Identification of Surface Proteins of *Helicobacter pylori* by Selective Biotinylation, Affinity Purification, and Two-dimensional Gel Electrophoresis*

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**Helicobacter pylori** is a widespread human pathogen that can cause gastric ulcers and cancer. To identify surface proteins that may play a role in pathogen-host interactions and represent potential targets for the control of this infection, we selectively biotinylated intact *H. pylori* with the hydrophilic reagent sulfosuccinimidyl-6-(biotinamido)-hexanoate and purified the labeled proteins by membrane isolation, solubilization, and affinity chromatography. After separation of 82 biotinylated proteins on two-dimensional gels, 18 were identified with comparison to proteome data and peptide mass fingerprinting. Among the identified proteins, 9 have previously been shown to be surface-exposed, 7 are associated with virulence, and 11 are highly immunogenic in infected patients. In conclusion, this generally applicable combined proteome approach facilitates the rapid identification of promising targets for the control of *H. pylori* and might be applicable to numerous other human pathogens although larger biotinylation reagents might be required in some cases to prevent permeation of porin channels in the outer membrane.

**Helicobacter pylori** is a micro-aerophilic, Gram-negative bacterium that colonizes in the stomach of ~50% of the world human population (1, 2). Infection results in chronic inflammation of the gastric mucosa, which in most cases remains asymptomatic. However, in 10% of those infected severe pathological consequences develop including gastric and duodenal ulcer, atrophic gastritis, adenocarcinoma, or mucosa-associated lymphoid tissue (MALT) lymphoma.

The surface of *H. pylori* provides an important interface for pathogen-host interactions including flagella-driven motility in the stomach mucus layer, local buffering of stomach acid by lymphoid tissue (MALT) lymphoma, and the ability to adhere to host cells. Host immune responses and to drug therapy and thus could be used to control this important human pathogen. Because of its outstanding importance, several approaches have been developed to identify and characterize additional *H. pylori* envelope proteins.

Global theoretical predictions of outer membrane proteins based on the two completely sequenced genomes and physicochemical properties and sequence similarities to proteins with known surface localization have yielded 64 putative candidates (12). Several of these have been experimentally verified, but it is unclear if all of the predicted surface proteins are expressed. Moreover, several proposed surface proteins including urease, Hsp60, Hsp70, flagellar sheath protein, and catalase do not share common properties of surface proteins and are therefore neglected by theoretical predictions.

Surface proteins including outer membrane proteins can be experimentally identified by selective solubilization and sucrose gradient centrifugation for enrichment of outer membrane fractions. In *H. pylori*, these techniques yielded enriched outer membrane fractions, and many putative surface proteins have been found. However, these preparations are heavily contaminated by inner membrane components (13, 14), so that independent evidence, such as specific antibody binding to intact bacteria, is necessary to verify true surface proteins (15). Five outer membrane proteins with the typical β-barrel structure were identified by their anomalous temperature-dependent migration in polyacrylamide gels (14), but surface proteins with different structures escaped this method.

Selective labeling with reactive reagents is an alternative approach to identify surface proteins. In *H. pylori* urease could be labeled with 125I demonstrating its surface localization (16). Labeling with a reactive sugar ligand confirmed the specific binding of the Lewis antigen to the surface-exposed *H. pylori* adhesin BabA (3). The hydrophilic reagent S-NHS-LC-biotin (sulfosuccinimidyl-6-(biotinamido)-hexanoate) allowed us to selectively biotinylate surface proteins of *Escherichia coli* although to a lesser extent periplasmic proteins were also labeled (17). Here, we confirmed the periplasmic labeling for the well characterized MalE protein of *E. coli* and showed that lysis followed by membrane purification removes all of the labeled MalE protein. We then applied this technique to identify surface proteins of *H. pylori*. Biotinylated membrane proteins of *H. pylori* were further enriched by avidin-affinity chromatography, separated by two-dimensional gel electrophoresis, and identified by peptide mass fingerprinting. This generally applicable approach led to the rapid experimental identification of...
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both new and previously known H. pylori envelope and putative surface proteins.

**EXPERIMENTAL PROCEDURES**

*E. coli* HB2151 was grown in M9 minimal medium supplemented with 0.2% maltose, to induce the mal operon (18), 0.1% hydroxylated casein, and 1 μM biotin to block active uptake of the biotinylation reagent. The cells were harvested at an OD600 nm of 0.6–0.7, corresponding to the mid-exponential growth phase.

*H. pylori* strain 26695 (19) was cultured in a saturated α-cyanocarboxylic acid ammonium at 37 °C in a micro-aerobic atmosphere (5% O2, 85% N2, and 10% CO2) on serum-agar plates (20) for 3 days, and grown for 1 additional day on fresh plates. The bacteria were harvested and suspended in ice-cold phosphate-buffered saline, 1 mM CaCl2, 0.5 mM MgCl2, 1.6 μM biotin at an OD600 nm of 2.5–3.5 (equivalent to 1–2 × 109 colony-forming units). The bacteria were surface-labeled by incubation with 200 μM (final concentration) S-NHS-LC-biotin (Pierce) for 20 min on ice. The reaction was stopped by adding two volumes of TNKM (50 mM Tris, pH 7.4, 100 mM NaCl, 27 mM KCl, 1 mM CaCl2, 0.5 mM MgCl2). After 10 min of incubation at room temperature, the bacteria were sedimented by centrifugation at 3,500 × g for 10 min and washed three times with TNKM.

The viability of the bacteria before and after labeling was determined by plating on serum agar and by flow cytometry as well as fluorescence microscopy using a membrane-permeable (Syto9) and a membrane-impermeable (propidium iodide) fluorophore according to the manufacturer’s instructions (LIVE/DEAD kit, Molecular Probes) except that an altered ratio in millimolar of 3:27 of the dyes Syto9/propidium iodide was used.

To isolate biotinylated membrane proteins, labeled bacteria were resuspended in 50 mM Tris-Cl, pH 7.4, 1 mM MgCl2, with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 1 μM leupeptin, 2.9 mM benzamidin) and disrupted by 4 passages through a French press at 15,000 lb/in2. After removal of intact bacteria by two centrifugations at 4,000 × g and 4 °C for 10 min, membranes were pelleted at 40,000 × g and 4 °C for 30 min and then washed, and resuspended in TKE (50 mM Tris-HCl, 150 mM KCl, 10 mM EDTA, proteinase inhibitors, see above). Membrane samples were adjusted to a concentration of 5 mg/ml, solubilized with 2% zwittergent 3-14 (Fluka), and incubated for 1 h at 4 °C with head-over-head mixing. Insoluble membranes were removed by ultra centrifugation for 1 h at 100,000 × g and 4 °C. The soluble fraction was purified by affinity chromatography on reversibly binding avidin-agarose according to the instructions of the manufacturer (Roche Molecular Biochemicals) with slight modifications. In brief, 20–24 mg of membrane proteins were diluted 10-fold in 100 mM NaPO4, 150 mM NaCl, pH 7.2 and mixed with 1 ml of avidin-agarose matrix equilibrated in washing buffer (100 mM NaPO4, 150 mM NaCl, pH 7.2, 0.2% zwittergent). After 30 min of incubation at room temperature, the matrix was washed five times with 2 ml of washing buffer. The biotinylated proteins were eluted by rising the avidin-agarose temperature to 55 °C for 15 min at 37 °C with washing buffer containing 5 mM T-biotin. Protein-containing fractions were pooled and concentrated by acetone precipitation.

One dimensional SDS-PAGE and blotting on polyvinylidene difluoride membranes were performed according to standard protocols. Biotinylated proteins were detected by plating, fluorescence microscopy, and detected incorporated biotin by neutravidin staining (data not shown). Almost all proteins of the labeled lysate were biotinylated suggesting that most *E. coli* proteins contain accessible free amino groups. In contrast, labeling of intact bacteria resulted in a small subset of biotinylated putative surface proteins. However, MalE, which is a well characterized periplasmic protein and can be detected with a commercial monoclonal antibody, was also heavily labeled.

We first tried to enhance the labeling selectivity by lowering the reagent concentration. A 5-fold lower amount (200 μM) compared with the original procedure still gave sufficiently strong biotinylation, but this did not prevent biotinylation of MalE. We then tried to increase selectivity by using a larger biotinylation reagent. As periplasmic labeling appears to be the result of permeation of the reagent through porin channels, enlargement of the reagent might prevent access to the periplasm resulting in higher selectivity. We increased the molecular weight by coupling sulfo-succinimidyl-6-[3-(2-pyridyldithio)-propionamido]hexanoate via its sulfhydryl group to (∗)-biotinyl-3-maleimidopropionamidyl-3,6-dioxoacetamide. The resulting biotinylation compound has a calculated molecular mass of 943 Da, which exceeds the exclusion limit of reported porin channels (< 600 Da, OmpF in *E. coli*, Refs. 22 and 23; < 800 Da, *Salmonella typhimurium*, Ref. 24). Indeed, this compound substantially reduced the relative biotinylation of MalE in comparison with other protein species, but the remaining MalE labeling was still not acceptable (data not shown). As the reagent was difficult to generate we discontinued its use.

As the various attempts to enhance selectivity were only partially successful, we decided to remove the contaminating periplasmic biotin-labeled proteins by cell lysis followed by membrane sedimentation. After this simple membrane enrichment, there remained no detectable MalE protein suggesting that the periplasmatic proteins were indeed efficiently removed (Fig. 1). Accordingly, the remaining biotinylated proteins most likely represent true surface proteins (see “Discussion”).

We then applied this modified procedure to *H. pylori*. In contrast to *E. coli*, this organism is especially prone to autolysis in vitro (25, 26), which could prevent interpretable results. To test if such unwanted lysis occurred during labeling, the viability of the bacteria was determined before and after labeling by plating, fluorescence microscopy, and flow cytometric analysis using a combination of membrane-permeable and non-permeable fluorescent dyes (LIVE/DEAD staining). Plating indicated that less than 10% of the bacteria were killed during incubation and washing. Moreover, only 2–5% of the bacteria had a compromised membrane integrity after labeling as determined by LIVE/DEAD staining. The small portion of killed
bacteria might have released some cytosolic material but most of this would be removed during subsequent washing steps. Therefore cytosolic components are not likely to contaminate the labeled proteins.

In \textit{H. pylori}, unlabeled samples contained only one weakly avidin-binding spot (apparent molecular mass of about 20 kDa, pI \(\approx 9.0\); data not shown) as shown by two-dimensional electrophoresis, blotting, and avidin-peroxidase conjugate staining. When \textit{H. pylori} lysates were labeled, almost all proteins that could be detected by staining with Coomassie Blue were also biotinylated, indicating that almost all proteins possess accessible amino groups (Fig. 2). Among the very few proteins that did not bind avidin, there were the previously identified citrate synthase and NapA (21). In addition, Hsp60 and the urease \(\beta\)-subunit were present in high amounts but gave comparatively weak signals in the neutravidin-peroxidase staining compared with other less abundant spots. This is surprising because Hsp60, NapA, and urease \(\beta\)-subunit contain many lysine residues. Possibly, these lysines are not readily accessible to the biotinylation reagent.

In contrast to the almost complete labeling of lysate proteins, labeled intact \textit{H. pylori} contained only 102 spots that were reproducibly biotinylated and resolved in three independent experiments (Fig. 3). Several species appeared as horizontal spot series instead of single spots, and such spot series might represent different modifications of a single protein species (see below). Taking this into account, 34 distinct protein species were resolved. Most species had isoelectric points in the alkaline range. A comparison of labeled intact cells versus labeled lysates (Figs. 2 and 3) revealed a high selectivity of biotinylation as expected for a hydrophilic reagent and intact membranes. In contrast, the uncharged sulphydryl-reactive reagent polyethylene oxide-iodoacetyl biotin labeled several known cytoplasmic proteins, indicating permeation of the bacterial membranes.

Based on our experience with labeled periplasmic proteins in \textit{E. coli} that can be effectively removed by membrane isolation (see above), we enriched putative biotinylated \textit{Helicobacter} surface proteins by lysis and membrane sedimentation. Surprisingly, in the case of \textit{Helicobacter} this did not result in a major change in the two-dimensional electrophoretic pattern of biotinylated proteins, and all spots were preserved suggesting that few if any periplasmic proteins had been labeled. One possible explanation could be that \textit{H. pylori} strain 26695 may lack large porin channels through which the biotinylation reagent can get access to the periplasm (see “Discussion”). Additional studies are required to clarify this issue.

To further enrich the biotinylated proteins, they were solubilized with zwittergent 3-14 and applied to a reversible biotin-binding avidin D column and washed and eluted with biotin. Two-dimensional electrophoresis of these purified labeled proteins revealed 162 spots corresponding to 59 different species (see above) as detected by silver staining (Fig. 4a) most of which (53 of 59, i.e. 90\%) were biotinylated (Fig. 4b). The remaining 6 species were considered minor contaminants of non-biotinylated proteins as indicated by their weak silver staining. Moreover, 76 additional spots corresponding to 29
different species appeared in the neutravidin-peroxidase staining but were not visualized by silver staining probably due to their low amount. Compared with whole biotinylated H. pylori, the overall avidin binding patterns of purified proteins were quite similar but more complex and difficult to assign because of longer spot series and more total detectable species (48 spots in addition). Possibly, these additional species escaped detection in non-enriched whole cell samples because of low abundance. In contrast, all but 2 of 34 biotinylated proteins from whole H. pylori could also be detected in the enriched protein fraction.

To tentatively identify the biotinylated proteins, we compared the avidin binding patterns with the H. pylori strain 26695 reference pattern in our data base (www.mpiib-berlin.mpg.de/2D-PAGE, Ref. 21). The relative positions of 18 biotinylated species matched with previously identified Helicobacter proteins (Table I). To confirm these tentative assignments, the 13 most abundant biotinylated species were cut out from the blots, digested, and identified by peptide mass fingerprinting. All of the direct identifications were consistent with the indirect assignments, which confirmed the validity of the indirect approach. For spots 6 and 10, sequence coverage was lower but all major mass peaks matched with HP1118 and HP1098, respectively. All 10 assignments and 2 additional proteins with weak sequence coverage (HP0231, HP0659) verified the previous identification based on comparison to the standard proteome pattern, suggesting the validity of this indirect approach.

Most unlabeled proteins of H. pylori appear as single spots in the two-dimensional pattern. In contrast, most labeled protein species appeared as horizontal spot series, which could represent the same protein with slight modifications that alter the pI. This was confirmed for three members of a biotinylated spot series corresponding to catalase (data not shown). Probably, a variable number of amino groups reacted with the biotinylation reagent resulting in a differential loss of protonable residues and stepwise decreasing pI values. The pI difference between consecutive members of spot series were largely constant and in the range of one protonable group per molecule (ΔpI of ~0.05), which is consistent with this hypothesis. Using a 25-fold greater amount of biotinylation reagent, many extensive spot series were obtained (data not shown). Moreover, acidic members of spot series were enriched during the avidin-affinity chromatography, which further enriches for highly biotinylated species.

FIG. 4. Two-dimensional blot of biotinylated membrane proteins purified from labeled intact H. pylori cells. A, silver staining; B, neutravidin staining. The original gel size was 23 × 30 cm.

**DISCUSSION**

Surface proteins of H. pylori mediate important pathogen-host interactions that are essential for colonization, adherence, survival, and virulence of this pathogen. To identify H. pylori surface proteins, several approaches have been used (see the Introduction). Using a global proteome approach, we combined a selective biotinylation of free amino groups with membrane isolation followed by affinity purification of solubilized proteins, two-dimensional electrophoresis, and peptide mass fingerprinting.

A prerequisite for this method is the presence of free amino groups that are exposed to the external medium. The genome sequences indicate that except for two predicted proteins all contain one or several lysine residues. Many of these residues

2 D. Frishman, personal communication.
seem to be on the outside of the corresponding protein structures, because almost all detectable protein species in *H. pylori* lysates can be labeled using the hydrophilic biotinylation reagent S-NHS-LC-biotin that reacts with free amino groups. In contrast, in intact bacteria only a subset of the proteome was labeled, indicating that the hydrophobic membranes limited the accessibility of most proteins for the highly hydrophilic biotinylation reagent. While the inner membrane is essentially impermeable for the reagent, previous studies (17), which we confirmed in this study, have shown that in *E. coli*, the outer membrane is partially permeable for S-NHS-LC-biotin, resulting in substantial labeling of periplasmic proteins. Outer membrane porins contain water-filled channels that permit the diffusion of hydrophilic molecules with molecular masses up to 800 Da (24, 27), which is well above the molecular mass of S-NHS-LC-biotin (560 Da). If porins are indeed the cause of periplasm access, enlargement of the biotinylation reagent above the exclusion limit could improve its selectivity for sur-

<table>
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<tr>
<th>Name</th>
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<th>Spot No.</th>
<th>Sequence coverage in %</th>
<th>Cellular localisation according to independent data</th>
<th>seroreactivity</th>
<th>putative role in virulence in <em>H. pylori</em> or other bacteria</th>
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\*a Sequence coverage of matching peptides in tryptic peptide mass fingerprints; c, identified by comparison with the standard proteome pattern.

\*b sur, surface; sec, secreted; per, periplasm; homologue, paralogue or orthologue in other organisms.

\*c Strongly recognized by sera from infected patients according to combined data from three studies (Refs. 41, 50, 51).

\*d nd, not determined.

\*e Footnote 3.
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face proteins. We synthesized a reagent with a calculated molecular mass of 943 Da, which showed increased but still not sufficient selectivity in a well defined E. coli model system. This result suggests that still larger reagents might be highly specific but because of the absence of commercially available suitable precursors we could not follow this approach. Instead, we observed that following the reaction with S-NHS-LC-biotin, unwanted periplasmic-labeled proteins could be completely removed by cell lysis with a simple membrane sedimentation.

When applied to H. pylori, this approach showed that only a few minor biotinylated spots were lost during membrane enrichment, suggesting that the outer membrane of H. pylori may possibly be less leaky for S-NHS-LC-biotin when compared with the outer membrane of E. coli. Indeed, single-channel conductance measurements have suggested smaller pore sizes for the H. pylori porins HopA, HopB, HopC, HopD (14), HopE (29), and HopV (30) compared with E. coli OmpF with a size exclusion limit of 600 Da. In contrast, Helicobacter HopX might have a larger pore, but this porin is apparently not expressed in detectable quantities (30).

The absence of large porin channels that permit permeation of the S-NHS-LC-biotin in H. pylori is also supported by the fact that the great majority of identified biotinylated proteins are likely to be true H. pylori surface proteins based on evidence obtained with independent methods (see below), which suggests that the labeling was indeed highly selective for surface proteins.

In total, 82 putative surface-exposed H. pylori protein species were found by selective labeling and membrane isolation, and 18 of these were identified. It is likely that H. pylori possesses some additional surface proteins that escaped labeling. Three prominent proteins (UreB, NapA, Hsp60) were only weakly labeled in lysates, indicating that their localization could not be assessed using this approach, although their surface localization has previously been demonstrated (31, 32, 55).

Among the 18 identified proteins, several have been already predicted to be surface-exposed in H. pylori based on results from various independent methods. Antibody staining indicated that urease A, catalase, and a homologue of HP0410, the flagellar sheath protein, are on the surface (25, 31, 33, 34). MsrA, the cell-binding factor 2, HP0231, HP1098, HP1350, Htra, carbonic anhydrase, and γ-glutamyltransferase have been found to be secreted into the extracellular medium (35, 36). The large overlap between surface-exposed, firmly bound proteins (this study) and previously characterized secreted proteins is consistent with the concept of re-adsorption of released proteins at the H. pylori surface in vitro (25, 26). HefA is homologous to ToIC, an outer membrane protein from E. coli (37, 38), while HP1564 is homologous to an outer membrane protein of Pasteurella multocida (39). In contrast, the ABC transporter of iron, CeuE, is likely to be localized in the periplasm based on data from E. coli (40). However, a subpopulation of this protein might still be surface-exposed like other classic periplasmic proteins that have been found in this and previous studies to be surface-exposed in H. pylori (Htra, γ-glutamyltransferase). In summary, the various evidence strongly supports that our methods almost exclusively identifies surface proteins, although independent evidence would still be needed for the several candidates that have not been previously characterized.

Interestingly, only 2 of 18 identified proteins (HP0605, HP1564) have been theoretically predicted to be surface proteins, and none of the hypothetical outer membrane proteins (HOPs) have yet been found. The strain HP26695 is known neither to express BabA nor several members of the HOP family (3, 5). HopC has been reported to be expressed in this strain (41) and might be among the yet unidentified labeled protein species.

Several surface proteins of H. pylori mediate important host-pathogen interactions. This is also the case for some of the 18 proteins that were identified in this study. Two of them have been previously described as essential virulence factors (urease, γ-glutamyltransferase). Moreover, the flagellar sheath protein is part of functional flagella that are also essential for virulence. Cag16 is a member of the Cag pathogenicity island that is known to enhance inflammatory responses to H. pylori. Cag16 is essential for the expression of the VirB7 homologue HP0532, which is supposedly a structural component of the type IV secretion system (42, 43). The HP1098 paralogue HcpA induces IL-12-dependent IFN-γ secretion in CD4+/-NK- cells (44). For other human pathogens, homologues of catalase (e.g. in Legionella, Ref. 45, Mycobacterium, Ref. 46, or Campylobacter, Ref. 47) and the protease HtrA (in Salmonella, Brucella, and Yersinia, Ref. 48) are important for virulence. Indeed all fresh human isolates of H. pylori express catalase, which is apparently essential for survival of the phagocyte oxidative burst in cell culture models (49). Information about a potential role for HtrA in H. pylori virulence is lacking. It would be interesting to functionally characterize HtrA and, in particular, the additional surface proteins with no known homologues in other organisms.

Surface proteins of H. pylori are particularly exposed to the host immune system and therefore might represent major antigens. Indeed, 11 of 18 identified proteins are among the previously described 32 antigens that are recognized by a majority of sera from infected humans (41, 50, 51). This indicates that our identification procedure strongly enriched for highly immunogenic antigens (11 antigens of 18 surface proteins versus 32 antigens of 1560 total proteins). This set of proteins therefore represents a basis to rationally select antigen candidates for vaccine development. Indeed, 2 of 18 identified proteins (catalase, urease) have previously been shown to induce protective immunity against a H. pylori challenge infection in the mouse model. We have recently shown that HP0231 and HP0410 are also highly protective in the mouse Helicobacter infection model. Additional candidate antigens are currently being tested.

In conclusion, a rapid proteome approach has been developed to identify surface proteins of H. pylori that are promising targets for the control of this important human pathogen. When larger biotinylation reagents become available, this approach should be generally applicable to characterize the surface of pathogens to identify new potential target proteins for drug therapy and vaccine development.

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