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Identification of Surface-Exposed Outer Membrane Antigens of *Helicobacter pylori*

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Despite the potential significance of surface-localized antigens in the colonization by and disease processes of *Helicobacter pylori*, few such components have been unequivocally identified and/or characterized. To further investigate the surface of this bacterium, monoclonal antibodies (MAbs) to a sarcosine-insoluble outer membrane fraction prepared from *H. pylori* NCTC 11637 were raised. MAbs were selected on the basis of their surface reactivity to whole cells by enzyme-linked immunosorbent assay, immunofluorescence, and immunoelectron microscopy. By use of this selection protocol, 14 surface-reactive MAbs were chosen. These MAbs were used to identify six protein antigens (molecular masses, 80, 60, 51, 50, 48, and 31 kDa), all of which were localized within or associated with the outer membrane. Two of the MAbs recognized the core region of lipopolysaccharide (LPS). Only these two anti-LPS MAbs also recognized the flagellar sheath, indicating a structural difference between the sheath and outer membrane. Three of the protein antigens (80, 60, and 51 kDa) were strain specific, while the other three antigens were present in other strains of *H. pylori*. Both the 51- and 48-kDa antigens were heat modifiable and likely are porins. A conserved 31-kDa protein may represent another species of porin. A method involving sucrose density ultracentrifugation and Triton extraction that allows the preparation of *H. pylori* outer membranes with minimal inner membrane contamination is described. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed that the protein content of the *H. pylori* outer membrane is similar structurally to those of other species of *Helicobacter* but markedly different from those of taxonomically related *Campylobacter* spp. and *Escherichia coli*. *H. pylori* also appeared to lack peptidoglycan-associated proteins.

Helicobacter pylori is a spiral-shaped, gram-negative, microaerophilic bacterium which colonizes the stomach of humans (29). This organism resides predominantly within the mucus overlying the gastric epithelium. However, a fraction of these bacteria penetrate the mucous layer and directly attach to the underlying epithelial cells (30). Despite an active systemic and mucosal humoral immune response, *H. pylori* colonization persists for years, possibly for life, in untreated individuals (4). Ultimately, such persistent colonization by *H. pylori* elicits an inflammatory response which in turn leads to damage of the host tissues. The pathology of infection by *H. pylori* ranges from chronic superficial type B gastritis to gastric and duodenal ulcers. *H. pylori* has also been implicated in the development of gastric cancer (5) and the occurrence of primary cell gastric mucosa-associated lymphoid tissue lymphoma (22). Epidemiological studies have shown that *H. pylori* infection occurs throughout the world, with 40 to 60% of all individuals being colonized by age 60 (46). Thus, *H. pylori* may be one of the most common pathogens of humans (29).

Despite the clinical significance and prevalence of *H. pylori*, relatively little is known about the bacterial components that allow it to colonize, to persist, and to promote an inflammatory response within its host. Surface-exposed components frequently influence the colonization and persistence of a pathogen, as well as the pathogenesis of the disease process, including the inflammatory response on the host. In the case of *H. pylori*, one macromolecular assembled protein component which is unequivocally located on the surface of the bacterium is the sheathed flagellum. The filament of this motility organelle is composed of two species of flagellins which differ with respect to their apparent subunit molecular weights (26). It is argued that the motility of *H. pylori*, conferred by these flagella, allows the bacterium to penetrate the mucus layer, possibly leading to mucus and epithelial colonization and a resulting persistence of the infection due in part to increased resistance to peristalsis (5, 29). Two other proteins which can be isolated in abundance from suspensions of *H. pylori* cells, but which are normally regarded as intracellular in other bacterial species, are urease and a GroEL analog, Hp60K (1, 11, 12, 19, 34). Whether these large macromolecular assemblies are surface proteins on *H. pylori* or are released as a result of cellular lysis remains in dispute. However, other intracellular proteins can be readily isolated from culture supernatants after mild shearing and extraction procedures, and these released proteins have initially been incorrectly interpreted as representing surface proteins (7, 8, 39).

In the case of gram-negative bacteria, many surface-exposed proteins are constituents of the outer membrane. The outer membrane has a structural role and also plays a major role in determining what enters the cell, what molecules are secreted from the cell, and how the cell interacts with its environment (38). Little is known about the outer membrane structure of *H. pylori* or the identities of its surface-exposed proteins. Only a few proteins have been identified as being unambiguously localized within the outer membrane (10, 24). This is partly because of high-copy-number assemblies of proteins such as urease and GroEL which have copurified with cell envelopes in the past, complicating analysis of the membrane components (36).

To unambiguously identify surface-exposed proteins of *H. pylori*, we have raised a panel of monoclonal antibodies

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(MAbs) which react to this bacterium's cell surface as monitored by enzyme-linked immunosorbent assay (ELISA), immunofluorescence, and immunogold electron microscopy. Using these MAbs, and by evaluating various outer membrane isolation procedures, we have identified six outer membrane proteins with surface-accessible domains, including three potential porin species. We have found that like those of *Campylobacter* spp. (42), the outer membrane of *H. pylori* is difficult to isolate free of inner membrane contamination and appears to be strongly associated with the inner membrane. We have also shown that similarities exist between the protein profiles of various *Helicobacter* spp., a property not shared with taxonomically related campylobacters. We also report that the outer membrane and sheath of *H. pylori* flagella differ with respect to protein composition, demonstrating that the sheath structure is not just a continuation of the outer membrane.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *H. pylori* strains examined in this study were endoscopic biopsy isolates CCUG (Culture Collection, University of Göteborg) 915 (Stockholm, Sweden, isolate); strains 5294, 5442, and 5155 (Sydney, Australia, isolates); NCTC (National Type Culture Collection) 11637 (type strain of species; Perth, Australia, isolate); and strains 7958 and 5790 (Canadian isolates). *Helicobacter mustelae* Hm180, Hm181, and Hm4298 (40), *Helicobacter felis* Hf1, *Campylobacter rectus* 33238 and 314 (a generous gift from S. C. Holt, University of Texas Health Science Center, San Antonio), *Campylobacter coli* VC167B (18), and *Escherichia coli* JM109 were also examined. Stock cultures were maintained at −70°C in 15% (vol/vol) glycerol-Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Cultures of *Helicobacter* spp. were grown at 37°C on chocolate blood agar plates in an atmosphere containing 10% (vol/vol) carbon dioxide for 48 h. *C. rectus* was grown on chocolate blood agar under anaerobic conditions at 37°C. *C. coli* was grown on Mueller-Hinton agar at 37°C in an atmosphere containing 10% (vol/vol) carbon dioxide. *E. coli* was grown at 37°C in Luria broth.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) was performed by the method of Laemmli (28) in a minislab apparatus or with the Phast gel electrophoresis system (Pharmacia, Baie d'Urfe, Quebec, Canada). Protein was stained with Coomassie blue R-250 or with silver according to the method of Tsai and Frasch (47).

When required, separated proteins were transferred from the polyacrylamide slab gel to a nitrocellulose membrane according to the method described by Bjerrum and Schafer-Neilsen (2). Electroblotting was carried out in a semidry transblot apparatus (LKB, Baie d'Urfe, Quebec, Canada) for 30 min at a field strength of approximately 0.8 mA/cm^2.

Western blotting (immunoblotting). After electroblotting, unreacted sites on the nitrocellulose membrane were blocked with a 2% (vol/vol) solution of skim milk in 10 mM Tris-HCl–0.9% NaCl, pH 7.5 (MTS). The nitrocellulose membrane was then incubated with an appropriate dilution of antibody for 2 h and then washed three times with 10 mM Tris-HCl–0.9% NaCl–0.05% Tween 20, pH 7.5 (TTBS). Alkaline phosphatase conjugated to goat immunoglobulin G (IgG) (anti-mouse IgG) (Southern Biotechnology Associates, Inc., Birmingham, Ala.) was then added in MTS and incubated for 1 h at room temperature. After incubation, the nitrocellulose membrane was washed three times in TTBS. The reactive bands were visualized as described by Blake et al. (3), using 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim Gmbh, Mannheim, Germany) as the alkaline phosphatase substrate and Nitro Blue Tetrazolium (Sigma Chemical Co., St. Louis, Mo.) as the color development reagent.

Assays. The succinate dehydrogenase (SDH) assay described by Osborn et al. (37) was used. The Bradford assay was used to measure protein, with bovine serum albumin (BSA) as a standard (6).

Sarcosine preparation of outer membranes. *H. pylori* was grown for 48 h on chocolate blood agar and harvested by suspension in 20 mM Tris, pH 7.5. Bacteria were collected by centrifugation (12,000 × *g*, 20 min, 4°C), and washed three times with 20 mM Tris, pH 7.5. Cells were suspended in 20 mM Tris, pH 7.5, and lysed by passing the suspension through a French pressure cell (15,000 lb/in^2) three times. DNase (0.1 mg) and RNase (0.5 mg) were added to the cell suspension, and the mixture was incubated at room temperature for 30 min. Unbroken cells were removed by centrifugation, and the supernatant was retained. Total membranes were then collected by centrifugation (40,000 × *g*, 30 min, 4°C). Membranes were resuspended in 20 mM Tris, pH 7.5, containing 2.0% (wt/vol) sodium lauryl sarcosine and incubated at room temperature for 30 min. Outer membranes were collected by centrifugation (40,000 × *g*, 30 min, 4°C) and washed three times with Milli Q water. The pellet was resuspended in Milli Q water, aliquoted, and stored at −20°C until use.

Triton preparation of outer membranes. Bacteria were grown and total membranes were prepared as described above except that 10 mM Tris, pH 7.5, was used in place of 20 mM Tris, pH 7.5. Total membranes were collected by centrifugation (40,000 × *g*, 30 min, 4°C) and suspended in 10 mM Tris–20% sucrose, pH 7.5. This suspension was layered on top of a 52–58–64–70% (wt/vol) sucrose step gradient. The gradient was then subjected to centrifugation at 150,000 × *g* for 18 h. Bands were removed, washed with 10 mM Tris (pH 7.5) three times, and assayed for protein content, SDH activity, and lipopolysaccharide (LPS) content. The fraction containing the least SDH activity and highest LPS content was the crude envelope preparation. Most of the remaining inner membrane was removed by extraction with 2% (vol/vol) Triton X-100 or Triton X-114 in 20 mM Tris, pH 7.5 (0.4 mg of protein per ml).

MAb production. BALB/c mice were immunized with sarcosine-prepared outer membranes of *H. pylori* NCTC 11637 (10 μg per mouse) and boosted three times. Cloned hybridomas secreting MAbs were then prepared by the procedure of Pearson et al. (43). The class and subclass of each MAb were determined by indirect ELISA (American Qualex International, Inc., La Mirada, Calif.).

LPS preparation. LPS was prepared from whole cells as described previously (33), using proteinase K to digest protein.

Peptidoglycan association. Proteins associated with the peptidoglycan were identified by the method of Rosenbusch (45), as described by Logan and Trust (31).

Absorption of antibodies. In order to remove antibodies directed to surface epitopes, MAbs were absorbed with whole cells of strain NCTC 11637. Washed bacteria were added to the antiserum and incubated at room temperature with shaking for 2 h. Cells were removed by centrifugation (13,000 × *g* for 2 min at room temperature). The absorption was then repeated twice more.

ELISA. To each well of a 96-well ELISA plate (Costar, Cambridge, Mass.), 100 μl of an *H. pylori* whole-cell suspension (A_{650} = 0.1) or Triton X-114-prepared outer membranes (10 μg/ml) was added and dried overnight at 37°C. Plates were then washed with TTBS (250 μl per well) and blocked with 1% BSA in 10 mM Tris-HCl–0.9% NaCl, pH 7.5 (TBS) (200 μl per well) for 1 h at 37°C. At this time the wells were washed three

TABLE 1. Summary of MAbs used in this study

MAb	Subclass	Antigen recognized	Surface reactivity[a]	Specificity
1G1	IgG2a	31 kDa	++++	All *H. pylori*, *C. coli* 50-kDa–54-kDa doublet
4B9	IgG2a	31 kDa	++++	All *H. pylori*, *C. coli* 50-kDa–54-kDa doublet
6H2	IgG2a	31 kDa	++++	All *H. pylori*
6A8	IgG1	48 kDa	+++	*H. pylori* 11637, 915
7D1	IgG2a	48 kDa	++	*H. pylori* 11637, 5442
8D1	IgG2a	48 kDa	++++	*H. pylori* 11637 specific
2H4	IgG2a	50 kDa	+++	All *H. pylori*
9E3	IgG2b	50 kDa	+++	All *H. pylori*
1H5	IgG1	51 kDa	++++	*H. pylori* 11637 specific
6F4	IgG1	60 kDa	+++	*H. pylori* 11637 specific
2H1	IgG2a	90 kDa	+++	*H. pylori* 11637 specific
11B3	IgG2b	90 kDa	+++	*H. pylori* 11637 specific
6A5	IgG3	LPS	++++	*H. pylori* 11637, 915, 5294, 5790
6C11	IgG1	LPS	++++	*H. pylori* 11637, 915, 5294, 5790

[a] Intensity was assessed by the indirect fluorescent-antibody technique: +, weakest; ++++, strongest.

times with TTBS. Primary antibody (100 μl) diluted in TTBS was then added to each well and incubated at 37°C for 2 h. Wells were then washed five times and goat IgG (anti-mouse IgG plus IgM plus IgA) conjugated to alkaline phosphatase (Southern Biotechnology Associates, Inc.) was added to each well. After 2 h, wells were washed five times. A substrate solution consisting of 1.0 M diethanolamine, 0.5 mM magnesium chloride, and 3.8 mM disodium *p*-nitrophenyl phosphate (Sigma), pH 9.5, was added to each well (100 μl per well). The A_{405} of each well was then determined.

Immunofluorescence microscopy. The indirect fluorescent-antibody technique was used with acetone-fixed bacterial cells and with living cells as previously described. MAbs were used as tissue culture supernatants at a 1:200 dilution. The second antibody was a fluorescein-conjugated goat IgG (anti-mouse IgG) (Caltag Laboratories, San Francisco, Calif.), and slides were observed by using a standard microscope with an epifluorescence attachment.

Electron microscopy. A grid covered with a Formvar film was floated on a 50-μl drop of bacterial cells in TBS for 5 min. The grids were stained by floating on a drop of 1% (wt/vol) ammonium molybdate, pH 7.5, and were examined in a Hitachi electron microscope operated at an accelerating voltage of 75 kV.

For immunoelectron microscopy the grid was floated on the sample and then removed and floated on a drop of TBS containing 1% (wt/vol) BSA for approximately 30 min. The grid was then incubated on a drop of TBS containing the diluted antibody for 1 h. After incubation, the grid was removed, and nonspecifically bound immunoglobulin was removed by floating the grid on 3 drops of TBS. The grid was then floated on a drop of TBS containing a 1:50 (vol/vol) dilution of 15-nm-diameter colloidal gold particles coated with goat IgG (anti-mouse IgG) (Amersham Canada LTD, Oakville, Ontario, Canada). After incubation for 30 min, the nonspecifically bound colloidal gold particles were removed by floating the grid on 3 drops of TBS. The grids were negatively stained and examined as described above.

RESULTS

MAB preparation and selection. BALB/c mice were primed by using a sarcosine-insoluble outer membrane preparation derived from *H. pylori* NCTC 11637. This preparation lacked the GroEL analog of *H. pylori* and contained only trace amounts of urease and flagellin as assessed by electron microscopy of negatively stained preparations and by Western immunoblotting. A total of 1,152 clones were selected for primary screening. Tissue culture supernatants of the hybridomas were screened for production of antibodies to surface antigens by ELISA, using formalin-fixed whole cells of strain NCTC 11637 as the antigen. One hundred thirty-seven clones were selected for further characterization. These clones were then screened by the indirect fluorescent-antibody technique to identify potential surface reactivity. Clones that produced a strong positive reaction were screened further by Western immunoblotting with outer membrane fractions prepared by Triton X-114 extraction as the antigen. Fourteen surface-reactive MAbs were selected by these selection criteria (Table 1). A number of additional MAbs recognizing urease subunits were also isolated but were not studied further.

Surface exposure of MAb-reactive antigens. Preliminary cell fractionation studies revealed that the 14 surface antigens were predominantly located within or associated with the sarcosine-insoluble outer membrane fraction. To confirm that the MAbs recognized antigens on the outer surface of the bacterium and to determine whether they recognized discernible surface structures (e.g., flagella), immunoelectron microscopy was performed. Consistent with the selection protocol, all 14 MAbs bound to the surface of cells of *H. pylori* NCTC 11637, albeit with various degrees of intensity. As a control, a MAb specific for an inner membrane protein was used and did not label the surface (9). However, labeling was generally uniform across the surface of the bacterium. This is well illustrated by the case of MAb 6A8 (Fig. 1C). MAbs 6A5 (Fig. 1A) and 6C11 (9) also labeled the flagellar sheath. None of the other MAbs labeled the sheath to any significant degree (Fig. 1B) (9).

To further confirm the surface localization of the MAb-reactive epitopes, MAbs were absorbed with whole cells of *H. pylori* NCTC 11637 and employed in an ELISA system with outer membrane preparations as the antigen. All MAbs exhibited a reduction in the end point titer of at least twofold after absorption (9).

MAb specificities. The molecular masses and cross-reactivities of the antigens recognized by the various MAbs are given in Table 1. MAbs 1G1, 4B9, and 6H2 recognized a 31-kDa antigen unique to all strains of *H. pylori* examined (Fig. 2; Table 1). These three MAbs also weakly recognized antigens with molecular masses of approximately 50 and 90 kDa, but they did not react with protein antigens present in other *Helicobacter* species. MAbs 1G1 (Fig. 2A) and 6H2 did,

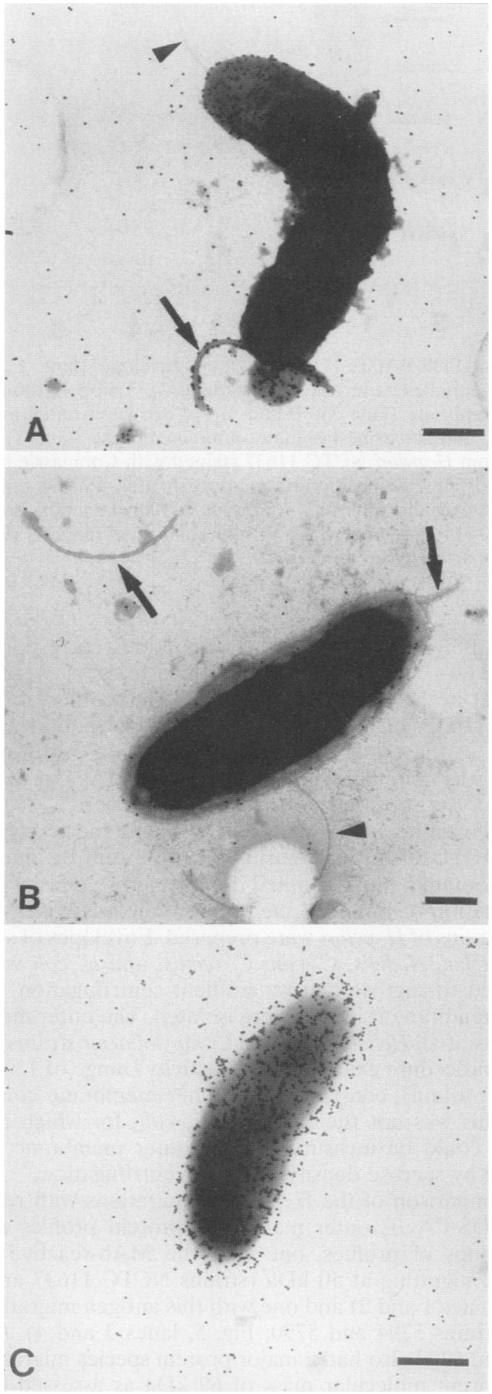

FIG. 1. Electron micrographs showing binding of MAbs to *H. pylori* NCTC 11637, using the immunogold labeling technique. In panels A and B arrows indicate sheathed flagella, while arrowheads indicate unsheathed flagellar filaments. The print exposure in these panels has been chosen to allow visualization of antibody binding to the flagellar sheath. (A) Gold labeling of the bacterial surface by using anti-LPS core MAb 6A5. The ability of this antibody to bind to the flagellar sheath is clearly visible. (B) Gold labeling of the bacterial surface by using anti-48-kDa protein MAb 8D1. (C) Gold labeling of the bacterial surface by using anti-48-kDa MAb 6A8. This print was underexposed to allow for better visualization of the gold particles binding to the cell surface. Bars, 100 nm.

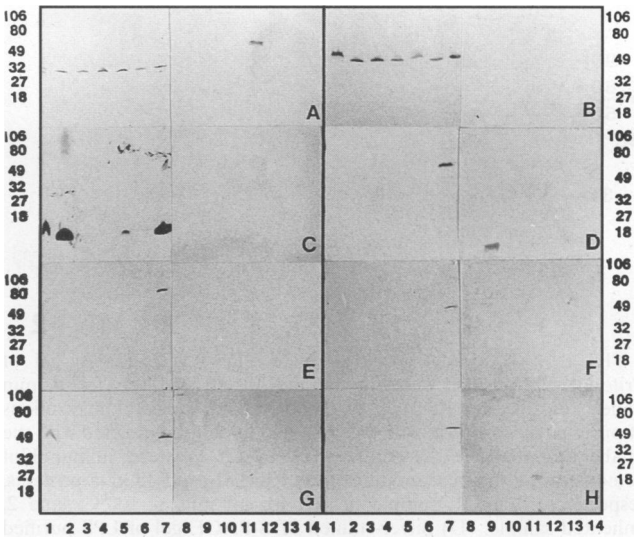

FIG. 2. Strain and species reactivities of various *H. pylori* outer membrane-directed MAbs. The loading of the strains in each plate was as follows: lane 1, *H. pylori* 915; lane 2, *H. pylori* 5155; lane 3, *H. pylori* 5294; lane 4, *H. pylori* 5442; lane 5, *H. pylori* 5790; lane 6, *H. pylori* 7980; lane 7, *H. pylori* NCTC 11637; lane 8, *H. felis* Hf1; lane 9, *H. mustelae* Hm180; lane 10, *H. mustelae* Hm181; lane 11, *H. mustelae* Hm4298; lane 12, *C. coli* 167A; lane 13, *C. rectus* 314; lane 14, *C. rectus* 33238. The blots were probed with the following MAbs: (A) anti-31-kDa 1G1; (B) anti-50-kDa 9E3; (C) anti-LPS core 6C11; (D) anti-60-kDa 6F4; (E) anti-90-kDa 11B3; (F) anti-48-kDa 8D1; (G) anti-48-kDa 6A8; (H) anti-51-kDa 1H5. The relative positions of molecular mass markers (in kilodaltons) are given on the right for panels B, D, F, and H and on the left for panels A, C, E, and G.

however, recognize a 50-kDa–54-kDa doublet band in *C. coli*. This doublet was found to be localized in the outer membrane of *C. coli* (9).

MAb 1H5 recognized a 51-kDa antigen (Fig. 2H), while MAbs 8D1 (Fig. 2F), 6A8 (Fig. 2G), and 7D1 reacted with a 48-kDa protein. None of these MAbs reacted with other species of *Helicobacter* or *Campylobacter* tested. Whereas 1H5 and 8D1 were specific for *H. pylori* NCTC 11637, 6A8 and 7D1 each reacted with a single other strain (strain 915 for 6A8 and strain 5442 for 7D1) (Table 1). Both the 51- and 48-kDa protein antigens were heat modifiable, migrating with apparent molecular masses of 39 and 37 kDa, respectively, when estimates were made prior to heating at a temperature greater than 60°C for 10 min in SDS-PAGE sample buffer (Fig. 3).

MAbs 2H4 and 9E3 (Fig. 2B) reacted with a 50-kDa antigen present in all *H. pylori* strains examined (Table 1). The apparent molecular mass of the antigen did exhibit some strain-to-strain variability, with the antigen migrating at a slightly higher apparent mass in strains 5294 and 5790. This antigen appeared to be species specific, because MAbs 2H4 and 9E3 did not recognize antigens present in other *Helicobacter* or *Campylobacter* species.

MAbs 6A5 (Fig. 3D) and 6C11 (Fig. 2C) were specific for a fast-migrating antigen with a low apparent molecular mass. By using purified LPS from strain NCTC 11637, these MAbs were shown to be specific for a region comprising the low-molecular-mass core region of the molecule (Fig. 3). These MAbs also reacted with *H. pylori* 915, 5294, and 5790 but did not react with the other *H. pylori* strains examined or with any of the strains of other *Helicobacter* or *Campylobacter* species tested (Fig. 2; Table 1).

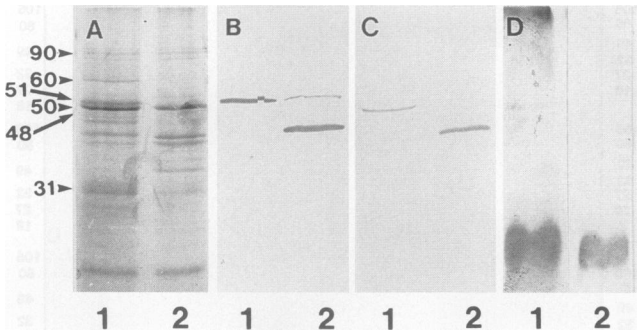

FIG. 3. (A) Coomassie blue-stained SDS-PAGE (15%) gel of Triton X-114-purified outer membrane heated at 100°C for 10 min (lane 1) or unheated (lane 2). Arrows on the left indicate the positions of proteins of various molecular mass (in kilodaltons) reacted with the MAbs described in this study. (B and C) Western immunoblot demonstrating the heat modification of the 51- and 48-kDa proteins, respectively. Lane 1, sample heated for 10 min at 100°C; lane 2, unheated sample. (D) Silver-stained SDS-PAGE gel of LPS purified from *H. pylori* NCTC 11637 (lane 1) and a Western immunoblot of this preparation with anti-LPS MAb 6A5 (lane 2). Identical results were obtained with MAb 6C11.

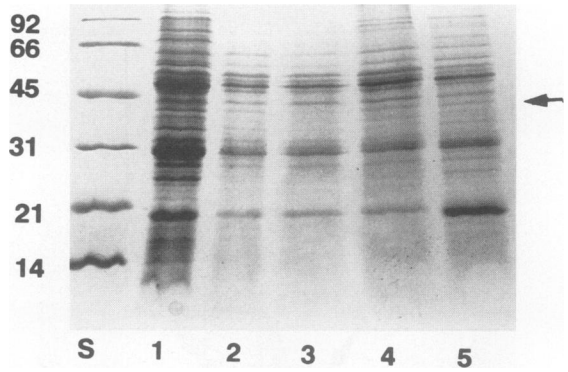

FIG. 4. SDS-PAGE (15%) of total envelope (lane 1), sucrose gradient-enriched outer membrane (lane 2), Triton X-100-extracted outer membrane (lane 3), Triton X-114-extracted outer membrane (lane 4), and sarcosine-insoluble outer membrane (lane 5), all prepared from *H. pylori* NCTC 11637 stained with Coomassie blue. The arrow indicates the position of an approximately 40-kDa polypeptide that was partially removed by Triton X-100 extraction but not by Triton X-114 extraction. Sizes of molecular mass markers (lane S; in kilodaltons) are given on the left.

MAbs 11B3 (Fig. 2E), 2H1, and 6F4 (Fig. 2D) recognized antigens specific to strain NCTC 11637 (Table 1). MAbs 2H1 and 11B3 reacted with a 90-kDa antigen, while 6F4 reacted with an approximately 60-kDa antigen.

Outer membrane analysis. The information gained from the surface labeling studies was then used to help develop a procedure for isolation of *H. pylori* outer membrane containing minimal inner membrane contamination. After preliminary experimentation, two methods of outer membrane isolation were chosen for comparison. By using a total envelope fraction (SDH activity = 33 U/g of protein), outer membranes were prepared by either loading them onto a sucrose gradient or extracting them with sarcosine. After separation of the total membrane preparation by density gradient centrifugation, the fraction migrating at approximately 54% (wt/vol) sucrose was found to contain the highest amount of LPS and lowest SDH activity (SDH activity = 8.1 U/g of protein). To further reduce the inner membrane contamination of this outer membrane-enriched preparation, the membranes were extracted with Triton X-100 or X-114. Both of these preparations exhibited reduced SDH activity, with the Triton X-114 sample showing the greatest reduction (SDH activity for the Triton X-100 extract = 4.6 U/g of protein; SDH activity for the Triton X-114 extract = 2.1 U/g of protein). Sarcosine-prepared outer membranes contained the highest level of SDH activity (5.4 U/g of protein).

SDS-PAGE comparison of the protein compositions of the outer membranes isolated by these different isolation procedures showed that there were 8 major polypeptide species, with at least 6 to 10 minor polypeptides (Fig. 4). Triton X-100- and X-114-extracted outer membrane preparations exhibited few differences between their protein profiles, the most notable being the loss of a minor 40-kDa polypeptide in the Triton X-114 extract (Fig. 4). More pronounced differences were noted between the Triton-extracted outer membranes and those prepared by the sarcosine differential solubilization method. There was a difference in the number of minor polypeptides, and the relative abundance of many of the major proteins also varied, especially among a group of proteins with apparent molecular masses of approximately 50 kDa and a 19-kDa polypeptide (Fig. 4). Polypeptides corresponding to the 90-, 60-, 51-, 50-, 48-, and 31-kDa antigens were readily apparent in the outer membrane protein profiles of *H. pylori* NCTC 11637 (Fig. 3). A major 19-kDa polypeptide was also present in the outer membrane preparations. Although this protein was immunogenic in mice (9), no MAbs were isolated by use of the selection protocol employed here.

To determine whether the outer membrane fraction of *H. pylori* NCTC 11637 was unusual in the extent of inner membrane contamination obtained during sucrose density gradient fractionation of total cell membranes, the envelopes of three other strains of *H. pylori* were prepared. Envelopes of strains of *H. mustelae*, *H. felis*, *C. coli*, *C. rectus*, and *E. coli* were also subjected to sucrose density gradient centrifugation, and the outer membrane fractions were isolated. The outer membrane fractions of all *Helicobacter* and *Campylobacter* strains isolated by this procedure exhibited SDH activity (range of 1.56 to 5.79 U/g of protein), consistent with inner membrane contamination. This was not the case with *E. coli*, for which no SDH activity could be measured in the outer membrane fraction isolated by sucrose density gradient centrifugation.

A comparison of the *H. pylori* preparations with respect to their SDS-PAGE outer membrane protein profiles revealed two groups of profiles, one with the MAb-reactive 50-kDa protein migrating at 50 kDa (strains NCTC 11637 and 5155; Fig. 5, lanes 1 and 2) and one with this antigen migrating at 55 kDa (strains 5294 and 5790; Fig. 5, lanes 3 and 4). *H. pylori* 5294 and 5790 also had a major protein species migrating with an apparent molecular mass of 69 kDa as assessed by SDS-PAGE. Despite having MAb-reactive surface antigens similar in mass to those of NCTC 11637, strain 5155 also possessed a major 40-kDa protein and a 25-kDa protein doublet. *H. mustelae* and *H. felis* possessed SDS-PAGE protein profiles similar to that of *H. pylori* NCTC 11637 (Fig. 5, lanes 5 and 6). The outer membrane protein profiles of *H. pylori* differed significantly from those of *Campylobacter* spp. and *E. coli*. Indeed, the outer membrane profile of *C. coli* is extremely simple (Fig. 6, lane 7) compared with those of the other bacteria examined and is dominated by the porin complex. By use of the method of Rosenbusch (45), no peptidoglycan-associated proteins were found in *H. pylori* (Fig. 6, lanes 1 to 6).

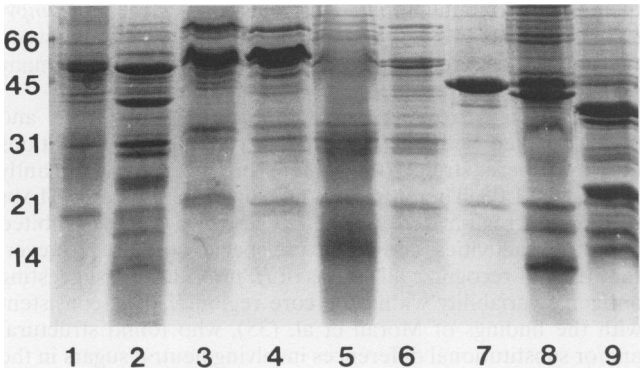

FIG. 5. SDS-PAGE (15%) of outer membrane preparations of various strains of *H. pylori* (strain NCTC 11637 [lane 1], strain 5155 [lane 2], strain 5294 [lane 3], and strain 5790 [lane 4]), *H. mustelae* Hm180 (lane 5), *H. felis* Hf1 (lane 6), *C. coli* 167B (lane 7), *C. rectus* 33238 (lane 8), and *E. coli* JM109 (lane 9). Sizes of molecular mass markers (in kilodaltons) are given on the left.

This is in clear contrast to the situation in *C. coli*, *C. rectus*, and *E. coli*, in which one or more protein species were peptidoglycan associated when examined by this procedure (Fig. 6, lanes 7 to 9).

DISCUSSION

The strategy of raising a panel of cell surface-binding MAbs has allowed the identification of six *H. pylori* outer membrane proteins which have surface-exposed domains. Five of these surface-exposed outer membrane proteins appear not to have been described previously. Although the immunizing antigen was enriched for outer membrane components and a total of 137 antibody-producing clones were screened in detail, only one of the outer membrane proteins, a 48-kDa species, appeared to be a strong immunogen. In addition to the three MAbs described in Results which react with surface-exposed epitopes of this 48-kDa protein (MAbs 8D1, 7D1, and 6A8), an additional 24 MAbs which were reactive with this protein were isolated (9). Nine of these additional MAbs recognized epitopes which were either poorly surface exposed or not surface exposed. This panel of MAbs will allow the topography of this membrane protein to be characterized once the protein sequence is determined.

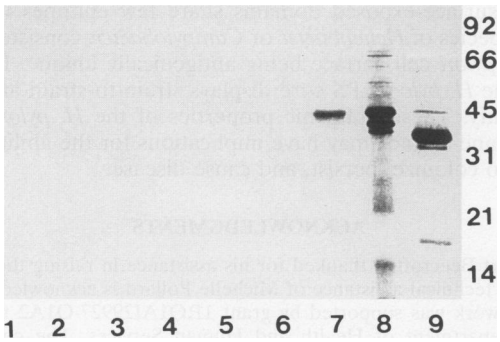

FIG. 6. Assessment of peptidoglycan-associated proteins of various strains of *H. pylori* (strain NCTC 11637 [lane 1], strain 5155 [lane 2], strain 5294 [lane 3], and strain 5790 [lane 4]), *H. mustelae* Hm180 (lane 5), *H. felis* Hf1 (lane 6), *C. coli* 167B (lane 7), *C. rectus* 33238 (lane 8), and *E. coli* JM109 (lane 9). Sizes of molecular mass markers (in kilodaltons) are given on the right.

Of the other MAbs raised in the study, only nine recognized surface-exposed epitopes on the other five proteins. This suggests that the surface-exposed regions of these five other *H. pylori* outer membrane proteins are poor immunogens, a property which could contribute to the apparent ability of the organism to persist in the stomach for years notwithstanding an apparently active and specific antibody response. In contrast to the case with these membrane proteins, and despite the fact that the immunizing antigen contained minimal urease contamination, a total of 30 MAbs were raised against urease, including 16 antibodies to the 66-kDa subunit and 14 to the 30-kDa subunit. This is a clear illustration of the strong immunogenicity of *H. pylori* urease. No MAbs to the putative fibrillar *N*-acetylneuraminyllactose-binding fibrillar hemagglutinin with a subunit M_r of 20,000 (HpaA) (13, 14) or to the putative 27-kDa *N*-acetylneuraminic acid (α-2-3)-sensitive hemagglutinin adhesin (20, 21) were isolated.

Perhaps the most important class of proteins in outer membranes is porins (38). These proteins form trans-outer membrane, water-filled channels, which permit the passage of small hydrophilic molecules with sizes below a given cutoff size. This ability to exclude molecules larger than a given size contributes sieving properties to the outer membrane. One characteristic of porin proteins is that during SDS-PAGE analysis, the protein exhibits a different apparent M_r depending upon the temperature of solubilization (45). This property is known as heat modification, and the immunodominant 48,000-M_r outer membrane species of *H. pylori* NCTC 11637 exhibited this behavior. Indeed, electrical conductance studies of this protein reconstituted into planar lipid bilayers have confirmed that the protein has the ability to produce channels (15). The 51-kDa species, which was present in higher copy number than the 48-kDa protein, was also heat modifiable, suggesting that it too is a porin. Both of these porin proteins displayed strain-to-strain antigenic variability in their surface-exposed epitopes. Despite the fact that heat-modifiable proteins with similar molecular masses occur in other strains of *H. pylori* and other *Helicobacter* species, MAb 1H5, which was specific for the 51-kDa porin of *H. pylori* NCTC 11637, failed to recognize any such protein in other helicobacters tested. Similarly, in the case of the 48-kDa porin, MAb 8D1 was strain specific, while MAbs 7D1 and 6A8 recognized the equivalent protein in only one other strain of *H. pylori* (a different strain in each case).

In addition to these 48- and 51-kDa porins, the *H. pylori* outer membrane appears to possess a third species of porin, the conserved 31-kDa protein. Along with the 50-kDa protein, this 31-kDa protein is one of the predominant surface-exposed proteins in the outer membrane of *H. pylori* and likely corresponds to the 30-kDa *H. pylori* protein recently reported by Tufano et al. (48). These workers showed that this 30-kDa protein formed trimers in its native state and on the basis of this property described their protein as a porin. MAbs to the 31-kDa protein of *H. pylori* NCTC 11637 weakly recognize antigens with apparent masses of 50 and 90 kDa in unheated membrane preparations, as well as the 31-kDa form (9). Although this antigenic reactivity could be due to the recognition of other protein antigens with higher masses, a more likely explanation is that the higher-mass antigens recognized by MAbs 1G1, 4B9, and 6H2 represent dimeric and trimeric forms of the lower-mass antigen. Another finding consistent with the 31-kDa species being a porin is that two of the three MAbs to the *H. pylori* NCTC 11637 protein that we isolated also reacted with a 50-kDa–54-kDa doublet present in the

outer membrane of *C. coli*. These *C. coli* proteins are heat modifiable (31) and constitute the porin complex of this species (23). This is the only outer membrane protein of *Helicobacter* and *Campylobacter* spp. which has been shown to share cross-reactive epitopes, suggesting that the two proteins are related and have a common function. These findings could have significance in the pathogenesis of the bacterium in light of the immunobiological activities ascribed to the 30-kDa protein described by Tufano et al. (48), in which this protein can induce release of cytokines from human lymphocytes-monocytes and modify the functional activity of polymorphonuclear leukocytes.

The porin content of the *H. pylori* outer membrane differs in several other respects from the porins of the taxonomically related campylobacters. For example, the outer membrane of *C. rectus* has two porin species of 43 and 51 kDa (25). On the basis Coomassie blue staining of SDS-PAGE gels, the relative copy numbers of these *C. rectus* porins appear to be higher than that of any of the three putative *H. pylori* porins of our reference strain *H. pylori* NCTC 11637. Certainly the *H. pylori* porin content appears to be significantly lower than those of *C. coli* and *Campylobacter jejuni*, in which the porin complex is the predominant species of outer membrane protein (31, 32, 41, 42). Porins are also often strongly but noncovalently associated with the peptidoglycan underlying the outer membrane (17), such that the complex, while dissociated upon boiling, is stable in 2% SDS at 60°C (45). This is certainly true of the porins of *C. jejuni* (31) and *C. coli* and *C. rectus* (Fig. 6). However, under conditions which show the peptidoglycan association of the porins of these and other species, the porins of *H. pylori* and other helicobacters display no association with peptidoglycan. Indeed, with 2% SDS at 60°C, no single *H. pylori* protein appeared to be peptidoglycan associated.

The other three *H. pylori* outer membrane proteins with surface-exposed domains described here have no readily ascribable function. The epitopes of both the 60- and 90-kDa proteins were strain specific, whereas the 50-kDa protein was present on all strains of *H. pylori* examined. The conservation of this 50-kDa protein may reflect an important role for the protein in outer membrane structure and/or function. SDS-PAGE analysis suggested that the outer membrane of *H. pylori* contained a number of other additional protein species to which MAbs were not isolated by our selection protocol. This could be due to their being poorly immunogenic. However, it should be noted that the polyclonal response of the hyperimmunized mice used to produce the MAbs recognized many other outer membrane antigens to which no MAbs were isolated. It is likely that these other proteins are not surface localized and that MAbs were not isolated because of the bias of the screening protocol towards the identification of surface-accessible MAbs.

Despite the differential solubilization procedures employed, some of the proteins present in the various outer membrane fractions were likely of inner membrane origin or represent proteins shared between the inner and outer membranes. For example, other studies have identified a 20-kDa lipoprotein which cofractionates with both the outer and inner membrane fractions of *H. pylori* (27). Recently, Poquet et al. (44) have proposed that certain lipoproteins may actually be superficially enriched in a distinct domain of the cell envelope that contains material from both the inner and outer membranes. Such domains could explain the difficulties encountered in the physical separation of the two *H. pylori* membranes. Indeed, some SDH activity was present in all outer membrane preparations obtained from all strains of *Helicobacter* and *Campylobacter* tested, indicating a greater or lesser degree of inner membrane contamination. This suggests that like *Campylobacter* species (42), *H. pylori* has a tight interaction between the inner and outer membranes, possibly mediated by many junction sites.

Two strongly reactive surface-directed MAbs (6A5 and 6C11) reacted with the LPS. Under the growth conditions employed here, strain NCTC 11637 produced predominantly rough-type LPS. Therefore, it is not surprising that only MAbs to the core region were isolated. These two MAbs exhibited identical reactivities, and both were species specific. However, they did not recognize all strains of *H. pylori* tested, suggesting antigenic variability within the core region. This is consistent with the findings of Moran et al. (35), who found structural and/or substitutional differences involving neutral sugars in the core compositions of the rough LPSs of various strains of *H. pylori*. Both anti-LPS MAbs reacted strongly with the flagellar sheath, confirming the LPS content of this structure. However, the lack of reactivity of other outer membrane-directed MAbs with the sheath suggests that the flagellar sheath is not just a simple extension of the outer membrane. There are considerable problems involved in determining the composition of a flagellar sheath because of the technical difficulties inherent in the physical separation of sheath from outer membrane fragments which are released from the cell surface during the shearing used to remove flagella from cells. Recently, the polypeptide content of crude sheath preparations of *H. pylori* has been reported (16). The authors could find no major protein bands characteristic of the sheath, and they reported that nearly all protein bands in the mass range of 200 to 30 kDa were present in both the sarcosine-insoluble outer membrane fraction and their sheath preparations, but in different amounts. Differences were observed in the 30- to 14-kDa range, where 22- and 21-kDa protein bands were enriched in the sarcosine-insoluble outer membrane fraction. The data presented here suggest that although the sheath has a typical bilayer structure (16), it is profoundly different from the outer membrane with respect to surface-accessible polypeptides, including proteins with apparent molecular masses greater than 30 kDa. For example, the sheath does not contain levels of the 48-kDa porin that can be detected by immunogold electron microscopic examination, suggesting that the flagellar sheath would have reduced permeability compared with the outer membrane.

In summary, this study has shown that the outer membrane of *H. pylori* NCTC 11637 contains eight major polypeptide species, at least three of which appear to be porins. Six of the outer membrane proteins have surface-exposed domains. These surface-exposed domains share few epitopes with related species of *Helicobacter* or *Campylobacter*, consistent with the *H. pylori* cell surface being antigenically unique. In addition, the *H. pylori* LPS core displays strain-to-strain antigenic variability. These antigenic properties of the *H. pylori* outer membrane surface may have implications for the ability of *H. pylori* to colonize, persist, and cause disease.

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