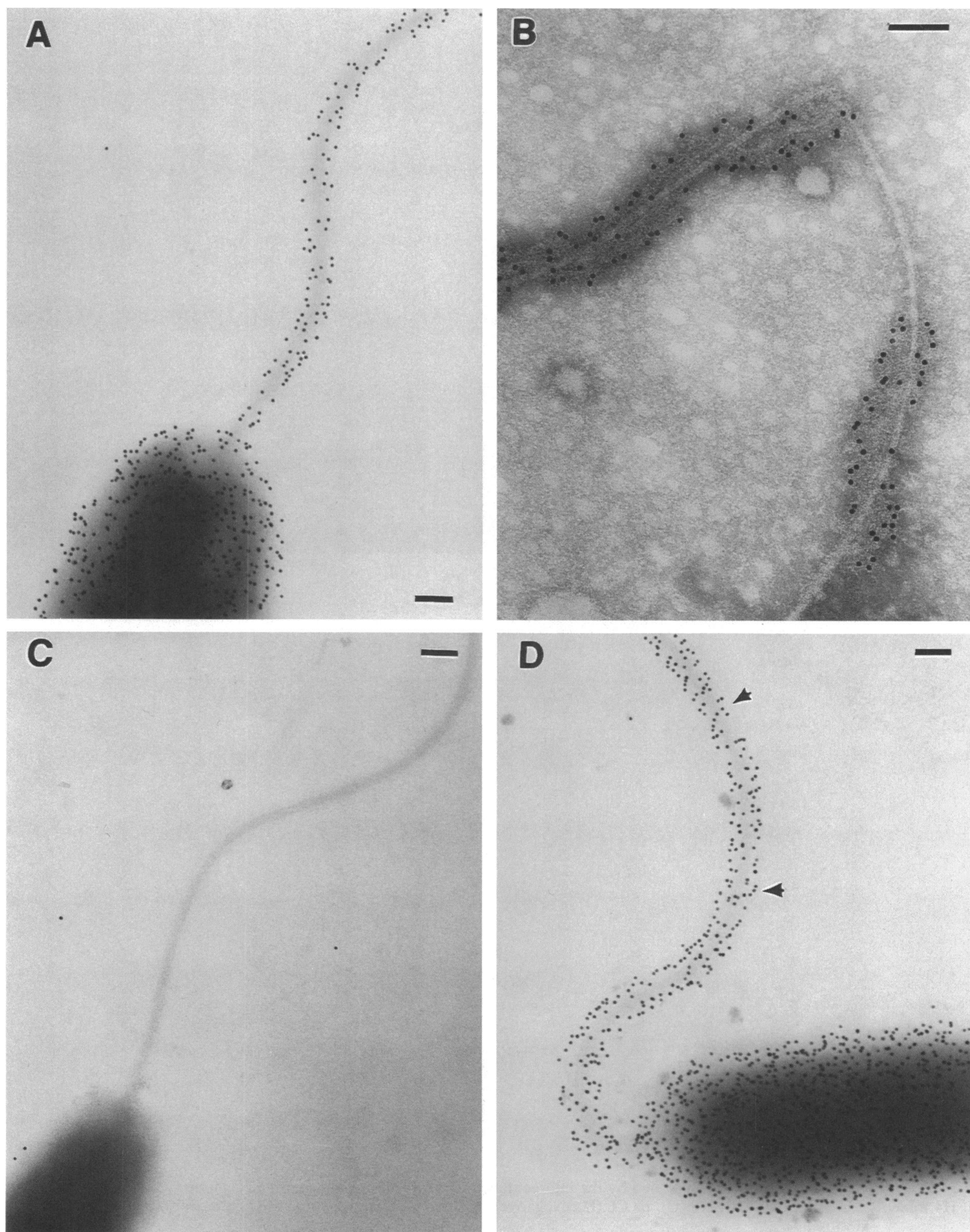

FIG. 2. *V. cholerae* UQM 2929, immunolabeled with the indirect labeling protocol, using as a primary antibody the monoclonal antibodies H4 (A and B), H8 (C), or C6 (D). Arrows indicate helical banding of gold particles around flagellum. Panels A, C, and D, Unstained; B, negatively stained with ammonium molybdate. Bar, 100 nm.

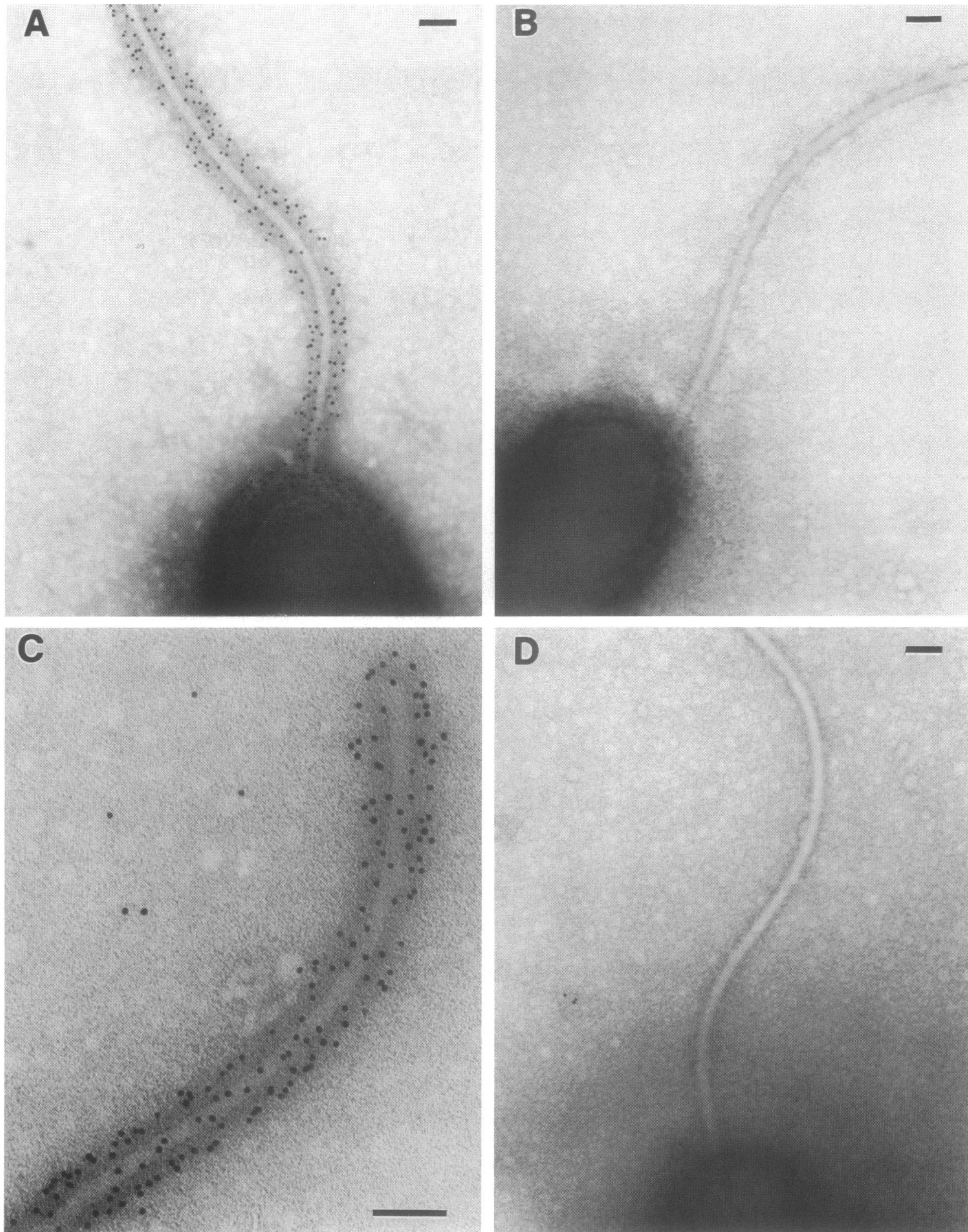

FIG. 3. *V. cholerae* UQM 2928, immunolabeled by the indirect labeling protocol, using as a primary antibody the monoclonal antibodies H4 (A), H4 mixed with Inaba LPS (B), H8 (C), or C6 (D). Negatively stained with ammonium molybdate. Bar, 100 nm.

membrane might be hindered if antibody reacted with LPS in the wall as well as in the flagellar sheath.

The presence of LPS in sheathed flagella of *V. cholerae* is in conformity with the demonstration by chemical methods of a substantial quantity of LPS in the flagellar sheath of *Bdellovibrio bacteriovorus* by Thomashow and Rittenberg (23). Results with bdellovibrios indicate, however, that although LPS may be present in both sheath and outer

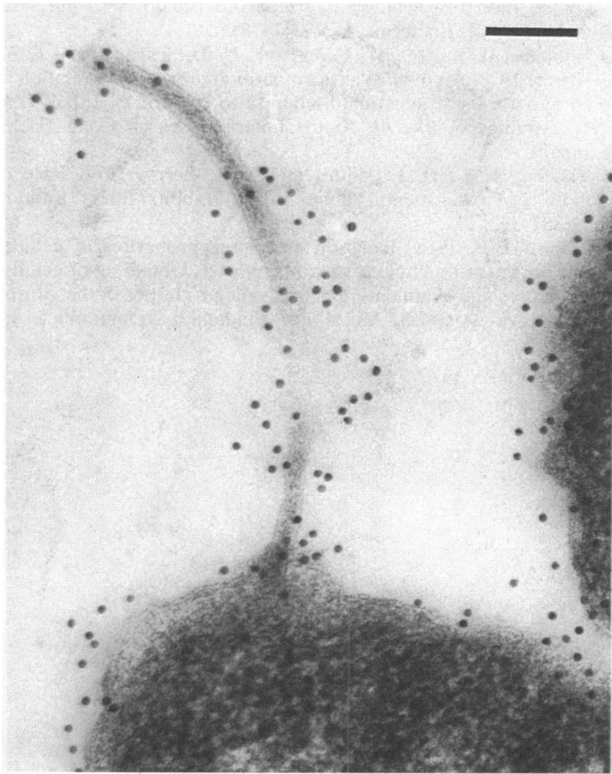

FIG. 4. *V. cholerae* UQM 2929, ultrathin sectioned and immunolabeled by the indirect protocol, using as a primary antibody the monoclonal antibody C6. Gold labeling of both the outer membrane of cell wall and the sheathed flagellum is displayed. Bar, 100 nm.

membrane, lipid A fatty acid composition of sheath LPS may differ substantially from that of outer membrane LPS. The immunolabeling experiments presented here do not distinguish whether sheath LPS differs in lipid A fatty acid composition, so that the question of whether the flagellar sheath in *V. cholerae* is a separate, stable, and more fluid domain relative to the outer membrane, as suggested for bdellovibrios by Thomashow and Rittenberg, is not resolved.

The demonstration of LPS on sheathed flagella of *V. cholerae* has implications with respect to mechanisms of pathogenesis, as LPS can now be considered as a possible adhesin contributing to colonization of the intestinal mucosa in cholera. This is so because the presence of the flagellum on *V. cholerae* cells is essential for in vitro attachment and enhances initial colonization of host intestinal surface in the infant mouse cholera model (1). The flagellum thus functions as a carrier for moieties promoting adherence. That LPS may be one of these moieties is suggested by the finding that LPS can inhibit adhesion of *V. cholerae* to intestinal mucosa (5), that anti-LPS antibodies can protect against experimental cholera (22), and that immunity against mucosal colonization in a rat model has been found to be accompanied at least in some cases by mucus-borne antibody against LPS (6). Non-LPS adhesins may also occur on the flagellum, however, including those which might be exposed where sheath is removed.

Thus, the demonstration of LPS on sheathed flagella of *V. cholerae* is consistent with the role of the flagellum as a carrier of adhesins and with the possible significance of LPS as one of these adhesins. Further work on the importance of LPS as an adhesin relative to other non-LPS adhesins (6) is necessary to define the importance to pathogenesis of flagellar sheath LPS in *V. cholerae*.

ACKNOWLEDGMENTS

We thank Bjorn Gustafsson for the generous donation of monoclonal antibodies and Phil Bird of the University of Queensland Dental School for donation of negative control anti-*Fusobacterium* monoclonal antibodies. We also thank Helen Ilett for technical assistance, Jane Westcott for assistance with photography, and Ric Webb and Jan Slot for assistance and expert advice in preparation of protein A-colloidal gold.

This work was supported by a Special Project Grant from the University of Queensland.

LITERATURE CITED

1. **Attridge, S. R., and D. Rowley.** 1983. The role of the flagellum in the adherence of *Vibrio cholerae*. J. Infect. Dis. **147**:864–872.
2. **Bayer, M. E., D. Weed, S. Haberer, and M. N. Bayer.** 1986. Localization of O9-lipopolysaccharide within the *Escherichia coli* K29 capsule. FEMS Microbiol. Lett. **35**:167–170.
3. **Benenson, A. S., M. R. Islam, and W. B. Greenough III.** 1964. Rapid identification of *Vibrio cholerae* by dark-field microscopy. Bull. WHO **30**:827–831.
4. **Chitnis, D. S., K. D. Sharma, and R. S. Kamat.** 1982. Role of bacterial adhesion in the pathogenesis of cholera. J. Med. Microbiol. **15**:43–51.
5. **Chitnis, D. S., K. D. Sharma, and R. S. Kamat.** 1982. Role of somatic antigen of *Vibrio cholerae* in adhesion to intestinal mucosa. J. Med. Microbiol. **15**:53–61.
6. **Cooper, G. N., and R. Narendranathan.** 1986. Antibacterial immunity to *Vibrio cholerae* in rats. J. Med. Microbiol. **22**:133–141.
7. **Das, J., and S. N. Chatterjee.** 1966. Electron microscopic studies on some ultrastructural aspects of *Vibrio cholerae*. Indian J. Med. Res. **54**:330–338.
8. **Follett, E. A. C., and J. Gordon.** 1963. An electron microscope study of vibrio flagella. J. Gen. Microbiol. **32**:235–239.
9. **Fuerst, J. A.** 1980. Bacterial flagella and the rotary motor model for the mechanism of bacterial motility. J. Theor. Biol. **84**:761–774.
10. **Glauert, A. M., D. Kerridge, and R. W. Horne.** 1963. The fine structure and mode of attachment of the sheathed flagellum of *Vibrio metchnikovii*. J. Cell Biol. **18**:327–336.
11. **Goding, J. W.** 1978. Use of staphylococcal protein A as an immunological reagent. J. Immunol. Methods **20**:241–253.
12. **Guentzel, M. N., and L. J. Berry.** 1975. Motility as a virulence factor for *Vibrio cholerae*. Infect. Immun. **4**:890–897.
13. **Gustafsson, B., and T. Holme.** 1983. Monoclonal antibodies against group- and type-specific lipopolysaccharide antigens of *Vibrio cholerae* O:1. J. Clin. Microbiol. **18**:480–485.
14. **Gustafsson, B., and T. Holme.** 1985. Rapid detection of *Vibrio cholerae* O:1 by motility inhibition and immunofluorescence with monoclonal antibodies. Eur. J. Clin. Microbiol. **4**:291–294.
15. **Gustafsson, B., A. Rosén, and T. Holme.** 1982. Monoclonal antibodies against *Vibrio cholerae* lipopolysaccharide. Infect. Immun. **38**:449–454.
16. **Holmgren, J., and A.-M. Svennerholm.** 1977. Mechanisms of disease and immunity in cholera: a review. J. Infect. Dis. **136**: S105–S112.
17. **Hranitsky, K. W., A. Mulholland, A. D. Larson, E. R. Eubanks, and L. T. Hart.** 1980. Characterization of a flagellar sheath protein of *Vibrio cholerae*. Infect. Immun. **27**:597–603.
18. **Jones, G. W., and R. Freter.** 1976. Adhesive properties of *Vibrio cholerae*: nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. Infect. Immun. **14**:240–245.
19. **Lam, J. S., M. Y. C. Lam, L. A. MacDonald, and R. E. W. Hancock.** 1987. Visualization of *Pseudomonas aeruginosa* O antigens using a protein A-dextran-colloidal gold conjugate with both immunoglobulin G and immunoglobulin M monoclonal

antibodies. J. Bacteriol. **169**:3531–3538.
20. **Manning, P. A., M. W. Heuzenroeder, J. Yeadon, D. I. Leavesley, P. R. Reeves, and D. Rowley.** 1986. Molecular cloning and expression in *Escherichia coli* K-12 of the O antigens of the Inaba and Ogawa serotypes of the *Vibrio cholerae* O1 lipopolysaccharides and their potential for vaccine development. Infect. Immun. **53**:272–277.
21. **Slot, J. W., and H. J. Geuze.** 1985. A new method of preparing gold probes for multiple-labelling cytochemistry. Eur. J. Cell Biol. **38**:87–93.
22. **Svennerholm, A. M., and J. Holmgren.** 1976. Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect. Immun. **13**:735–740.
23. **Thomashow, L. S., and S. C. Rittenberg.** 1985. Isolation and composition of sheathed flagella from *Bdellovibrio bacteriovorus* 109J. J. Bacteriol. **163**:1047–1054.
24. **Williams, H. R., Jr., W. F. Verwey, G. D. Schrank, and E. K. Hurry.** 1974. An *in vitro* antigen-antibody reaction in relation to an hypothesis of intestinal immunity to cholera, p. 161–173. *In* Proceedings of the 9th Joint Cholera Research Conf. (U.S.-Japan).
25. **Yancey, R. J., D. L. Willis, and L. J. Berry.** 1978. Role of motility in experimental cholera in adult rabbits. Infect. Immun. **22**:387–392.
26. **Zykin, L. F.** 1968. Isolation and some properties of cellular components of a cholera vibrio. Report 1. Obtaining of cellular structures and examination of antigenic properties of the vibrion of Ogawa type. Zh. Mikrobiol. Epidemiol. Immunobiol. **45**: 38–43.