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

Nicolas Sabarth, Stefanie Lamer, Ursula Zimny-Arndt, Peter R. Jungblut, Thomas F. Meyer‡, and Dirk Bumann

From the Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie, Schumannstraße 21/22, D-10117 Berlin, Germany

***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 urease, adhesion to gastric epithelial cells mediated by several adhesins including BabA (3), AlpA/AlpB (4), HopZ (5), translocation of CagA into host cells *via* the type IV secretion apparatus encoded in the Cag[1] (cytotoxin-associated genes) pathogenicity island (6–10), and adherence of neutrophils to endothelial cells induced by the neutrophil-activating protein (NAP) (11). Envelope proteins are particularly accessible to 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 ^{125}I demonstrating its surface localization (16). Labeling with a reactive sugar ligand confirmed the specific binding of the Lewis^b 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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[1] The abbreviations used are: Cag, cytotoxin-associated genes; S-NHS-LC-biotin, sulfosuccinimidyl-6-(biotinamido)hexanoate; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; IEF, isoelectric focusing; HOP, hypothetical outer membrane protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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% hydrolysated casein, and 1 μM biotin to block active uptake of the biotinylation reagent. The cells were harvested at an $OD_{600\,nm}$ of 0.6–0.7, corresponding to the mid-exponential growth phase.

H. pylori strain 26695 (19) was cultured at 37 °C in a micro-aerobic atmosphere (5% O_2, 85% N_2, and 10% CO_2) 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 $CaCl_2$, 0.5 mM $MgCl_2$, 1.6 mM biotin at an $OD_{600\,nm}$ of 2.5–3.5 (equivalent to 1–2 $\times$ 10^9 colony-forming units). The bacteria were surface-labeled by incubation with 200 μM (final concentration) S-NHS-LC-biotin (Pierce) for 30 min on ice. The reaction was stopped by adding two volumes of TNKCM (50 mM Tris, pH 7.4, 100 mM NaCl, 27 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$). After 10 min of incubation at room temperature, the bacteria were sedimented by centrifugation at 3,500 $\times$ *g* for 10 min and washed three times with TNKCM.

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-HCl, pH 7.4, 1 mM $MgCl_2$ 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 $\times$ *g* and 4 °C for 10 min, membranes were pelleted at 40,000 $\times$ *g* and 4 °C for 30 min and then washed, and resuspended in TKE (50 mM Tris-HCl, 150 mM KCl, 10 mM EDTA, protease inhibitors, pH 7.4). Membranes were adjusted to a protein concentration of 5 mg/ml, solubilized with 2% zwitterion 3-14 (Fluka), and incubated for 1 h at 4 °C with head-over-head mixing. Insoluble membranes were removed by ultracentrifugation for 1 h at 100,000 $\times$ *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 $NaPO_4$, 150 mM NaCl, pH 7.2 and mixed with 1 ml of avidin-agarose matrix equilibrated in washing buffer (100 mM $NaPO_4$, 150 mM NaCl, pH 7.2, 0.2% zwitterient). 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 five times for 15 min at 37 °C with washing buffer containing 5 mM D-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 on the blots using neutravidin-peroxidase staining (Pierce) and chemiluminescent visualization (ECL, Amersham Biosciences) according to the manufacturer's instructions. The maltose-binding protein MalE was detected with a monoclonal antibody (Sigma) and a polyclonal anti-mouse IgG-horseradish peroxidase conjugate (Nordic).

Two-dimensional gel electrophoresis and protein identification by MALDI-MS were performed as described previously (21). In brief, protein samples were solubilized for 30 min at ambient temperature in 9 M urea, 1% CHAPS, 70 mM dithiothreitol, 2% Servalyte pI 2–4 (Serva). For the resolution of protein samples a 23 $\times$ 30 cm two-dimensional gel electrophoresis system was used. For the identification of proteins 200–300 μg (total cell samples) or 50–70 μg (biotinylated proteins) were applied to the anodic side of the IEF gel. Proteins were identified by peptide mass fingerprinting after in-gel tryptic digestion of excised spots. The peptide mixture was mixed (1:1) with a saturated α-cyano-4-hydroxycinnamic acid solution in 50% acetonitrile, 0.3% trifluoroacetic acid, and 2 μl were applied to the sample template of a MALDI mass spectrometer (Voyager Elite, Perseptive). Peptide mass fingerprints were searched with the program MS-FIT (prospector.ucsf.edu/ucsfhtml/msfit.htm) using all *H. pylori* proteins in the NCBI data base allowing a mass accuracy of 100 ppm for the peptide masses. Partial enzymatic cleavages leaving two cleavage sites, oxidation of methionine, pyroglu-

tamic acid formation at the N-terminal glutamine, and modification of cysteine by acrylamide were considered in these searches.

RESULTS

Surface proteins may mediate important pathogen-host interactions in *H. pylori* infections. To identify novel *Helicobacter* surface proteins, we adapted a method that had been originally developed for *E. coli* (17). In this method, bacteria are treated with the hydrophilic biotinylation reagent S-NHS-LC-biotin that cannot permeate lipid bilayers. As a result of its limited access, this compound reacts preferentially with free amino groups of surface proteins. However, some of the reagent obviously enters the periplasm possibly through water-filled channels of *E. coli* outer membrane porins, resulting in substantial labeling of periplasmic proteins, which potentially confuses the localization of previously uncharacterized proteins.

To confirm these studies, we labeled *E. coli* lysate and intact cells, separated the samples by two-dimensional gel electrophoresis, 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-maleimidoproprionamidyl-3,6-dioxaoctane-diamine. 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
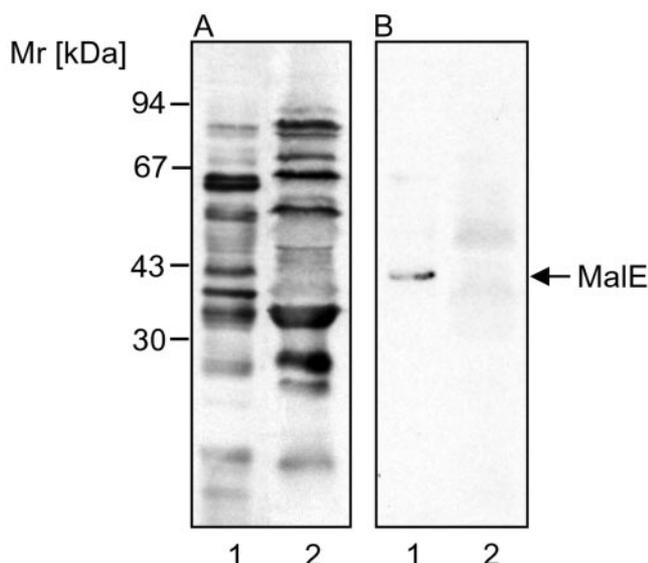
FIG. 1. **One-dimensional blot of biotinylated intact *E. coli* cells.** *A*, neutravidin staining. *B*, anti-MalE staining. *Lane 1*, complete *E. coli* cells; *lane 2*, membrane fraction. The *arrow* indicates the MalE protein.

FIG. 2. **Two-dimensional blot of biotinylated *H. pylori* lysates stained with neutravidin-coupled peroxidase.** In contrast to Figs. 3 and 4, a small gel (8 × 7 cm) was used for this control experiment.

FIG. 3. **Two-dimensional blot of biotinylated intact *H. pylori* cells stained with neutravidin-coupled peroxidase.** *Marked spots* were identified. Numbers correspond to the numbers in Table 1. The original gel size was 23 × 30 cm.

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 *H. pylori,* unlabeled samples contained only one weakly avidin-binding spot (apparent molecular mass of about 20 kDa, pI >9.0; data not shown) as shown by two-dimensional electrophoresis, blotting, and avidin-peroxidase conjugate staining. When *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 β-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 β-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 *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 sulfhydryl-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 *E. coli* that can be effectively removed by membrane isolation (see above), we enriched putative biotinylated *Helicobacter* surface proteins by lysis and membrane sedimentation. Surprisingly, in the case of *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
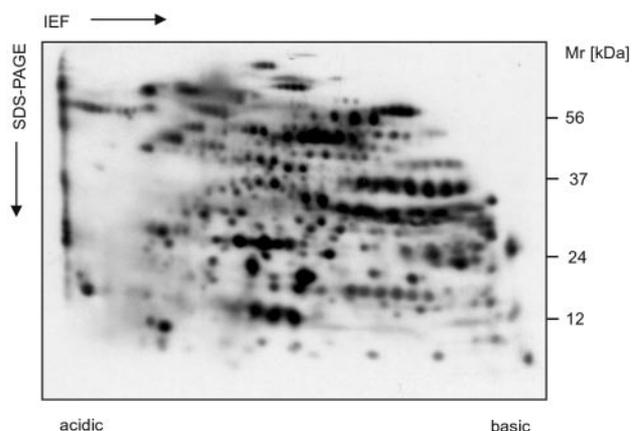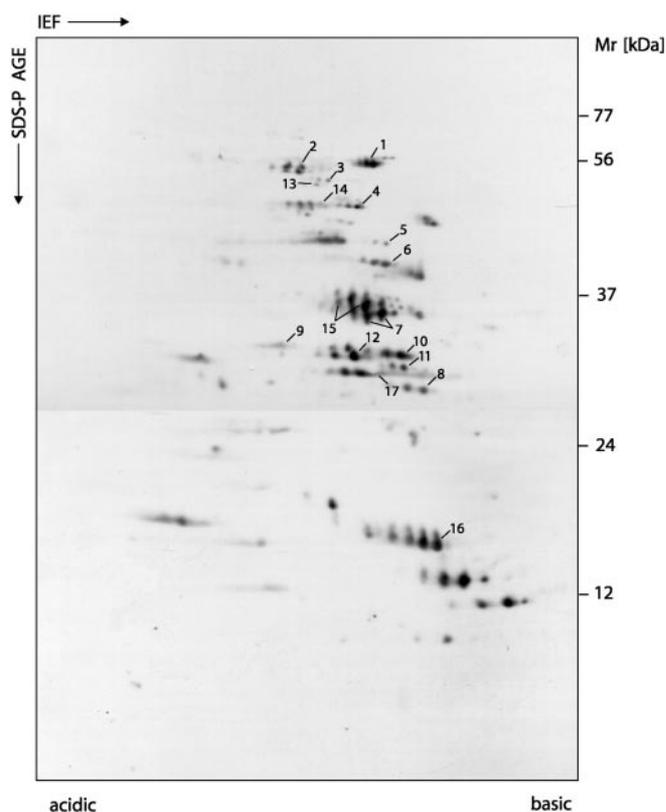
possible explanation could be that *H. pylori* strain 26695 may lack large porin channels through which the biotinylation reagent can get access to the periplasm (see "Discussion"). Additional studies are required to clarify this issue.

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. 4*a*) most of which (53 of 59, *i.e.* 90%) were biotinylated (Fig. 4*b*). 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
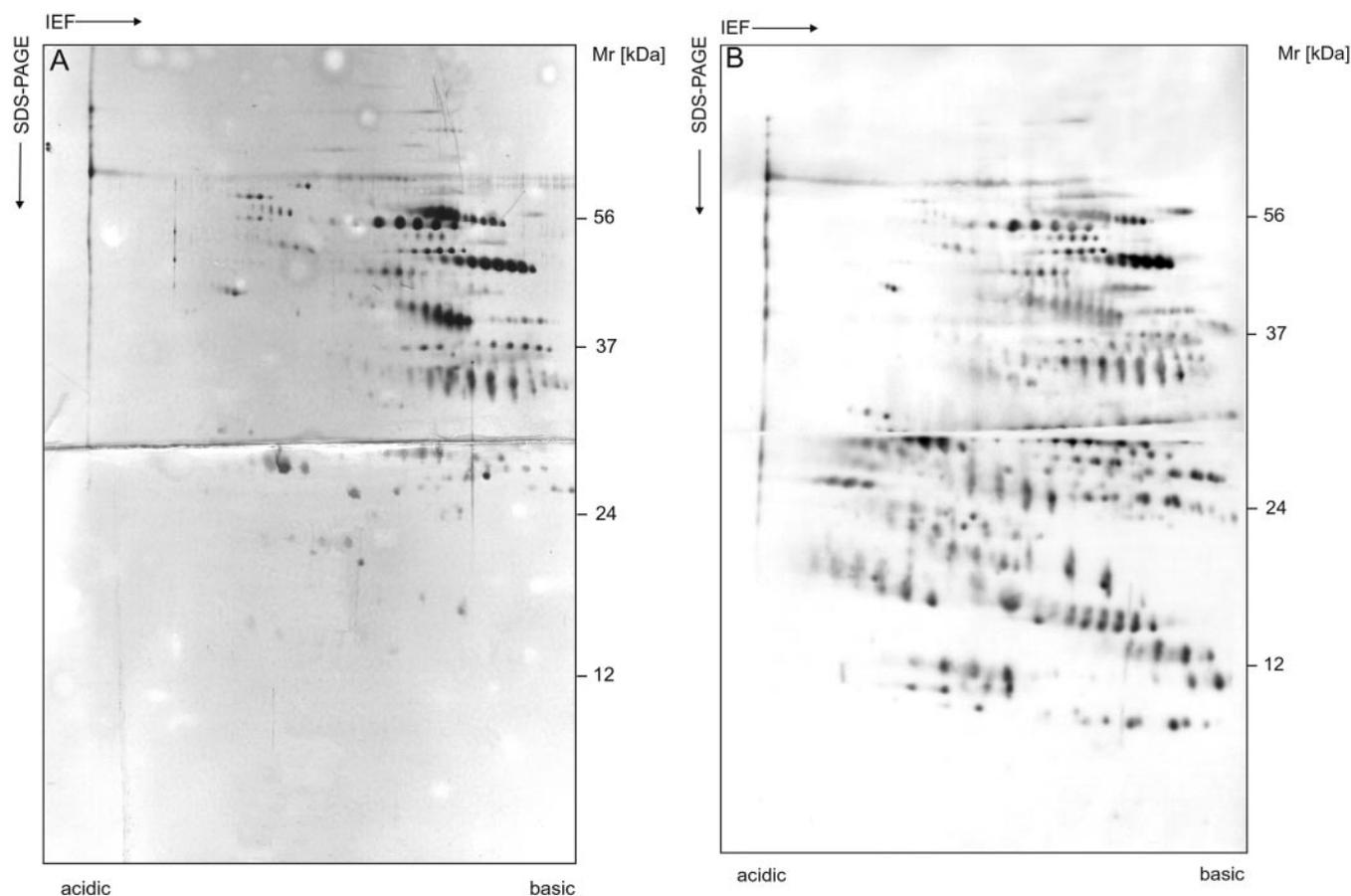
FIG. 4. **Two-dimensional blot of biotinylated membrane proteins purified from labeled intact *H. pylori* cells.** *A*, silver staining; *B*, neutravidin staining. The original gel size was 23×30 cm.

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 8 of 13 proteins, tryptic peptide masses that fit theoretical masses covered more than 30% of the corresponding protein sequence. This allowed confident identification. 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 $\sim$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.

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.[2] Many of these residues

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[2] D. Frishman, personal communication.

TABLE I
Surface-exposed proteins of H. pylori

Name	locus	Spot No.	Sequence coverage in %[a]	Cellular localistion according to independent data[b]	seroreactivity[c]	putative role in virulence in *H. pylori* or other bacteria	protective immunity in mice[d]
Catalase	HP0875	1	48, c	sur + cyto [53]	+	neutralizes reactive oxygen species [45;46;47]	+ [53]
Serine protease (HtrA)	HP1019	2	41, c	sec [36]	+	survival in mice and/or macrophage [48]	nd
Predicted coding region	HP0659	3	15, c	unknown	-	unknown	nd
Protease	HP1350	4	30, c	sec [36]	+	unknown	nd
Cag pathogenicity island protein (Cag16)	HP0537	5	29, c	unknown	+	essential for expression of VirB7 homologue HP0532 [43]	nd
γ-glutamyltranspeptidase (GGT)	HP1118	6	12, c	sec [36]	-	colonization factor [54]	nd
Cell binding factor 2	HP0175	7	33, c	sec [36]	+	unknown	nd
Conserved hypothetical secreted protein	HP1285	8	38, c	unknown	+	unknown	nd
Putative neuraminyllactose-binding hemagglutinin homologue	HP0410	9	67, c	paralogue: sur [33]	+		+ [e]
Conserved hypothetical secreted protein	HP1098	10	19, c	sec [36]	+	Hp1098 paralogue HcpA induces IFN-γ secretion [44]	nd
Urease alpha subunit	HP0073	11, 12	64, c	sur + cyto [55]	+	colonization factor [56]	+ [57]
Predicted coding region	HP0231	12	10, c	sec [36]	+	unknown	+ [e]
Putative outer membrane protein (HefA)	HP0605	13	c	homologue: sur [37;38]	-	unknown	nd
Peptide methionine sulfoxide reductase (MsrA)	HP0224	14	c	sec [35]	-	protects bacteria against oxidative damage from reactive nitrogen intermediates [28]	nd
Iron ABC transporter (CeuE)	HP1561/2	15	c	homologue: per [52]	-	unknown	nd
Predicted coding region	HP0721	16	c	unknown	-	unknown	nd
Outer membrane protein	HP1564	17	c	homologue: sur [39]	+	unknown	nd
Carbonic Anhydrase	HP1186	11	c	sec [36]	-	unknown	nd

[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.

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-

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-glutamyltranspeptidase 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 TolC, an outer membrane protein from *E. coli* (37, 38), while HP1564 is homologous to an outer membrane protein of *Pasteurella hemolytica* (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-glutamyltranspeptidase). 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-glutamyltranspeptidase). 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.[3] 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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Nicolas Sabarth, Stefanie Lamer, Ursula Zimny-Arndt, Peter R. Jungblut, Thomas F. Meyer and Dirk Bumann

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