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Identification, Characterization, and Spatial Localization of Two Flagellin Species in *Helicobacter pylori* Flagella

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Flagellar filaments were isolated from *Helicobacter pylori* by shearing, and flagellar proteins were further purified by a variety of techniques, including CsCl density gradient ultracentrifugation, pH 2.0 acid disassociation-neutral pH reassociation, and differential ultracentrifugation followed by molecular sieving with a Sephacryl S-500 column or Mono Q anion-exchange column, and purified to homogeneity by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to an Immobilon membrane. Two flagellin species of pI 5.2 and with apparent subunit molecular weights (M_rs) of 57,000 and 56,000 were obtained. N-terminal amino acid analysis showed that the two *H. pylori* flagellin species were related to each other and shared sequence similarity with the N-terminal amino acid sequence of *Campylobacter coli*, *Bacillus*, *Salmonella*, and *Caulobacter* flagellins. Analysis of the amino acid composition of the predominant 56,000-M_r flagellin species isolated from two strains showed that it was comparable to the flagellins of other species. The minor 57,000-M_r flagellin species contained a higher content of proline. Immunoelectron microscopic studies with polyclonal monospecific *H. pylori* antiflagellin antiserum and monoclonal antibody (MAb) 72c showed that the two different-M_r flagellin species were located in different regions of the assembled flagellar filament. The minor 57,000-M_r species was located proximal to the hook, and the major 56,000-M_r flagellin composed the remainder of the filament. Western immunoblot analysis with polyclonal rabbit antisera raised against *H. pylori* or *Campylobacter jejuni* flagellins and MAb 72c showed that the 56,000-M_r flagellin carried sequences antigenically cross-reactive with the 57,000-M_r *H. pylori* flagellin and the flagellins of *Campylobacter* species. This antigenic cross-reactivity did not extend to the flagellins of other gram-negative bacteria. The 56,000-M_r flagellin also carried *H. pylori*-specific sequences recognized by two additional MAbs. The epitopes for these MAbs were not surface exposed on the assembled inner flagellar filament of *H. pylori* but were readily detected by immunodot blot assay of sodium dodecyl sulfate-lysed cells of *H. pylori*, suggesting that this serological test could be a useful addition to those currently employed in the rapid identification of this important pathogen.

Helicobacter pylori (14) is a curved or spiral gram-negative microaerophilic organism which was first isolated from a human gastric biopsy specimen in 1983 (49). Since this first isolation, it has become apparent that the organism may be one of the most common bacterial pathogens of humans. Epidemiological evidence has shown that *H. pylori* colonizes the upper gastrointestinal tract of more than one in two individuals during their life spans, and in many of these persons, the organism is associated with disease of this upper gastrointestinal tract. Indeed, *H. pylori* appears to be causally associated with active and chronic gastritis, as well as peptic and duodenal ulcers, and may also be associated with carcinoma of the stomach (2, 3, 5, 18, 20, 25, 32, 51). Additional evidence for the pathogenic activity of *H. pylori* has been provided by studies with gnotobiotic and barrier-born pigs (22, 24) and rhesus monkeys (38a) and two human volunteer studies (33, 38).

Although first classified as a species of the genus *Campylobacter*, the organism differs from the true campylobacters in a number of phenotypic and genotypic characteristics (15, 42, 45). In particular, *H. pylori* produces multiple polar sheathed flagella rather than the single unsheathed flagellum produced by true campylobacters (15). It has been argued that the motility imparted by these sheathed flagella facilitates the ability of *H. pylori* to colonize the viscous mucous environment coating the gastric mucosa (20, 25). However, there has been some controversy about the identity of the *H. pylori* flagellin subunit. Early studies by Lee and coworkers (26) showed that only one protein of *H. pylori* was consistently cross-reactive antigenically between *H. pylori* and enteropathogenic campylobacters. This protein had a subunit M_r of 56,000 to 59,000 in *H. pylori* and displayed antigenic cross reactivity in Western immunoblots with an antiserum produced against *Campylobacter jejuni* flagellin (26). This finding led to the suggestion that the antigenically cross-reactive 56- to 59-kDa protein was *H. pylori* flagellin. This was supported by Newell (39) and Mills et al. (36). However, Dunn and coworkers (9) and Geis et al. (12) suggested that *H. pylori* flagellin might not in fact be the protein which displayed antigenic cross-reactivity with anti-*C. jejuni* flagellin antiserum or might have a subunit M_r significantly lower than that of protein first identified by Lee et al. (26). Most recently, Luke et al. (31) reported that the flagellar filament of *H. pylori* consists of a single 54,000-M_r flagellin species.

To clarify the situation, we isolated flagellar filaments from two strains of *H. pylori* and examined the structure and composition of the proteins contained in these filaments. In this paper we present biochemical and immunochemical evidence for the presence of two flagellin species with subunit M_rs of 57,000 and 56,000 in *H. pylori* flagella and immunomorphological evidence for the differential spatial location of these two flagellins within the filament. We also report that both flagellin species share epitopes with the flagellins of other campylobacterlike organisms, while the

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56,000-M_r subunit also carries amino acid sequences which are specific for *H. pylori*.

MATERIALS AND METHODS

Bacterial strains. *H. pylori* strains examined in this study were endoscopic-biopsy isolates 915, CCUG 17874 (Culture Collection University of Göteborg, Göteborg, Sweden [= type strain of *H. pylori* subsp. *pylori* subsp. nov. NTCC 11637, National Type Culture Collection, Colindale, London, United Kingdom], originally Royal Perth Hospital isolate 13487 [Australia]), 5155, 5294, and 5442 (A. Lee, University of New South Wales, Kensington, Australia), 52, 66, 95, and 253 (Culture Collection University of Lund, Lund, Sweden). Rodent ileal colonizing spiral isolate T1 (A. Lee, University of New South Wales) was also used. Stock cultures were maintained at −70°C in 15% (vol/vol) glycerol–Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Cultures were grown at 37°C on chocolate blood agar plates containing 0.001% vancomycin, 0.005% trimethoprim, and 0.00075% colymycin in an atmosphere containing 10% carbon dioxide. *Campylobacter coli* VC80, VC148, VC167B, and VC211; *C. jejuni* VC27, VC74, VC150, and VC210; *Campylobacter lari* VC81 (formerly NCTC 11352; also known as LIO34, the Lior *C. lari* biotype 2 reference strain); and *Campylobacter fetus* VC78 and VC119 from the University of Victoria *Campylobacter* collection were also grown on chocolate Mueller-Hinton agar plates supplemented with *Campylobacter* antibiotics. Plates were incubated in a Forma CO_2 incubator at 37°C with a CO_2 concentration of 10% (vol/vol) for 24 h. Cultures of *Vibrio cholerae* MW13, *Proteus vulgaris*, *Salmonella typhimurium*, *Salmonella enteriditis* 2, *Aeromonas hydrophila* TF7, and *Escherichia coli* 122 from the University of Victoria collection were grown on Trypticase soy agar plates and incubated in air at 37°C for 24 h.

Flagellum isolation. Cells were harvested into distilled water and homogenized, and deflagellated cells removed by centrifugation according to the protocol of Logan et al. (28). The bacterium-free supernatant was centrifuged at 100,000 × *g* for 1 h, and the pellet of flagella was retained and subjected to CsCl equilibrium density gradient centrifugation in 1.3-g/cm^3 (final density) CsCl (Pharmacia Fine Chemicals, Uppsala, Sweden) at 180,000 × *g* for 20 h, essentially by the protocol outlined by Chandler and Gulasekharam (4). Unsheathed flagellum filaments formed a band near the center of the gradient and were collected by puncturing the tube with a 26-gauge needle and aspirating the contents into a syringe. Flagellum preparations were diluted 20 times in distilled water and centrifuged at 100,000 × *g* for 1 h. For some experiments, flagellar filaments were treated with 1 mg of trypsin (Boehringer GmbH, Mannheim, Federal Republic of Germany) per ml in 0.02 M Tris hydrochloride, pH 7.8, containing 0.02 M $CaCl_2$ for 1 h at 37°C prior to CsCl density gradient centrifugation. The reaction was stopped by adding an excess of soybean trypsin inhibitor (Boeringher GmbH). Flagella were also isolated by the ultracentrifugation-acid disassociation-differential centrifugation-pH reassociation outlined by Logan et al. (28). Flagella were first pelleted by centrifugation at 100,000 × *g* for 1 h at 4°C. The pellet was suspended in distilled water, adjusted to pH 2.0 with HCl, and held on ice for 15 min. Material insoluble at pH 2.0 was removed by centrifugation at 100,000 × *g* for 1 h. The supernatant was then adjusted to pH 7.0 with NaOH and left on ice for 30 min to allow for flagellar reassociation.

Flagellin purification. Further purification of flagellum filaments obtained from CsCl density gradients was achieved by molecular sieving on a Sephacryl S-500 HR column (1.5 by 50 cm; Pharmacia). The system was run in 20 mM Tris hydrochloride (pH 7.4) at a flow rate of 60 ml/h. The peaks eluted were monitored at 214 nm. After chromatography, the peak of interest was collected, dialyzed against distilled water, and concentrated by evaporation. Anion-exchange chromatography was also employed to further purify flagellin obtained by the ultracentrifugation-acid disassociation-differential centrifugation-pH reassociation procedure. A Mono Q anion-exchange column (Pharmacia) was run isocratically at 0.5 ml/min for 10 min in 10 mM Tris, pH 6.2, and then a 0 to 10% gradient of 1 M NaCl in 10 mM Tris, pH 6.2, was applied to the column for 5 min. The column was run isocratically at this concentration of NaCl for 10 min, during which time the flagellin eluted as a single major peak. The peaks eluted were monitored at 214 nm, and the peak of interest was collected, dialyzed against distilled water, and concentrated by evaporation.

Glycine extraction of flagellin. Small-scale rapid extraction of flagellin was achieved by acid pH disassociation of flagellar filaments (17). A 0.3-cm loopful of bacteria, or a single colony, was suspended in 100 μl of 0.2 M glycine-hydrochloride (pH 2.2) and allowed to sit at room temperature for 5 min. Bacteria were then removed by centrifugation at 12,000 × *g* for 3 min.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (23) in a minislab apparatus (Bio-Rad Laboratories, Richmond, Calif.). Protein solubilized in sample buffer was stacked in a 4.5% (wt/vol) acrylamide (100 V, constant voltage) and separated with 7.5 or 12.5% (wt/vol) acrylamide (200 V). Protein was stained with Coomassie blue R-250.

Two-dimensional PAGE with isoelectric focusing (IEF) in the first dimension and SDS-PAGE in the second dimension was performed according to the method of O'Farrell (40) with a Mini-Protean II 2-D Cell (Bio-Rad Laboratories). IEF gels containing 4% (wt/vol) acrylamide, 8 M urea, 2% (vol/vol) Nonidet P-40, and 2% (vol/vol) total ampholytes (1.6% [vol/vol] Bio-Lyte 4/6 ampholyte and 0.4% [vol/vol] Bio-Lyte 3/10 ampholyte; Bio-Rad Laboratories) were poured into capillary tubes and allowed to polymerize overnight. Samples containing acid-disassociated flagella were neutralized, concentrated by evaporation, and then mixed with an equal volume of lysis buffer (9.5 M urea, 5% [vol/vol] 2-mercaptoethanol, 2% [vol/vol] Nonidet P-40, and 2% [vol/vol] ampholytes of the same pH range used to prepare the IEF gels). The mixture was applied to the IEF gel and overlaid with sample overlay buffer (5 M urea, 2% [vol/vol] Nonidet P-40, 2% [vol/vol] ampholytes). Electrophoresis was performed at room temperature for 10 min at 500 V and then at to 750 V for 3.5 h. The cathode solution was 0.1 N NaOH, and the anode solution was 0.06% (vol/vol) phosphoric acid. The pH gradient was measured by the method of Marshall et al. (34). For the second dimension, the IEF gel was placed on the top of a 7.5% SDS-PAGE gel and overlaid with sample buffer, and electrophoresis was performed by the method of Laemmli (23).

When required, separated proteins were transferred from the slab to nitrocellulose paper (NCP) by the methanol Tris-glycine system described by Towbin et al. (48). Electroblotting was carried out in a transblot apparatus (Bio-Rad Laboratories) for 18 h at 60 V. Nondenaturing IEF gels were run in a minislab gel apparatus according to the method of

Robertson et al. (44) and were stained with Coomassie blue according to the Bio-Rad IEF standards instruction sheet.

Amino acid composition analysis. Flagellin was electroblotted to an Immobilon membrane (Millipore Corp., Bedford, Mass.) by the method of LeGendre and Matsudaira (27), hydrolyzed by 6 N HCl vapors, and analyzed for amino acid composition in an Applied Biosystems model 420 analyzer (Applied Biosystems, Foster City, Calif.). The method used was that described by the manufacturer. Cysteine content was determined by pyridylethylating potential cysteine residues (10). Tryptophan content was analyzed after oxidation of these residues was prevented by reaction with vapors of 5% thioglycolic acid (52).

N-terminal sequence analysis. Flagellin was electroblotted to an Immobilon membrane. The blotted proteins were stained with Coomassie blue, and amino acid sequencing was performed on an Applied Biosystems model 470A gas phase sequenator running a standard operating program. Phenylthiohydantoin derivatives were separated by using an on-line phenylthiohydantoin analyzer (Applied Biosystems model 120A). Data handling employed an Applied Biosystems model 900.

Antibody production. Antiserum MK1 was prepared by immunizing an adult New Zealand White rabbit intravenously with increasing doses (from 5×10^7 to 1×10^{10}) of live cells of *H. pylori* 915 once a week over a 5-week period. Antiserum JB1 was raised by intramuscular injection of a rabbit with 10^9 Formalin-killed cells of 48-h cultures of *H. pylori* 5294, emulsified in Freund complete adjuvant. Booster doses were given in Freund incomplete adjuvant on days 22 and 42. To prepare antiserum JB2, a rabbit was immunized subcutaneously and intramuscularly with approximately 100 μg of linearized 56,000-M_r flagellin in acrylamide, obtained by SDS-PAGE separation of Mono Q-purified flagellin, cutting the protein band out of the gel, and then homogenizing the protein-acrylamide preparation in Freund complete adjuvant. A booster dose of 10 μg of linearized 56,000-M_r flagellin was given on day 32. Antiserum JB3 was raised in an adult New Zealand White rabbit by subcutaneous and intramuscular injection with 100 μg of Mono Q-purified flagellin in Freund complete adjuvant. Booster doses of 50 and 25 μg in Freund incomplete adjuvant were given on days 31 and 59. In all cases, the sera were collected on day 90 and stored at −20°C. Control nonimmune sera were obtained before the first injection. Antiserum SML2 produced against SDS-linearized *C. jejuni* VC74 flagellin was prepared as previously described (29).

Production of MAb to flagellin. Doubly cloned hybridomas secreting monoclonal antibodies (MAbs) to the Mono Q-purified protein of *H. pylori* 5294 were prepared by the procedure of Pearson et al. (41). The class and subclass of each MAb were determined by indirect enzyme-linked immunosorbent assay (American Qualex International, Inc., La Mirada, Calif.). MAbs were used as ascitic fluids. To confirm that the three MAbs used in the study recognized different epitopes, flagellin was subjected to in-gel cleavage with endoproteinase Glu-C (*Staphylococcus aureus* V8 protease [Boehringer GmbH]), a peptide mapped by the method of Cleveland et al. (6), and Western blotted with the appropriate MAb.

Western blotting. After electroblotting, unreacted sites on the NCP were blocked with a 1% (vol/vol) solution of skim milk in 10 mM Tris hydrochloride–0.9% NaCl (pH 7.4) (MTS) for 1 h at room temperature. The NCP was then incubated with an appropriate dilution of antiserum or MAb in the same buffer for 2 h. The NCP was washed five times with 10 mM Tris hydrochloride–0.9% NaCl (pH 7.4). Alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse antibody (Caltag Laboratories, South San Francisco, Calif.) was then added in MTS buffer and incubated for 1 h at room temperature. After incubation, the NCP was washed five times in 10 mM Tris hydrochloride–0.9% NaCl (pH 7.4). The reactive bands were visualized as described by Blake et al. (1) by using 5-bromo-4-chloro-3-indolyl phosphate (Boehringer GmbH) as the alkaline phosphatase substrate and Nitro blue tetrazolium (Sigma) as the color development reagent.

Immunodot blotting. Dot blotting was performed by transferring 10 μg of whole-cell lysate protein of each strain to be tested to NCP. After drying at 37°C for 1 h, the NCP was blocked with MTS, reacted with MAb, washed, and developed by the procedures described for Western blotting.

Indirect fluorescent antibody testing. Indirect fluorescent antibody testing was performed as previously described (29)

Electron microscopy. A grid covered with a Formvar film was floated on a 50-μl drop of bacterial cells or purified flagella for 5 min. The grids were stained by floating them on a drop of 1% (wt/vol) ammonium molybdate containing 0.1% (vol/vol) glycerol, pH 7.5, and were examined in a JEOL JEM-1200EX or Philips EM-300 electron microscope operated at an accelerating voltage of 60 kV. Images were recorded on 70-mm Fine Grain Release Film (Kodak, Rochester, N.Y.).

For immunoelectron microscopy, the grid was floated on the sample, removed, and floated on a drop of 10 mM Tris containing 150 mM NaCl and 2% (wt/vol) nonfat skim milk (Tris-NaCl-milk) for approximately 30 min. The grid was then incubated on polyclonal serum diluted in Tris-NaCl-milk for 1 h. After incubation, the grid was removed, and nonspecifically bound immunoglobulin G (IgG) was removed by floating the grid on 3 drops of Tris-NaCl. The grid was then floated on a drop of Tris-NaCl-milk containing a 1:10 dilution of 15-nm colloidal gold particles coated with protein A (Jannsen Biotech, Olen, Belgium). After incubation for 1 h, the nonspecifically bound colloidal gold particles were removed by floating the grid on 2 drops of Tris-NaCl and 1 drop of distilled water. The grids were negatively stained and examined as described above.

For labeling with MAbs, the grid was floated on the sample, blocked with 10 mM Tris, pH 7.5, containing 150 mM NaCl and 0.1% (wt/vol) bovine serum albumin (BSA) and incubated on a drop of a 1:100 dilution of MAb (as ascitic fluids) in Tris-NaCl-BSA for 1 h. The grid was washed on 3 drops of Tris-NaCl and then incubated on a drop of rabbit polyclonal anti-mouse IgG1 (ICN Immunobiologicals, Costa Mesa, Calif.) diluted 1:100 in Tris-NaCl-BSA. In some cases, the grid was then incubated with colloidal gold-protein A as described above. The grid was then washed on 3 drops of Tris-NaCl and 1 drop of distilled water. The grids were negatively stained and examined as described above.

RESULTS

Isolation and purification. Typically, *H. pylori* produces up to five sheathed flagella from a single pole of the cell. To identify the protein(s) composing the inner flagellin filament of these sheathed flagella, flagella were removed from *H. pylori* 915 and 5294 cells by shearing, deflagellated cells were removed by centrifugation, and the flagella were collected by ultracentrifugation. SDS-PAGE analysis showed that the major contaminating protein in this crude flagellar preparation was the multimeric high-M_r enzyme urease (19), which

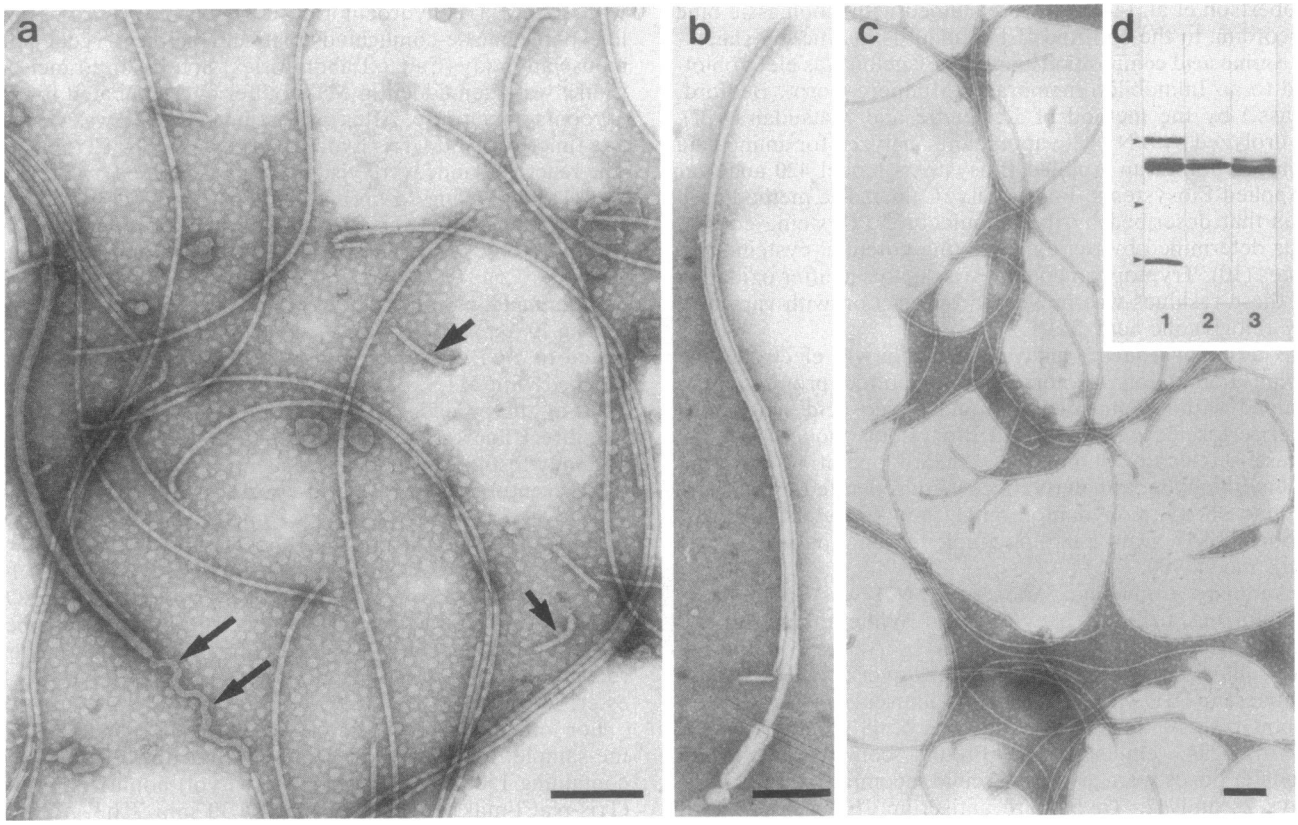

FIG. 1. Electron micrographs of *H. pylori* flagella negatively stained with 1% (wt/vol) ammonium molybdate containing 0.1% (vol/vol) glycerol, pH 7.5. (a) Upper band obtained during CsCl density gradient centrifugation, showing the presence of sheathed and unsheathed flagella, flagellar hooks (short arrows), fragments of outer membrane, and sheath material (long arrows). Bar = 200 nm. (b) Detail showing flagellar sheath peeling from the inner flagellar protein filament. Bar = 100 nm. (c) Lower band obtained during CsCl density gradient centrifugation, showing trypsin-treated purified preparation of unsheathed filaments. Bar = 200 nm. (d) SDS-PAGE (7.5% acrylamide) stained by Coomassie blue and Western blot analysis of CsCl density gradient centrifugation preparations of *H. pylori* flagella. Lane 1, A sample which was not preincubated with trypsin and contains contaminating urease subunits at approximate M_rs of 66,000 and 29,000; lane 2, a similar preparation which was treated with trypsin before CsCl density gradient centrifugation to remove contaminating urease; lane 3, Western blot of the preparation shown in panel c reacted with a 1:5,000 dilution of MAb 72c to demonstrate the presence of two flagellin species of different apparent M_rs. The higher copy number of the 56,000-M_r flagellin species is readily apparent in all lanes. Arrowheads on the left indicate M_r standards (from top to bottom): 66,200, 45,000, and 31,000.

was also released from the *H. pylori* cell surface by the shearing procedure and pelleted by the ultracentrifugation. However, preincubation with trypsin allowed for digestion of the contaminating urease with no digestion of the flagellar filaments. Further purification was then achieved by the CsCl equilibrium density gradient ultracentrifugation protocol of Chandler and Gulasekharam (4). After ultracentrifugation, two bands were seen in the CsCl gradient. Electron microscopy of negatively stained preparations showed that the upper band contained fragments of both sheathed and unsheathed flagella (Fig. 1a). The intact sheathed *H. pylori* filament had a diameter of 25.4 ± 0.5 nm (Fig. 1a and b). The sheath appeared to be relatively delicate in nature and in some instances appeared to peel from the filament (Fig. 1b). In other instances the sheath appeared to stretch off the end of the inner flagellin filament (Fig. 1a). The inner *H. pylori* flagellin filament had a diameter of 13.5 ± 0.2 nm. In fields showing the proximal end of the unsheathed flagellin filament, typical hook structures were seen (Fig. 1a). The upper band was also contaminated with membrane fragments and blebs. Negative staining of the lower band obtained in CsCl gradients revealed the presence of unsheathed flagellin filaments (Fig. 1c). SDS-PAGE analysis confirmed the removal of contaminating urease by preincubation with trypsin (Fig. 1d, lanes 1 and 2) and revealed the presence in the CsCl flagellar band of two flagellins with apparent subunit M_rs of 57,000 and 56,000. Western blot analysis with antiserum SML2 produced against SDS-linearized *C. jejuni* flagellin, polyclonal flagellin antiserum JB2, or MAb 72c (Fig. 1d, lane 3) prepared against purified *H. pylori* flagellin showed that both proteins were immunoreactive. The 56,000-M_r protein was present in higher copy number.

Separation of the subunit 57,000- and 56,000-M_r flagellins from strain 915 from contaminating urease (19) could also be achieved by molecular sieving on a Sephacryl S-500 column. The two flagellins eluted in the first peak (Fig. 2), while urease was contained in the second peak. Again, the subunit 56,000-M_r flagellin was present in higher copy number. In the case of strain 915, attempts to further separate these subunit 57,000- and 56,000-M_r flagellins by molecular sieving on a Superose 12 column, anion-exchange chromatography on a Mono Q column, and reverse-phase chromatography on a ProRPC column were unsuccessful. Small amounts of each protein were therefore purified to homogeneity by the Im-

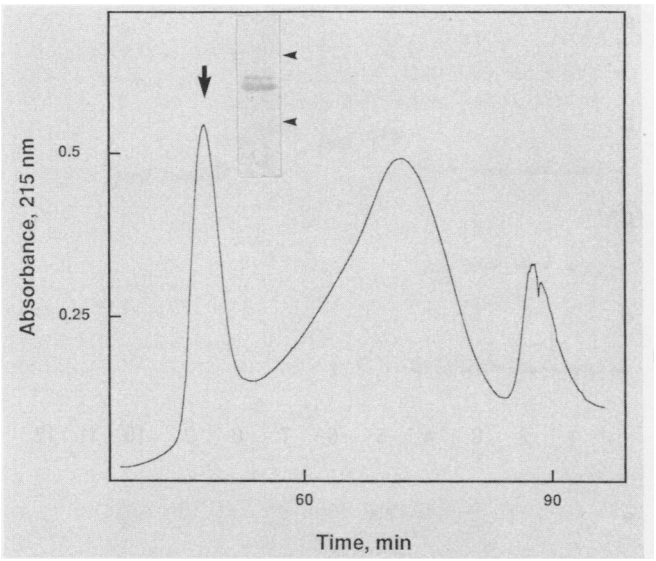

FIG. 2. Purification elution profile of *H. pylori* flagellin by using Sephacryl S-500 HR. The flagellin-containing peak is indicated by the arrow. The inset shows a Western blot of a 7.5% SDS-PAGE gel of the flagellin peak reacted with a 1:5,000 dilution of MAb 72c. The higher copy number of the 56,000-M_r flagellin species is again readily apparent. Arrowheads on right indicate M_r standards at 66,200 (top) and 45,000 (bottom).

TABLE 1. Amino acid composition of *H. pylori* flagellins

Amino acid	No. of residues/flagellin subunit in:					
	H. pylori			*C. coli* VC167 P1[a]	*S. typhimurium*[b]	*C. crescentus*[c]
	915		5294 56K			
	57K	56K				
Asx	58	80	78	86	79	42
Thr	24	35	38	40	55	22
Ser	50	54	47	73	40	17
Glx	55	49	52	53	47	25
Pro	15	4	0	0	4	7
Gly	79	60	51	68	39	24
Ala	60	61	57	72	61	40
Val	42	42	44	29	31	17
Met	5	9	6	14	3	4
Ile	35	36	38	50	27	13
Leu	50	44	44	37	40	35
Tyr	11	8	8	8	13	1
Phe	19	12	11	19	6	5
His	7	4	2	4	1	2
Lys	22	28	34	27	27	10
Arg	22	21	22	17	14	12
Cys	NT[d]	0	0	0	0	0
Try	NT	0	0	0	0	0
No. of residues/mol	554	543	532	598	489	276
Apparent M_r (10^3)	57.0	56.2	56.1	61.5	51.2	28.5
% Hydrophobic residues[e]	41	38	38	37	35	43.8

[a] Data from Logan et al. (30); calculation based on the DNA sequence of the *flaA* gene of *C. coli* VC167. P1 is antigenic phase 1 flagellin.
[b] Data from Joys (21); calculation based on the DNA sequence of the *H-1*[i] gene.
[c] Data from Gill and Agabian (13); calculation based on the DNA sequence.
[d] NT, Not tested.
[e] V, M, I, L, A, F, W, and P.

mobilon transfer method of LeGendre and Matsudaira (27) after separation of the subunit 57,000- and 56,000-M_r proteins by two-dimensional PAGE.

The subunit 56,000-M_r flagellin from strain 5294 was also purified by the ultracentrifugation-acid disassociation-differential centrifugation-pH reassociation procedure outlined by Logan et al. (28) followed by anion-exchange chromatography employing a Mono Q column. Flagellin eluted as a single peak with 0.1 M NaCl in 10 mM Tris, pH 6.2. The protein in this peak was used, both directly and after linearization by SDS-PAGE, to produce two different polyclonal antiflagellin antisera. The Mono Q-purified protein was also used to immunize mice for the production of MAbs.

Biochemical analysis. The isoelectric points of the *H. pylori* 915 and 5294 flagellins were determined by two-dimensional PAGE. In both strains, the 57,000- and 56,000-M_r proteins focused into a single spot at a pI equal to 5.2 (data not shown), showing that both species of *H. pylori* flagellins were acidic proteins. Amino acid composition analysis of the 56,000-M_r flagellins purified from *H. pylori* 915 and 5294 showed that the calculated relative hydrophobicity (assuming Val, Met, Ile, Leu, Ala, Phe, Try, and Pro) of the two proteins was approximately 38%. The predicted M_r of 56,100 to 56,200 (Table 1) were in keeping with the 56-kDa size estimated by SDS-PAGE. Asx, Ala, Gly, Ser, Glx, Val, Leu, and Ile were prominent, and the absence of cysteine and tryptophan residues was confirmed by repeat analysis of samples in which potential cysteine residues were pyridylethylated and potential tryptophan residues were protected from oxidation. Also characteristic of the two 56,000-M_r flagellins was their low proline content. Indeed, the amino acid composition of the *H. pylori* 56,000-M_r proteins was consistent with compositions reported for the flagellins of *C. coli* (30) and *S. typhimurium* (21) (Table 1). The composition of the 57,000-M_r flagellin purified from *H. pylori* 915 was different in the content of individual amino acids such as Pro, Asx, Gly, Leu, and Thr compared with the composition of the 56-kDa *H. pylori* flagellins, although the overall content of the various classes of amino acids was similar in both M_r species.

The N-terminal amino acid sequences of the 56,000-M_r *H. pylori* 915 and 5294 flagellins were determined for the first 24 residues, while the N-terminal amino acid sequence of the 57,000-M_r *H. pylori* 915 flagellin was determined for the first 20 residues (Table 2). The first 24 residues of the 56,000-M_r flagellin contained approximately 50% hydrophobic amino acids, including a methionine at residue 20, as well as 46% neutral amino acids. None of the first 24 residues were charged. Similarly, the first 20 residues of the 57,000-M_r flagellin of *H. pylori* 915 contained 40% hydrophobic and 45% neutral residues and only two charged residues. Table 2 also shows the alignment of the N-terminal sequences of the flagellins of *H. pylori* 5294, *C. coli* VC167, *S. typhimurium*, and *Campylobacter crescentus*.

Immunochemical analysis. Several polyclonal antisera were used in this study. Antisera MK1 and JB1 were raised against whole cells of *H. pylori* 915 and 5294, respectively. Antisera JB2 and JB3 were raised to Mono Q-purified flagellin from *H. pylori* 5294 and SDS-PAGE-linearized 5294 flagellin, respectively. Antiserum SML2 was raised against SDS-PAGE-linearized *C. jejuni* VC74 flagellin. Western blot analysis of glycine extracts of *H. pylori* strains and other mucus-colonizing spiral bacteria with monospecific polyclonal antiserum JB3 showed that the flagellins of these

TABLE 2. N-terminal amino acid sequence of *H. pylori* and other flagellins

Organism	M_r (10^3)	Residues[a]
		10 20
H. pylori 915	56	AFQVNTNINA MNAXVQSALT QNALKT
	57	S·RI····A· LTSHAVGV— ··
H. pylori 5294	56	····· ···G····· ····
C. coli VC167[b]		G·RI···VA· L··KAN·D·N SRS·
S. typhimurium[c]		·QVI···SLS LLTQNNLNKS ·S··
B. subtilis (8)		RI·H·IA· L·TLNRLSSN NS·S
C. crescentus (37)	28.5	·LS····QP· LI·LQNLNR· NDDM
	27	LNSI···PG· LV·LQ
	25	ALNSI···AG· ·I·LQ

[a] Amino acid residues are designated by the single letter nomenclature. ·, Residue homologous with *H. pylori* 915 sequence; —, gap introduced to facilitate sequence alignment. Conservative changes are underlined.
[b] Sequence derived from DNA sequence (30).
[c] Sequence derived from DNA sequence of *H-1*[i] gene (21).

organisms contained cross-reactive epitopes (Fig. 3a, lanes 1 to 10). Reaction was also seen with the flagellins of *C. lari* VC81 and *C. fetus* VC78 (data not shown). This antigenic cross-reactivity did not extend to the flagellins of *V. cholerae* MW13, *S. enteritidis* 2 (Fig. 3a, lanes 11 and 12), *P. vulgaris*, *S. typhimurium*, *E. coli* 122, and *A. hydrophila* TF7 (data not shown). Identical results were obtained with monospecific polyclonal antiserum JB2 against *H. pylori* flagellin, monospecific polyclonal antiserum SML2 against *C. jejuni* flagellin, and both antisera prepared against cells of *H. pylori* 915 and 5294 (data not shown). With each polyclonal antiserum, examination of the blot reaction with *H. pylori* strains showed the presence of two immunoreactive proteins with approximate subunit M_rs of 57,000 and 56,000 (Fig. 3a, lanes 1 to 4). Immunogold electron microscopy with antiserum JB2 showed the presence of antibodies capable of binding to the surface of unsheathed portions of *H. pylori* flagellin filaments of both homologous and heterologous strains (Fig. 4). No antibody binding to the flagellar sheath (Fig. 4b) or with the flagella of *C. jejuni* VC74 or *C. coli* VC167B cells (data not shown) was observed. Identical results were obtained with polyclonal antiserum JB3. Indirect fluorescent antibody testing with antisera JB2 or JB3 further showed that very few flagellar filaments displayed immunofluorescence, even in a dense smear of *H. pylori* cells, indicating that the vast majority of flagellar sheaths in a population of *H. pylori* cells were intact.

To extend these findings, isotype IgG1 MAbs 72c, 220a, and 104a were used. As was the case with the various polyclonal antisera tested, Western blot analysis with MAb 72c showed that this MAb was cross-reactive with the flagellins of all of the *Campylobacter*-like bacteria tested (Fig. 3b, lanes 1 to 10) but not with the flagellins of *V. cholerae* MW13, *S. enteritidis* 2 (Fig. 3b, lanes 11 and 12), *P. vulgaris*, *Salmonella typhimurium*, *E. coli* 122, and *A. hydrophila* TF7 (data not shown). In the case of the *H. pylori* strains tested, the epitope for MAb 72c was present on both of the subunit 57,000- and 56,000-M_r flagellins (Fig. 3B, lanes 1 to 4). In contrast, MAbs 220a and 104a were reactive with only the 56,000-M_r flagellin subunit of *H. pylori* (Fig. 3c and d, lanes 1 to 4) and did not react with the flagellins of other campylobacters (lanes 5 to 10) or noncampylobacters (lanes 11 and 12) tested. Immunoelectron microscopy showed that MAb 72c bound to the hook-proximal region of unsheathed assembled flagellar filaments of *H. pylori* 915 (Fig. 5a and b)

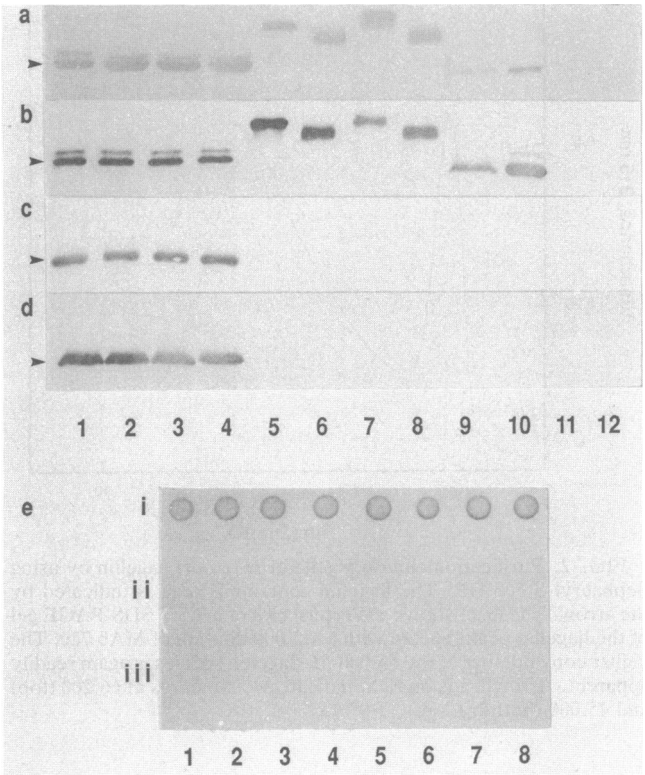

FIG. 3. Western blot of glycine extracts reacted with 1:1,000 dilution of polyclonal antiserum JB3 (a) and 1:5,000 dilutions of MAbs 72c (b), 104a (c), and 220a (d). Lane 1, *H. pylori* 915; lane 2, *H. pylori* 5155; lane 3, *H. pylori* 5294; lane 4, *H. pylori* 5442; lane 5, *C. coli* VC167B; lane 6, *C. coli* VC148; lane 7, *C. jejuni* VC74; lane 8, *C. jejuni* VC150; lane 9, *C. fetus* VC119; lane 10, rodent ileal colonizing spiral isolate T1; lane 11, *V. cholerae* MW13; lane 12, *S. enteritidis* 2. Arrowheads indicate the 56,000-M_r flagellin subunit. (e) Immunodot blot of whole-cell lysate extracts (10 μg of protein) reacted with a 1:5,000 dilution of MAb 220a. Rows (letters) and columns (numbers) as follows: i1, *H. pylori* 915; i2, *H. pylori* 5155; i3, *H. pylori* 5294; i4, *H. pylori* 5442; i5, *H. pylori* 52; i6, *H. pylori* 66; i7, *H. pylori* 95; i8, *H. pylori* 253; ii1, *C. coli* VC80; ii2, *C. coli* VC148; ii3, *C. coli* VC167B; ii4, *C. coli* VC211; ii5, *C. jejuni* VC27; ii6, *C. jejuni* VC74; ii7, *C. jejuni* VC150; ii8, *C. jejuni* VC210; iii1, *C. fetus* VC119; iii2, *V. cholerae* MW13; iii3, *E. coli* 122; iii4, *S. enteritidis* 2.

and 5294 but not to the unsheathed flagella of *C. jejuni* VC74 or *C. coli* VC167B cells (data not shown). In contrast to MAb 72c, neither of the other two MAbs bound to unsheathed flagellin filaments (data not shown). However, immunodot blot assay of whole-cell lysates showed that these two MAbs which recognized different epitopes could be used to specifically identify a diverse selection of *H. pylori* strains. The results obtained with MAb 220a are shown in Fig. 3e.

DISCUSSION

In the cases of most of the well-studied swimming bacteria, plain flagellar filaments containing only a single flagellin species are produced. This is not the case with *H. pylori*. This study has provided morphological, biochemical, and immunochemical evidence that the inner filament of the sheathed *H. pylori* flagellum contains two flagellins, one with a subunit M_r of 57,000 and the other with a subunit M_r of

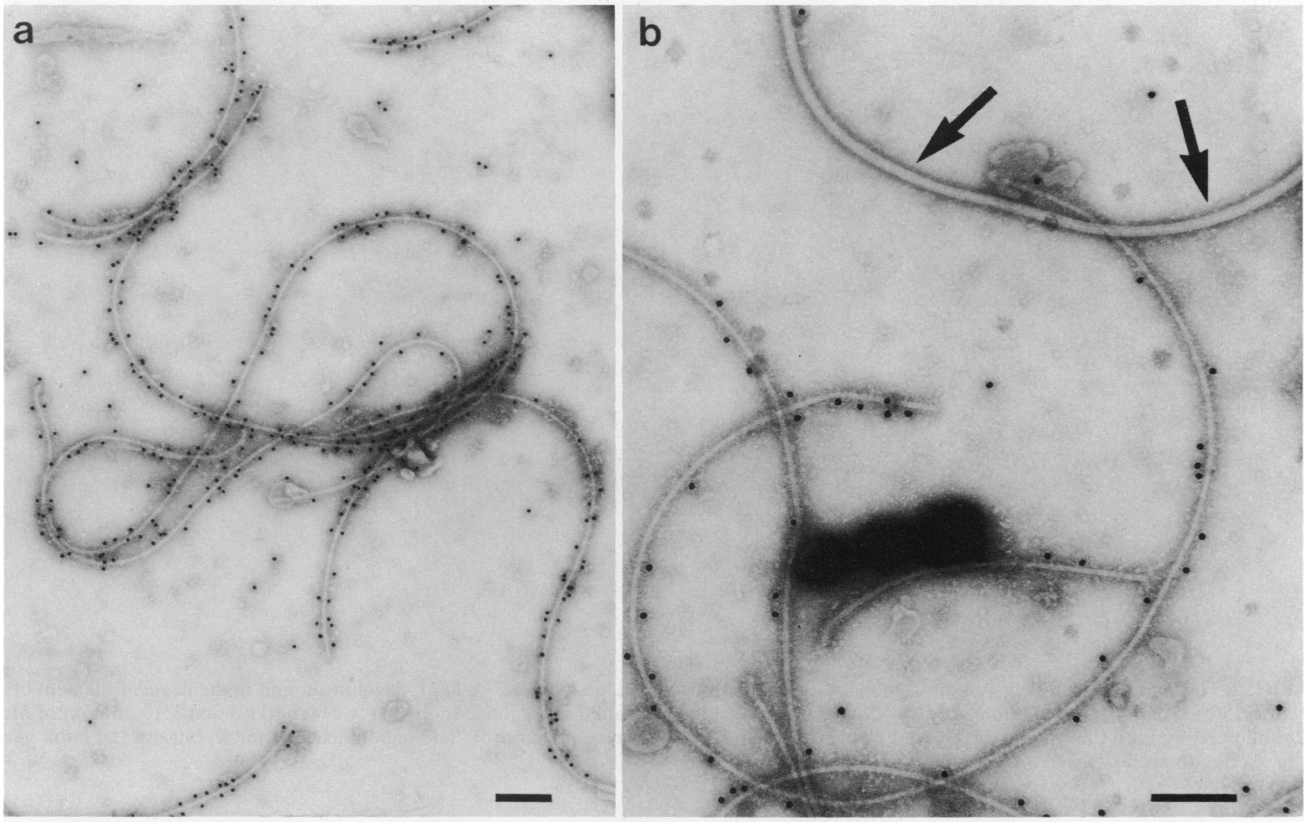

FIG. 4. Immunogold demonstration of inner-filament labeling of *H. pylori* flagella by using a 1:10 dilution of polyclonal antiserum JB3 prepared against the purified 56,000-M_r flagellin of *H. pylori* 5294. The antibody clearly labels the unsheathed flagellar filaments of the heterologous strain 915 (a) and the homologous strain 5294 (b). The flagellar sheath was not labeled by antibody (arrows). Bars = 500 nm.

56,000. Immunoelectron microscopy suggests that the 57,000-M_r flagellin is assembled proximal to the hook and forms the base portion of the filament, while the predominant 56,000-M_r flagellin composes the distal remainder of the filament. This organization of different flagellin species into separate regions of the flagellar filament is also seen in the case of *Caulobacter* species, in which there are three flagellin species with subunit M_rs of 25,000 to 29,000 in the flagellar filament (13, 37, 50). Like the 57,000-M_r flagellin of *H. pylori*, two of the *Caulobacter* flagellins are thought to be hook proximal, while the third makes up the majority of the filament (13, 37, 50). In this property of differential spatial localization of flagellins in the filament, the flagella of *H. pylori* and *Caulobacter* species are unusual. For example, in the case of *Rhizobium meliloti*, it is proposed that the complex flagellar filaments are composed of heterodimeric subunits that require stoichiometric amounts of the two flagellins (43), while in other cases in which there are multiple flagellin species produced by an organism, their organization in the filament has not been determined.

Previous studies have overlooked the presence of two flagellin species in the *H. pylori* flagellar filament. For example, neither Geis et al. (12) nor Luke et al. (31) made mention of the presence of a second species in their characterization of *H. pylori* flagella. In the case of the strains studied here, this is presumably because the 56,000-M_r subunit is present in the flagellar filament in much larger amounts than the 57,000-M_r subunit. Indeed, in crude flagellum preparations, simple Coomassie blue staining of SDS-PAGE gels containing >10% acrylamide does not readily distinguish the 57,000-M_r subunit from the 56,000-M_r subunit. The two flagellins are best visualized by Western blot analysis of low-acrylamide-content gels (e.g., 7.5% acrylamide). The two *H. pylori* flagellins are also copurified by a variety of techniques, making it especially difficult to purify the 57,000-M_r subunit on a preparative scale. To ensure purification to homogeneity of both flagellin species, we used SDS-PAGE and electroblotting to Immobilon membranes (27).

Amino acid composition analysis of the predominant 56,000-M_r flagellin purified from two strains of *H. pylori* showed that this protein was similar to other flagellins. The 57,000-M_r protein isolated from *H. pylori* 915 showed differences in the numbers of certain amino acids but was similar to the 56,000-M_r flagellins in overall content. For example, both the 57,000- and 56,000-M_r *H. pylori* flagellins had pIs of 5.2. Also, when the compositional relatedness was assessed by the algorithm of Metzger et al. (35), a difference index of approximately 9.5 was obtained. By this algorithm, two proteins with no amino acids in common have a difference index of 100, while two proteins with the same composition have a difference index of 0. The most notable difference in the 57,000-M_r species was its high proline content. In particular, the proline content of the 57-kDa *H. pylori* flagellin at 2.7% of the total residues per molecule was closer to the 3.0% proline content of the hook-proximal 28,500-M_r *C. crescentus* flagellin (13) than to that of other flagellins which make up either the majority or all of the flagellar filament.

N-terminal sequence analysis of the 56,000-M_r flagellins

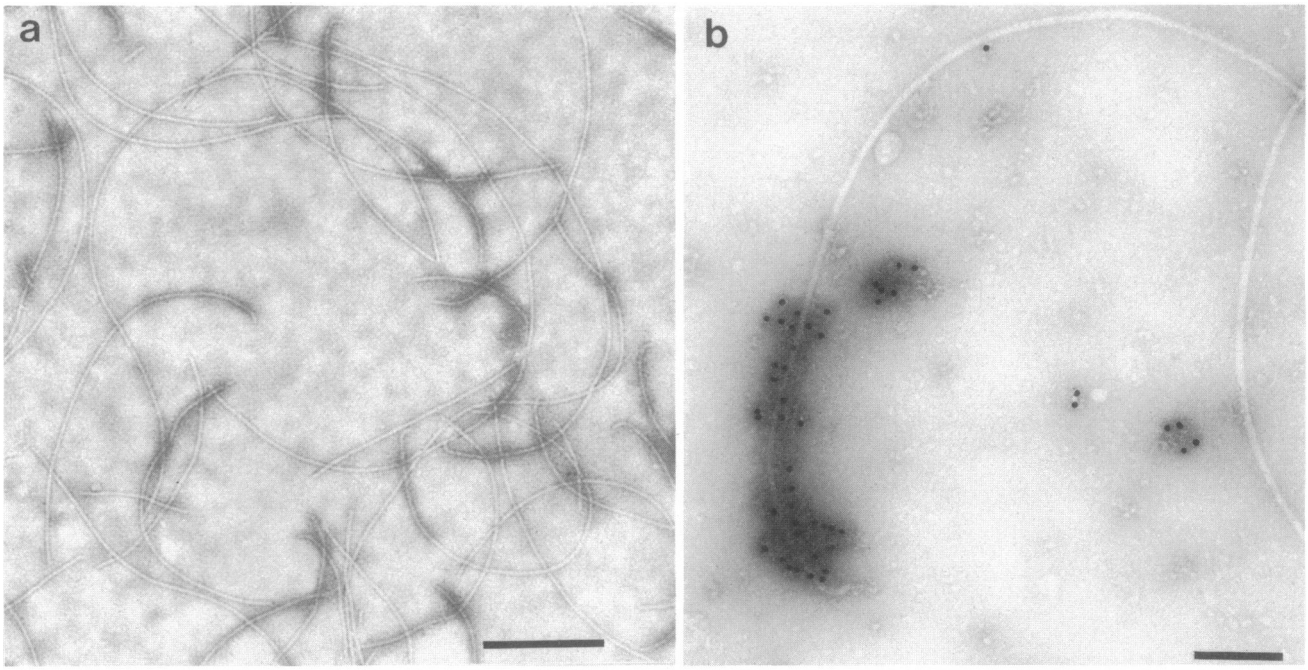

FIG. 5. Immunoelectron microscopic demonstration of hook-proximal location of 57,000-M_r flagellin subunit in the flagellar filament of *H. pylori* 915 by using a 1:100 dilution of MAb 72c and a 1:100 dilution of rabbit anti-mouse IgG1 (bar = 500 nm) (a) and 1:10 dilution of MAb 72c followed by rabbit anti-mouse IgG1 and protein A-colloidal gold (bar = 200 nm) (b). The region near the hook, but not the hook itself, is labeled with antibody.

isolated from the two diverse strains used in this study showed that in this region of the molecule, the two 56,000-M_r flagellins were virtually identical, indicative of strong conservation of the predominant *H. pylori* flagellar filament protein. The first 10 residues of the 57,000-M_r protein of strain 915 shared 6 identical residues with the 56,000-M_r flagellin, 1 conservative replacement at residue 4, and a highly significant mean homology score of 109 (7). Thereafter, the 57,000-M_r flagellin sequence diverged from the 56,000-M_r flagellin. Of the first 11 N-terminal residues of the 57,000-M_r protein, 9 are shared with the *C. coli* sequence, with a conservative replacement of isoleucine for valine at residue 8 and a highly significant mean homology score of 118 (7). The 57,000-M_r *H. pylori* flagellin also shows a high homology to *Bacillus subtilis* flagellin in this region of the molecule, with 8 of the first 11 residues being shared and a mean homology score of 107. Of the first 11 residues, 5 are also shared with *Salmonella* flagellin. By this Dayhoff algorithm (7), the N-terminal 20 residues of the predominant 56,000-M_r *H. pylori* flagellins and the *C. coli* flagellin (30) also have a significant mean homology score of 101. Sequence alignment showed that of the first 24 amino acids identified in the N-terminal sequence of the 56,000-M_r flagellin of *H. pylori* 5294, 11 were identical with the N-terminal sequence of *C. coli* VC167 flagellin. Of the remaining nonidentical residues, two replacements are conservative. There is a conservative replacement of isoleucine for valine in the *H. pylori* sequence at residue 4 and a replacement of valine for isoleucine at residue 8. In this region of the molecule, the 56,000-M_r *H. pylori* flagellin sequence was more closely related to the *C. coli* flagellin than it was with either the *Salmonella* or *Bacillus* flagellin. However, in the first 24 residues, the predominant 56,000-M_r *H. pylori* flagellin did share 7 homologous residues with *Salmonella* flagellin (21) and 5 homologous residues with *Bacillus* flagellin (8). In the case of the complex flagellum-forming bacterium *C. crescentus*, sequence similarities with the *H. pylori* proteins at the N terminus were again seen (37).

Western blot analysis with polyclonal antisera produced against *H. pylori* flagellin and antisera against *C. jejuni* flagellin showed that the *H. pylori* proteins were antigenically cross-reactive with each other and with the flagellins of a wide range of *Campylobacter*-like organisms but were not antigenically cross-reactive against the *Escherichia*, *Vibrio*, *Aeromonas*, or *Salmonella* flagellins. Immunogold analysis showed that the cross-reactive epitopes recognized by the polyclonal antisera prepared against *C. jejuni* flagellin were buried in both *Helicobacter* and *Campylobacter* filaments. In contrast, immunogold analysis with polyclonal antiserum JB3 showed that in the case of *H. pylori* flagella, there were cross-reactive epitopes exposed on the surface of the assembled filament, while in the case of the *C. jejuni* and *C. coli* flagella we tested, the cross-reactive epitopes were buried. This indicates that the flagellin subunits are assembled differently in *Helicobacter* and *Campylobacter* flagellar filaments. Interestingly, the 57,000- and 56,000-M_r *H. pylori* flagellins also appear to be assembled differently in the *H. pylori* filament, because although Western blotting showed that the epitope for MAb 72c was shared by these two flagellins, immunoelectron microscopy showed that the epitope for this antibody was exposed on the surface of the assembled filament in the case of the 57,000-M_r protein but masked in the case of the 56,000-M_r protein. Importantly, immunoelectron microscopy with antiflagellin antibodies also showed that neither the 57,000-M_r flagellin nor the 56,000-M_r flagellin was present in the sheath enclosing the *H. pylori* flagellar filament. This is similar to the situation in *Bdellovibrio bacterivorus* (46) and *Vibrio cholerae* (11), in

which the sheath has a membranelike structure and contains lipopolysaccharides.

In addition to the sequences shared by the 57,000- and 56,000-M_r flagellins of *H. pylori*, the two flagellins also contain sequence differences. This was illustrated both by endoproteinase Glu-C peptide mapping (data not shown) and by the inability of MAbs 104a and 220a to react with the 57,000-M_r protein. The sequences of the 56,000-M_r protein recognized by these two MAbs are buried in the assembled filament and map to different regions of the flagellin molecule (data not shown). Both epitopes appear to be *H. pylori* specific, since both MAbs reacted with all nine *H. pylori* strains tested but with no other organisms, including the related rodent ileal colonizing strain T1. Although the epitopes for these MAbs were not surface exposed, both MAbs were successfully employed to identify *H. pylori* in whole-cell lysates by a simple immunodot blot technique. This could be a useful addition to current laboratory identification procedures for *H. pylori*. SDS lysis is easy to perform and can be done on a small scale, such as in microcentrifuge tubes, and immunodot blot assay is within the capabilities of most laboratories and is simple to perform on a routine basis.

From a phylogenetic perspective, the related N-terminal sequence, together with the presence of shared antigenicity, suggests that the *Helicobacter* and *Campylobacter* flagellins may have evolved together and differently from other flagellins. Recent evidence also indicates that like the *H. pylori* flagellum, the *Campylobacter* flagellum contains two flagellins, one of which is present in higher copy number than the other (16). However, Southern blot analysis using cloned *C. coli* flagellin genes has shown that while the *C. coli* gene hybridizes to the flagellin genes of most of the true campylobacters tested, the *C. coli* gene does not hybridize to the *H. pylori* genes (47), suggesting that there are significant differences at the nucleotide level and that differential evolution has occurred within this group of flagellins. The nucleotide sequences of the genes coding for the two *Campylobacter* flagellins have been determined previously (17), and efforts are under way to clone and determine the nucleotide sequences of the genes coding for the two *H. pylori* flagellins so that the evolution of the *Helicobacter* and *Campylobacter* group of flagellins can be better understood.

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REFERENCES

1. **Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich.** 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. **136**:175–179.
2. **Blaser, M. J.** 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J. Infect. Dis. **161**:626–633.
3. **Blaser, M. J.** 1990. Epidemiology and pathophysiology of *Campylobacter pylori* infections. Rev. Infect. Dis. **12**:S99–S106.
4. **Chandler, H. M., and J. Gulasekharam.** 1974. The protective antigen of a highly immunogenic strain of *Clostridium chauvoei* including and evaluation of its flagella as a protective antigen. J. Gen. Microbiol. **84**:128–134.
5. **Cheng, S., C. Sanderson, T. Waters, and C. Goodwin.** 1987. *Campylobacter pyloridis* in patients with gastric carcinoma. Med. J. Aust. **147**:202.
6. **Cleveland, D. W., S. G. Fisher, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
7. **Dayhoff, M. O.** 1978. Atlas of protein sequence and structure. National Biomedical Research, Washington, D.C.
8. **Delange, R. J., J. Y. Chang, J. H. Sharper, and A. M. Glazer.** 1976. Amino acid sequence of flagellin of *Bacillus subtilis* 168. J. Biol. Chem. **254**:705–711.
9. **Dunn, B. E., G. I. Perez-Perez, and M. J. Blaser.** 1989. Two-dimensional gel electrophoresis and immunoblotting of *Campylobacter pylori* proteins. Infect. Immun. **57**:1825–1833.
10. **Dupont, D., P. Keim, A. Chui, R. Bello, and K. Wilson.** 1987. Cysteine/cystine modification for amino acid analysis. Applied Biosystems User Bulletin 1, p. 1–7. Applied Biosystems, Foster City, Calif.
11. **Fuerst, J. A., and J. W. Perry.** 1988. Demonstration of lipopolysaccharide on sheathed flagella of *Vibrio cholerae* O:1 by protein A-gold immunoelectron microscopy. J. Bacteriol. **170**:1488–1494.
12. **Geis, G., H. Leying, S. Suerbau, U. Mai, and W. Opferkuch.** 1989. Ultrastructure and chemical analysis of *Campylobacter pylori* flagella. J. Clin. Microbiol. **27**:436–441.
13. **Gill, P. R., and N. Agabian.** 1983. The nucleotide sequence of the Mr = 28,500 flagellin gene of *Caulobacter crescentus*. J. Biol. Chem. **258**:7395–7401.
14. **Goodwin, C. S., J. A. Armstrong, T. Chilvers, M. Peters, M. D. Collins, L. Sly, W. McConnell, and W. E. S. Harper.** 1989. Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov., respectively. Int. J. Syst. Bacteriol. **39**:397–405.
15. **Goodwin, C. S., R. K. McCulloch, J. A. Armstrong, and S. H. Wee.** 1985. Unusual cellular fatty acids and distinctive ultrastructure in a spiral bacterium (*Campylobacter pyloridis*) from the human gastric mucosa. J. Med. Microbiol. **19**:257–267.
16. **Guerry, P., S. M. Logan, S. Thornton, and T. J. Trust.** 1990. Genomic organization and expression of *Campylobacter* flagellin genes. J. Bacteriol. **172**:1853–1860.
17. **Harris, L. A., S. M. Logan, P. Guerry, and T. J. Trust.** 1987. Antigenic variation of *Campylobacter* flagella. J. Bacteriol. **169**:5066–5071.
18. **Hazell, S. L., A. Lee, L. Brady, and W. Hennessy.** 1986. *Campylobacter pyloridis* and gastritis: association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. J. Infect. Dis. **153**:658–663.
19. **Hu, L.-T., and H. L. T. Mobley.** 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori*. Infect. Immun. **58**:992–998.
20. **Jiang, S. J., W. Z. Liu, D. Z. Zhang, Y. Shi, S. D. Xiao, Z. N. Zhang, and D. Y. Lu.** 1987. *Campylobacter*-like organisms in chronic gastritis, peptic ulcer, and gastric carcinoma. Scand. J. Gastroenterol. **22**:553–558.
21. **Joys, T. M.** 1985. The covalent structure of the phase-1 flagellar filament protein of *Salmonella typhimurium* and its comparisons with other flagellins. J. Biol. Chem. **247**:5180–5193.
22. **Krakowska, S., D. R. Morgan, W. G. Kraft, and R. D. Leunk.** 1987. Establishment of gastric *Campylobacter pylori* infection in the neonatal piglet. Infect. Immun. **55**:2789–2796.
23. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
24. **Lambert, J. R., M. Borromeo, K. J. Pinkard, H. Turner, C. B. Chapman, and M. L. Smith.** 1987. Colonisation of gnotobiotic pigs with *Campylobacter pylori*—an animal model? J. Infect. Dis. **155**:1344.
25. **Lee, A., and S. L. Hazell.** 1988. *Campylobacter pylori* in health and disease: an ecological perspective. Microb. Ecol. Health Dis. **1**:1–16.
26. **Lee, A., S. M. Logan, and T. J. Trust.** 1987. Demonstration of a

flagella antigen shared by a diverse group of spiral-shaped bacteria that colonize intestinal mucus. Infect. Immun. **55**:828–831.

27. **LeGendre, N., and P. Matsudaira.** 1988. Direct protein microsequencing from Immobilon-P transfer membrane. Biotechniques **6**:154–159.
28. **Logan, S. M., L. A. Harris, and T. J. Trust.** 1987. Isolation and characterization of *Campylobacter* flagellins. J. Bacteriol. **169**: 5072–5077.
29. **Logan, S. M., and T. J. Trust.** 1986. Location of epitopes on *Campylobacter jejuni* flagella. J. Bacteriol. **168**:739–745.
30. **Logan, S. M., T. J. Trust, and P. Guerry.** 1988. *Campylobacter* flagellin: evidence for posttranslational modification and gene duplication. J. Bacteriol. **171**:3031–3038.
31. **Luke, C. J., E. Kubiak, A. Cockayne, T. S. J. Elliot, and C. W. Penn.** 1990. Identification of flagellar and associated polypeptides of *Helicobacter* (formerly *Campylobacter*) *pylori*. FEMS Microbiol. Lett. **71**:225–230.
32. **Marshall, B. J.** 1990. *Campylobacter pylori*: its link to gastritis and peptic ulcer disease. Rev. Infect. Dis. **12**:S87–S93.
33. **Marshall, B. J., J. A. Armstrong, D. B. McGechie, and R. J. Glancy.** 1985. Attempt to fulfill Koch's postulates for pyloric *Campylobacter*. Med. J. Aust. **142**:436–439.
34. **Marshall, T., K. M. Williams, and O. Vesterburg.** 1984. Two-dimensional electrophoresis of proteins in human serum: improved resolution by use of narrow pH gradients and prolonged electrophoresis. Clin. Chem. **30**:2008–2013.
35. **Metzger, H., M. B. Shapiro, J. E. Mosimann, and J. E. Vinton.** 1968. Assessment of compositional relatedness between proteins. Nature (London) **219**:1166–1168.
36. **Mills, S. D., L. A. Kurjanczyk, and J. L. Penner.** 1988. Identification of an antigen common to different species of the genus *Campylobacter*. J. Clin. Microbiol. **26**:1411–1413.
37. **Minnich, S. A., and A. Newton.** 1987. Promoter mapping and cell cycle regulation of flagellin gene transcription in *Caulobacter crescentus*. Proc. Natl. Acad. Sci. USA **84**:1142–1146.
38. **Morris, A., and G. Nicholson.** 1987. Ingestion of *Campylobacter pylori* causes gastritis and raised fasting gastric pH. Am. J. Gastroenterol **82**:192–199.

38a. **Newell, D., and A. Baskerville.** 1987. Proc. IVth Int. Workshop *Campylobacter* Infect., abstr. 252.

39. **Newell, D. G.** 1987. Identification of the other membrane proteins of *Campylobacter pyloridis* and antigenic cross-reactivity between *C. pyloridis* and *C. jejuni*. J. Gen. Microbiol. **133**:163–170.
40. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
41. **Pearson, T. W., M. Pinder, G. Roelants, S. K. Kar, L. B. Lundin, K. S. Mayor-Witney, and R. S. Hewett.** 1980. Methods for the derivation and detection of anti-parasitic monoclonal antibodies. J. Immunol. Methods **34**:141–154.
42. **Penner, J. L.** 1988. The genus *Campylobacter*: a decade of progress. Clin. Microbiol. Rev. **1**:157–172.
43. **Pleier, E., and R. Schmitt.** 1989. Identification and sequence analysis of two related flagellin genes in *Rhizobium meliloti*. J. Bacteriol. **171**:1467–1475.
44. **Robertson, E. F., H. K. Dannelly, P. J. Malloy, and H. C. Reeves.** 1987. Rapid isoelectrofocusing in a vertical polyacrylamide minigel system. Anal. Biochem. **167**:290–294.
45. **Romaniuk, P. J., B. Zoltowska, T. J. Trust, D. J. Lane, G. J. Olsen, N. R. Pace, and D. A. Stahl.** 1987. *Campylobacter pylori*, the spiral bacterium associated with human gastritis, is not a true *Campylobacter* sp. J. Bacteriol. **169**:2137–2141.
46. **Thomashow, L. S., and S. C. Rittenberg.** 1985. Isolation and composition of sheathed flagella from *Bdellovibrio bacteriovorus* 109J. J. Bacteriol. **163**:1047–1054.
47. **Thornton, S. A., S. M. Logan, T. J. Trust, and P. Guerry.** 1990. Polynucleotide sequence relationships among flagellin genes of *Campylobacter jejuni* and *Campylobacter coli*. Infect. Immun. **58**:2686–2689.
48. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **74**:4350–4354.
49. **Warren, J. R., and B. Marshall.** 1983. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet **i**:1273–1275.
50. **Weissborn, A., H. M. Steinman, and L. Shapiro.** 1982. Characterization of the proteins of the *Caulobacter crescentus* flagellar filament. J. Biol. Chem. **257**:2066–2074.
51. **Wyatt, J. I.** 1989. The role of *Campylobacter pylori* in the pathogenesis of peptic ulcer disease. Scand. J. Gastroenterol. **24**:7–11.
52. **Yokote, Y., K. M. Arai, and K. Akahane.** 1986. Recovery of tryptophan from 25-minute acid hydrolysates of protein. Anal. Biochem. **152**:245–249.