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# Targeted Identification of Glycosylated Proteins in the Gastric Pathogen *Helicobacter pylori* (Hp)<sup>\*§</sup>

Kanokwan Champasa<sup>‡¶</sup>, Scott A. Longwell<sup>‡¶</sup>, Aimee M. Eldridge<sup>‡</sup>, Elizabeth A. Stemmler<sup>‡</sup>, and Danielle H. Dube<sup>‡§</sup>

Virulence of the gastric pathogen *Helicobacter pylori* (Hp) is directly linked to the pathogen's ability to glycosylate proteins; for example, Hp flagellin proteins are heavily glycosylated with the unusual nine-carbon sugar pseudaminic acid, and this modification is absolutely essential for Hp to synthesize functional flagella and colonize the host's stomach. Although Hp's glycans are linked to pathogenesis, Hp's glycome remains poorly understood; only the two flagellin glycoproteins have been firmly characterized in Hp. Evidence from our laboratory suggests that Hp synthesizes a large number of as-yet unidentified glycoproteins. Here we set out to discover Hp's glycoproteins by coupling glycan metabolic labeling with mass spectrometry analysis. An assessment of the subcellular distribution of azide-labeled proteins by Western blot analysis indicated that glycoproteins are present throughout Hp and may therefore serve diverse functions. To identify these species, Hp's azide-labeled glycoproteins were tagged via Staudinger ligation, enriched by tandem affinity chromatography, and analyzed by multidimensional protein identification technology. Direct comparison of enriched azide-labeled glycoproteins with a mock-enriched control by both SDS-PAGE and mass spectrometry-based analyses confirmed the selective enrichment of azide-labeled glycoproteins. We identified 125 candidate glycoproteins with diverse biological functions, including those linked with pathogenesis. Mass spectrometry analyses of enriched azide-labeled glycoproteins before and after cleavage of O-linked glycans revealed the presence of Staudinger ligation-glycan adducts in samples only after beta-elimination, confirming the synthesis of O-linked glycoproteins in Hp. Finally, the secreted colonization factors urease alpha and urease beta were biochemically validated as glycosylated proteins via Western blot analysis as well as by mass spectrometry analysis of cleaved glycan products. These data set the stage for the development of glycosylation-based therapeutic strategies, such as new vaccines based on

natively glycosylated Hp proteins, to eradicate Hp infection. Broadly, this report validates metabolic labeling as an effective and efficient approach for the identification of bacterial glycoproteins. *Molecular & Cellular Proteomics* 12: 10.1074/mcp.M113.029561, 2568–2586, 2013.

*Helicobacter pylori* (Hp)<sup>1</sup> infection poses a significant health risk to humans worldwide. The Gram-negative, pathogenic bacterium Hp colonizes the gastric tract of more than 50% of humans (1). Approximately 15% of infected individuals develop duodenal ulcers and 1% of infected individuals develop gastric cancer (2). Current treatment to clear infection requires “triple therapy” (3), a combination of multiple antibiotics that is often associated with negative side effects (4). Because of poor patient compliance and the evolution of antibiotic resistance, existing antibiotics are no longer effective at eradicating Hp infection (4). New treatment methods are needed to eliminate Hp from the human gastric tract.

Recent work has focused on gaining insights into the pathogenesis of Hp to aid the development of new treatments. The most recent findings in this area have conclusively revealed that glycosylation of proteins in Hp is required for pathogenesis. Hp use complex flagella, comprised of flagellin proteins, to navigate the host's gastric mucosa (5, 6). The flagellin proteins are heavily glycosylated with the unusual nine-carbon sugar pseudaminic acid, found exclusively in mucosal-associated pathogens (Hp (7), *Campylobacter jejuni*

<sup>1</sup> The abbreviations used are: Hp, *Helicobacter pylori*; MOE, metabolic oligosaccharide engineering; Ac<sub>4</sub>GlcNAz, peracetylated N-azidoacetylglucosamine; Ac<sub>4</sub>GlcNAc, peracetylated N-acetylglucosamine; GlcNAc, N-acetylglucosamine; mudPIT, multidimensional protein identification technology; PBS, phosphate buffered saline; ureA, urease alpha subunit; ureB, urease beta subunit; CAZy, carbohydrate active enzymes; Phos-FLAG, phosphine conjugated to a FLAG peptide (DYKDDDDK); Phos-FLAG-His<sub>6</sub>, phosphine conjugated to a FLAG-His<sub>6</sub> peptide (DYKDDDDKHHHHHH); TCEP, tris(2-carboxyethyl)phosphine; HBFA, heptafluorobutyric acid; FBS, fetal bovine serum; HPLC-Chip/Q-TOFMS, High Performance Liquid Chromatographic-Chip Quadrupole Time-of-Flight Mass Spectrometer; nanoESI, nano-electrospray ionization; HexNAz, N-azidoacetylhexosamine; HexNAc, N-acetylhexosamine; CID, collision-induced dissociation.

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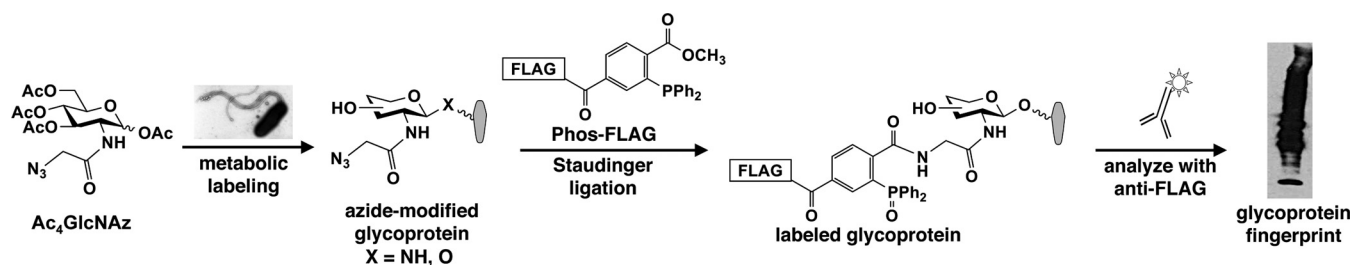


FIG. 1. **Metabolic oligosaccharide engineering facilitates labeling and detection of *Hp*'s glycoproteins.** Supplementation of *Hp* with  $Ac_4GlcNAz$  leads to metabolic labeling of *Hp*'s N-linked and O-linked glycoproteins with azides. Azide-modified glycoproteins covalently labeled with Phos-FLAG can be detected via Western blot analysis with anti-FLAG antibody to yield *Hp*'s glycoprotein fingerprint, which contains a large number of as-yet unidentified glycoproteins.

(8) and *Pseudomonas aeruginosa* (9)). This modification is absolutely essential for the formation of functional flagella on *Hp* (7, 10). Deletion of any one of the enzymes in the pseudaminic acid biosynthetic pathway results in *Hp* that lack flagella, are nonmotile, and are unable to colonize the host's stomach (7). Although pseudaminic acid is critical for *Hp* virulence, it is absent from humans (11, 12). Therefore, insights into *Hp*'s pathogenesis have revealed that *Hp*'s glycan pseudaminic acid is a bona fide target of therapeutic intervention. This is one of a number of examples linking protein glycosylation to virulence in medically significant bacterial pathogens (13, 14).

Despite these findings, *Hp*'s glycome remains poorly understood overall. Only the two flagellin glycoproteins have been firmly characterized in *Hp* (7) to date. Nine other candidate glycoproteins have been identified in *Hp*, but their glycosylation status has not been biochemically confirmed (15). The relative paucity of information regarding *Hp*'s glycoproteins is due in part to the previously held belief that protein glycosylation could not occur in bacteria (13, 16, 17). However, even after Szymanski (18, 19), Koomey (20), Guerry (21), Logan (7), Comstock and others (13, 16, 17) disproved this belief by firmly establishing the synthesis of glycoproteins in bacteria, the study of bacterial glycoproteins has presented unique challenges for analytical study (14, 22). For example, the unusual structures of bacterial glycans, which often contain amino- and deoxy-carbohydrates exclusively found in bacteria (12, 23–25), hampers their identification using existing tools. Though methods such as the use of glycan-binding reagents (20, 24, 26, 27) and periodic acid/hydrazide glycan labeling (15) have successfully detected glycoproteins in a range of bacteria, they present limitations. Glycan binding-based methods are often limited because of the unavailability of lectins or antibodies with binding specificity for glycosylated proteins in the bacteria of interest (14, 22). Periodic acid/hydrazide-based labeling is plagued by a lack of specificity for glycosylated proteins (15). Thus, an efficient and robust approach to discover *Hp*'s glycoproteins is needed.

In previous work, we established that the chemical technique known as metabolic oligosaccharide engineering (MOE), which was developed by Bertozzi (28, 29), Reutter (30),

and others for the study of mammalian glycoproteins, is a powerful approach to label and detect *Hp*'s glycoproteins (31). Briefly, *Hp* metabolically processes the unnatural, azide-containing sugar peracetylated *N*-azidoacetylglucosamine ( $Ac_4GlcNAz$ ) (32), an analog of the common metabolic precursor *N*-acetylglucosamine (GlcNAc), into cellular glycoproteins (Fig. 1). Elaboration of azide-labeled glycoproteins via Staudinger ligation (33) with a phosphine probe conjugated to a FLAG peptide (Phos-FLAG) (34) followed by visualization with an anti-FLAG antibody (Fig. 1) revealed a glycoprotein fingerprint containing a large number of as-yet unidentified *Hp* glycoproteins that merit further investigation (31).

Here we describe a glycoproteomic identification strategy for the selective detection, isolation, and discovery of *Hp*'s glycoproteins. In particular, we demonstrate that glycan metabolic labeling coupled with mass spectrometry analysis is an efficient and robust chemical approach to identify novel glycoproteins in *Hp*. This work characterizes glycosylated virulence factors in *Hp*, thus opening the door to new vaccination and antibiotic therapies to eradicate *Hp* infection. Broadly, this work validates metabolic oligosaccharide engineering as a complementary method to discover bacterial glycoproteins.

#### EXPERIMENTAL PROCEDURES

**Materials**—Protease inhibitor mixture, antibiotics, anti-FLAG antibodies and anti-FLAG agarose were purchased from Sigma (St. Louis, MO). Sequencing grade trypsin was from Promega (Madison, WI), and fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA). Nitrocellulose paper, Ni-NTA resin, zinc stain, and 4–16% Ready Gel Tris-HCl gels were from BioRad (Hercules, CA). GlcNAz,  $Ac_4GlcNAc$ ,  $Ac_4GlcNAz$ , Phos-FLAG (Phos-DYKDDDDK) and Phos-FLAG-His<sub>6</sub> (Phos-DYKDDDDKHHHHHH) were prepared as previously described (29). *Hp* strain 26695 was provided by Dr. Manuel Amieva (Stanford, CA). Azidoacetyl ethyl ester was a gift from Dr. Jennifer Prescher (Irvine, CA).

**Metabolic Labeling of *Hp***—*Hp* strain 26695 was grown on horse blood agar plates for 3–4 days in a microaerophilic environment (14% CO<sub>2</sub>) at 37 °C. The bacteria were then transferred to Brucella broth containing 10% FBS, 6 μg/ml vancomycin, and 1 mM peracetylated *N*-acetylglucosamine ( $Ac_4GlcNAc$ ) or  $Ac_4GlcNAz$ , as previously described (31). Alternatively, the bacteria were transferred to Brucella broth containing 10% FBS and 6 μg/ml vancomycin supplemented with 5 mM sodium azidoacetate or 5 mM azidoacetyl ethyl ester. Liquid

cultures were grown for 3–5 days in a microaerophilic environment with gentle rocking at 37 °C, then harvested as described below.

**Harvesting *Hp* to Probe for Total Cellular Azide-Labeled Glycoproteins**—*Hp* grown in the presence of Ac<sub>4</sub>GlcNAc, Ac<sub>4</sub>GlcNAz, sodium azidoacetate, or azidoacetyl ethyl ester were centrifuged at 3500 rpm using a Sorvall Legend RT<sup>+</sup> centrifuge (Thermo Scientific) and washed two times with PBS. The cells were resuspended in ice cold lysis buffer (20 mM Tris-HCl, pH 7.4; 1% Igepal; 150 mM NaCl; 1 mM EDTA) containing protease inhibitors (protease inhibitor mixture, Sigma) for 5 mins at room temperature. Protein concentration of the samples was measured using BioRad's D<sub>C</sub> protein concentration assay (BioRad) per manufacturer's instructions. All samples were standardized to 3.0 mg/ml. To probe for azide incorporation, the lysates were diluted 1:1 with 500 μM Phos-FLAG (34), reacted overnight at room temperature, and then analyzed via Western blot with anti-FLAG antibody (Sigma) or via SDS-PAGE with zinc stain (BioRad).

**Subcellular Fractionation of Glycoproteins**—*Hp* grown in the presence of Ac<sub>4</sub>GlcNAc or Ac<sub>4</sub>GlcNAz were harvested from Brucella broth by centrifugation at 6000 × *g*, and the conditioned medium was set aside to access secreted proteins. The harvested cells were fractionated as described by Hoshino *et al.* to obtain periplasmic, inner membrane-associated, and cytoplasmic protein fractions (35). Secreted proteins were isolated from the conditioned Brucella broth via TCA-precipitation as described by Bumann *et al.* (36). An acid-glycine extraction was performed as described previously to isolate *Hp*'s surface-associated proteins (37). An *N*-lauroylsarcosine extraction was performed as reported by Hopf *et al.* to access outer-membrane *Hp* proteins (15, 38). The protein concentration of subcellular fractions was standardized to 3.0 mg/ml using a BioRad D<sub>C</sub> Protein Assay (BioRad). Standardized samples were incubated 1:1 with 500 μM Phos-FLAG (34) overnight at room temperature to label azide-containing glycoproteins, and then analyzed by Western blot analysis with anti-FLAG antibody (Sigma) or via SDS-PAGE with zinc stain (BioRad). In addition, standardized periplasmic and cytoplasmic samples were assayed for malate dehydrogenase (35) activity alongside a positive control (malate dehydrogenase, Sigma) according to the manufacturer's (Sigma's) instructions to confirm the efficacy of subcellular fractionation.

**Preparation and Analysis of Enriched Azide-Labeled Glycoproteins**—*Hp* metabolically labeled with 1 mM Ac<sub>4</sub>GlcNAc or Ac<sub>4</sub>GlcNAz were centrifuged at 3000 × *g* and washed two times with phosphate buffered saline (PBS). The cells were lysed according to Koenigs *et al.* and centrifuged at 3000 × *g* for 10 min to remove insoluble debris (31). Following the protocol by Laughlin *et al.*, Phos-FLAG-His<sub>6</sub> was added as a solid directly to the lysate at a final concentration of 500 μM, and Staudinger ligation was run at room temperature for 24 h under argon (29, 39). The unreacted Phos-FLAG-His<sub>6</sub> was removed by Bio-Rad P-10 size-exclusion column according to the manufacturer's protocol. Flow-through fractions with A<sub>280</sub> > 0.050 were combined and concentrated using a centrifugal filter device with 30 kDa molecular weight cutoff (Millipore). FLAG-His<sub>6</sub>-tagged glycoproteins were enriched using 2 ml of α-FLAG agarose beads (Sigma) followed by 5 ml of nickel-nitrilotriacetic acid (Ni-NTA)-agarose resin (Bio-Rad), as previously described (29, 39). After rinsing away nonbinders, the bound glycoproteins were eluted from the nickel column with one column volume of a solution of 8 M urea, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, 10 mM Tris (pH 8.0), and 250 mM imidazole. Samples containing 15 μg of material from Ac<sub>4</sub>GlcNAc and Ac<sub>4</sub>GlcNAz-treated *Hp* both before purification (input) and after purification (eluent) were analyzed by SDS-PAGE to confirm successful enrichment. In addition, 150 μg of *Hp*'s enriched azide-labeled glycoproteins were suspended in 10 mM Tris (pH 8) and focused on 11 cm pH 3–10 nonlinear IPG strips (GE Healthcare) using GE Healthcare's Multiphor™ II system. These strips were reduced,

alkylated and loaded onto 8–18% polyacrylamide gels (GE Healthcare), which were run at 10 °C and stained with zinc stain (BioRad).

**Protein Identification by MudPIT**—An azide-labeled enriched glycoprotein sample containing 150 μg of protein and a mock enriched control (which was labeled with Ac<sub>4</sub>GlcNAc, reacted with Phos-FLAG-His<sub>6</sub>, and enriched via anti-FLAG and Ni-NTA affinity chromatography, as described above) were concentrated using a Microcon centrifugal filter device (Millipore) to ~10 μl, suspended in 8 M urea and 100 mM Tris-HCl (pH 8.5), reduced with 5 mM tris(2-carboxyethyl)phosphine (TCEP), and reacted with 10 mM iodoacetamide. The samples were diluted by a factor of four with 100 mM Tris-HCl (pH 8.5) and 1 mM CaCl<sub>2</sub>, and then digested with 0.25 μg of trypsin (Promega) overnight at 37 °C. The digests were mixed with formic acid to a final concentration of 5%, desalted using C<sub>18</sub> Spec tips (Varian), fully dried by speed vacuum at room temperature, and analyzed by multidimensional protein identification technology (mudPIT) (40). The Vincent J. Coates Proteomics/Mass Spectrometry Laboratory at University of California Berkeley performed mass spectrometry analyses of the enriched, azide-labeled sample and mock-enriched control sample. A nano LC column was packed in a 100 μm inner diameter glass capillary with an emitter tip. The column consisted of 10 cm of Polaris C18 5 μm packing material (Varian), followed by 4 cm of Partisphere 5 SCX (Whatman). The column was loaded by use of a pressure bomb and washed extensively with buffer A (see below). The column was then directly coupled to an electrospray ionization source mounted on a Thermo LTQ XL linear ion trap mass spectrometer. An Agilent 1200 HPLC equipped with a split line so as to deliver a flow rate of 30 nl/min was used for chromatography. Peptides were eluted using an 8-step MudPIT procedure (40). Buffer A was 5% acetonitrile/0.02% heptafluorobutyric acid (HBFA); buffer B was 80% acetonitrile/0.02% HBFA. Buffer C was 250 mM ammonium acetate/5% acetonitrile/0.02% HBFA; buffer D was same as buffer C, but with 500 mM ammonium acetate.

The programs SEQUEST, as embodied in BIOWORKS BROWSER (version 3.3.1 SP1), (41) and DTASELECT (version 1.9) (42) were used to identify peptides and proteins from a database consisting of all proteins (1560 total) from *Hp* 26695 plus a collection of 160 common contaminants (1720 protein entries in the database actually searched). The *Hp* database was the newest available from NCBI RefSeq on October 7, 2010. Search parameters were altered to DTA generation 600.00–4000.00 with an absolute threshold of 500. The search was restricted to tryptic peptides and allowed three missed cleavages. Carboxyamidomethylation of cysteines as a fixed modification and oxidation of methionines as a variable modification were specified. The mass tolerance for precursor ions was 1.4 and for fragment ions was 1.0. Individual peptides were accepted with Xcorr of 1.8 for charge state +1, 2.2 for charge state +2 and 3.5 for charge state +3. These values have given < 1% false positives over many data sets. Proteins were further required to have at least two peptides meeting acceptance criteria. Data was considered high confidence if assigned spectra were present in the Ac<sub>4</sub>GlcNAz samples but absent from the mock-enriched Ac<sub>4</sub>GlcNAc control samples.

**Prediction of Subcellular Localization**—Initial predictions of the subcellular localization of *Hp* proteins identified by mudPIT were based on literature reports, where available. Because of the shortage of subcellular location information for hypothetical proteins and functionally uncharacterized proteins, each identified protein was also subjected to PSORTb v.3.0 to predict subcellular location (43).

**Accessing Synthetic Staudinger Ligation-Glycan Standards**—Staudinger ligation was performed overnight at 37 °C by mixing 1:1 solutions of GlcNAz (100 mM in H<sub>2</sub>O) and Phos-FLAG-His<sub>6</sub> (100 mM in PBS) to produce GlcNAz-Phos-FLAG-His<sub>6</sub>. Similarly, overnight incubation at 37 °C of solutions of GlcNAz (100 mM in H<sub>2</sub>O) and Phos-FLAG-His<sub>6</sub> (500 μM in PBS) mixed 1:1 produced GlcNAz-Phos-FLAG-

His<sub>6</sub>. Successful synthesis was confirmed by high-resolution mass spectrometry analysis as described below (see HPLC-Chip/Q-TOF mass spectrometric analysis).

**Phos-FLAG Labeling to Produce "Tagged Lysates"**—*Hp* metabolically labeled with 1 mM Ac<sub>4</sub>GlcNAc or Ac<sub>4</sub>GlcNAz were centrifuged at 3000 × *g* and washed two times with phosphate buffered saline (PBS). The cells were lysed according to Koenigs *et al.* (31). Lysates were rinsed extensively with PBS using a Microcon centrifugal filter device (Millipore, 10000 MWCO) to remove residual Ac<sub>4</sub>GlcNAc or Ac<sub>4</sub>GlcNAz. Lysates were reacted with 250 μM Phos-FLAG at room temperature overnight. The unreacted Phos-FLAG was removed by extensive rinsing with ddH<sub>2</sub>O and passage of reacted lysates through a Microcon centrifugal filter device (Millipore, 10000 MWCO) to yield samples of "tagged lysates." These lysates were then subjected to beta-elimination, as described below.

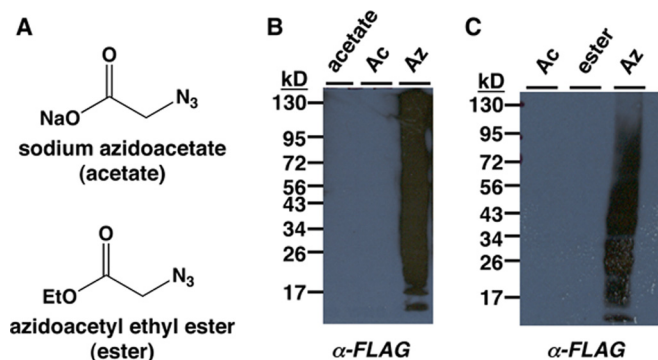
**Biochemical Purification of Azide-Labeled Urease**—Urease was purified from *Hp* strain 26695 using ion exchange chromatography (IEC) and size exclusion chromatography (SEC) based on previously described purification schemes by Hu and Mobley (44, 45) and Rokita *et al.* (46). A 250 ml culture of *Hp* metabolically labeled with 1 mM Ac<sub>4</sub>GlcNAz was inoculated and grown as described above. Cells were harvested by centrifugation at 10,000 × *g* and the resultant pellet was frozen at −20 °C. The cells were re-suspended in ice-cold buffer (20 mM sodium phosphate, pH 6.9) containing protease inhibitors (protease inhibitor mixture, Sigma) and 10 μg/ml DNase and then sonicated to achieve complete cell lysis. Concentrated stock solutions of EDTA and β-mercaptoethanol (βME) were added to the lysis solution to make it consistent with the initial IEC buffer (20 mM sodium phosphate, pH 6.9, 1 mM EDTA, 1 mM βME). Cellular debris was removed from the sample through centrifugation at 14,000 × *g*. The supernatant was loaded onto a Q-Sepharose Fast Flow column (25 ml, 3 cm diameter, GE Healthcare) that had been pre-equilibrated with the above IEC buffer. Urease was eluted from the column at ~150 mM NaCl using a linear gradient from 0 M to 0.5 M NaCl in IEC buffer. The presence of urease in the elution fractions was detected qualitatively using a phenol-red colorimetric assay (44). Fractions that exhibited urease activity were further analyzed by SDS-PAGE with zinc-stain (BioRad) and via Western with anti-ureA and anti-ureB antibodies (Santa Cruz Biotechnology) to verify the presence of ureA and ureB. Fractions with the most urease and fewest contaminants were pooled for subsequent purification by SEC. The pooled fractions were exchanged into the SEC buffer (20 mM sodium phosphate, pH 6.9, 150 mM NaCl, 1 mM EDTA, 1 mM βME) using a 9000 Da MWCO filter (Pierce) and concentrated to a final volume of 7 ml. This concentrated sample was loaded onto a pre-equilibrated Sepharose CL-6B (GE Healthcare) SEC column (200 ml total volume, 3 cm diameter) and eluted at 1 ml/min. Fractions containing urease were identified using the phenol-red assay and subsequently analyzed by SDS-PAGE. The purest urease-containing fractions (see supplemental Fig. S7) were pooled, concentrated to 0.5 mg/ml via ultrafiltration (Millipore, 100,000 MWCO), and mixed 1:1 with 500 μM Phos-FLAG at room temperature overnight. The unreacted Phos-FLAG was removed by extensive rinsing with 50% MeOH in ddH<sub>2</sub>O, 15% acetonitrile in ddH<sub>2</sub>O, and 100% ddH<sub>2</sub>O over a Microcon centrifugal filter device (Millipore, 10000 MWCO) to yield samples of "tagged urease." This sample was then subjected to beta-elimination, as described below.

**Beta-Elimination of *Hp*'s O-Linked Glycans**—*Hp*'s enriched azide-labeled glycoproteins, Phos-FLAG-reacted Ac and Az lysates ("tagged lysates"), and purified azide-labeled urease ("tagged urease") were subjected to beta-elimination to remove glycans overnight at 4 °C using GlycoProfile Beta-elimination Kit (Sigma-Aldrich) according to manufacturer's instructions. Released glycans were then analyzed by mass spectrometry, as described below.

**HPLC-Chip/Q-TOF Mass Spectrometric Analysis**—Samples of *Hp*'s enriched azide-labeled glycoproteins, Phos-FLAG-reacted Ac and Az lysates ("tagged lysates"), and purified azide-labeled urease ("tagged urease") were analyzed before and after beta-elimination of O-linked glycans using a 6530 High Performance Liquid Chromatographic-Chip Quadrupole Time-of-Flight Mass Spectrometer (HPLC-Chip/Q-TOFMS; Agilent Technologies, Santa Clara, CA). Chromatographic separation and nano-electrospray ionization (nanoESI) was performed with a 1260 Chip Cube system (Agilent Technologies) using a ProtiD-chip with a 40 nL enrichment column and a 150 mm × 75 μm analytical column (Agilent Technologies). The enrichment and analytical columns were packed with 300 Å, 5 μm particles with C18 stationary phase. The mobile phases were 0.1% formic acid/H<sub>2</sub>O (A) and 0.1% formic acid/acetonitrile (B). Samples (0.2–2 μl) were loaded on the enrichment column using 98:2 (A:B) at 4 μl/min and eluted through the analytical column using a gradient from 98:2 (A:B) to 80:20 (A:B) over 2 min to 2:98 (A:B) over a period of 8 min at 0.3 μl/min. Mass spectra (MS and MS/MS) were collected in positive ion mode; the ionization voltage ranged from 1850–1950 V and the ion source temperature was held at 350 °C. The fragmentor and collision cell voltages, as well as other tuning parameters, were optimized to minimize metastable water losses associated with ion sampling and transmission to the TOF analyzer. This was of particular importance for the analysis of HexNAz-Phos-FLAG-His<sub>6</sub> (1), for which metastable water losses became increasingly prominent for higher charge state ions. A similar effect, impacting metastable losses of sulfate from sulfated oligosaccharides, has been reported by Zaia and co-workers (47). Spectra were internally calibrated using methyl stearate (C<sub>17</sub>H<sub>35</sub>CO<sub>2</sub>CH<sub>3</sub>) or dibutyl phthalate (C<sub>16</sub>H<sub>22</sub>O<sub>4</sub>) and hexakis(1H, 1H, 4H-hexafluorobutyl)phosphazine (HP-1221; C<sub>24</sub>H<sub>18</sub>O<sub>6</sub>N<sub>3</sub>P<sub>3</sub>F<sub>36</sub>), continuously introduced and detected as [M+H]<sup>+</sup>. Collision-induced dissociation (CID)-MS/MS experiments were executed with precursor selection determined using a targeted or data-dependent approach. Precursor ions were subjected to CID using nitrogen as the target gas with collision energies ranging from 20–35 V.

**Immunopurification of *Hp*'s ureA and ureB**—Lysate from metabolically labeled *Hp* was standardized to 4.4 mg/ml and incubated 1:1 with 500 μM Phos-FLAG overnight at room temperature to label azide-containing glycoproteins. Insoluble membrane material was separated from soluble proteins by centrifugation at 12,000 × *g* for 5 min. To the soluble protein samples, 1 μl of α-*Hp* ureA or ureB (Santa Cruz Biotechnology) was added per 100 μl of protein volume. The samples were incubated at 4 °C overnight with gentle shaking. After completion of the overnight incubation, 100 μl of a 50% Protein-G agarose suspension in HNTG washing buffer (20 mM HEPES buffer pH 7.5, containing 150 mM NaCl, 0.1% (w/v) Triton X-100, and 10% (w/v) glycerol) was added to *Hp* lysates and allowed to incubate for 90 min at 4 °C with gentle shaking. The immunoprecipitated complexes were collected via centrifugation at 3000 × *g* for 5 min at 4 °C and the pellet was washed three times with ice-cold HNTG washing buffer. The final pellet was re-suspended in 40 μl of ice-cold HNTG washing buffer, diluted with an equal volume of 2 × SDS-loading buffer, and heated at 95 °C for 5 min. The boiled samples were centrifuged at 12,000 × *g* for 30 s to separate the agarose beads from the immunoprecipitated proteins. Immunoprecipitated proteins were then analyzed by Western blot with anti-FLAG (Sigma) or anti-urease antibody (Santa Cruz Biotechnology).

**Gel and Western Blot Analyses**—All lanes were loaded with 10–15 μg of protein sample unless otherwise noted. Western blots were treated with HRP-conjugated anti-FLAG antibody (1:3000, Sigma), rabbit polyclonal anti-*Hp* ureA (1:3000, Santa Cruz Biotechnology), or rabbit polyclonal anti-*Hp* ureB antibody (1:3000, Santa Cruz Biotechnology), followed by goat α-rabbit IgG-HRP (1:10000, Santa Cruz

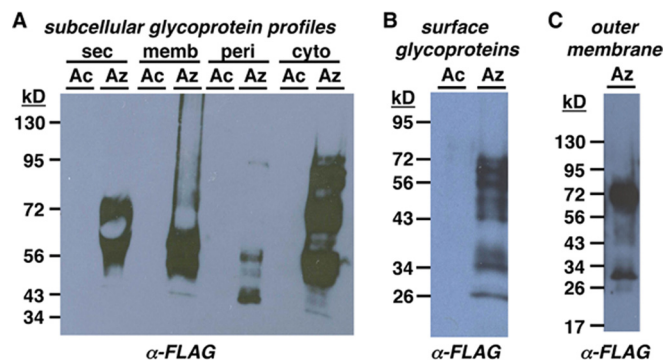


**FIG. 2. Metabolic labeling of *Hp*'s proteins with azides.** A, Azidoacetyl compounds used in metabolic feeding experiments with *Hp*. B, Western blot analysis of lysate from *Hp* treated with 5 mM sodium azidoacetate (acetate), 1 mM  $Ac_4GlcNAz$  (Az), or with 1 mM of the azide-free control sugar  $Ac_4GlcNAc$  (Ac) for 4 days. After lysis, samples were reacted with Phos-FLAG and visualized by Western blot with anti-FLAG antibody. C, Western blot analysis of lysate from *Hp* treated with 5 mM azidoacetyl ethyl ester (ester), 1 mM  $Ac_4GlcNAz$  (Az), or with 1 mM of the azide-free control sugar  $Ac_4GlcNAc$  (Ac) for 4 days. After lysis, samples were reacted with Phos-FLAG and visualized by Western blot with anti-FLAG antibody.

Biotechnology), then developed with chemiluminescent substrate (Pierce). Polyacrylamide gels were visualized with zinc stain (BioRad).

#### RESULTS AND DISCUSSION

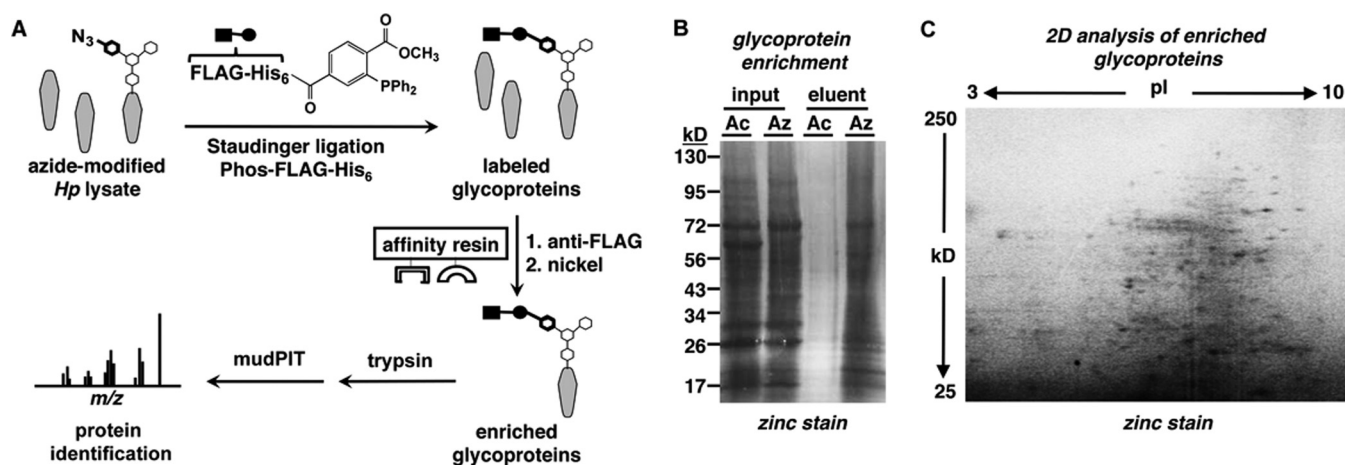
**Metabolic Labeling of *Hp*'s Proteins With Azides Does Not Appear to be Catabolic**—Here we employed metabolic glycan labeling to characterize *Hp*'s glycoproteins and unveil molecules that have the potential to serve as the basis of novel anti-*Hp* treatments. In previous work, our laboratory demonstrated that supplementation of *Hp*'s media with 1 mM  $Ac_4GlcNAz$  for three to 5 days leads to incorporation of azide-dependent signal into a large number of glycoproteins (31). The majority, though not all, of this azide-dependent signal can be removed by glycosidases that catalyze the cleavage of certain *N*-linked and *O*-linked glycans (31). Thus, we sought to assess whether the glycosidase-resistant azide signal is because of an alternative metabolic fate of  $Ac_4GlcNAz$  in *Hp*; specifically, catabolism of the azidosugar to the azidoacetyl unit followed by subsequent activation and covalent addition to proteins. This type of azidosugar catabolism has been observed in some other organisms. If this process were to occur in *Hp*, then supplementation of *Hp*'s media with the azidoacetyl moiety would likely lead to metabolic labeling of *Hp*'s proteins with azides. To assess this possibility, *Hp* cells were grown in media supplemented with 5 mM sodium azidoacetate or with the more bioavailable azidoacetyl ethyl ester (Fig. 2A); Hang and coworkers demonstrated that structurally analogous compounds are cell permeable (48, 49).  $Ac_4GlcNAz$  or the azide-free sugar peracetylated  $GlcNAc$  ( $Ac_4GlcNAc$ ) were employed as positive and negative controls of metabolic labeling, respectively.  $Ac_4GlcNAz$  treatment resulted in robust azide-dependent signal in lysates, whereas no azide-labeled proteins were observed in lysates from cells



**FIG. 3. Subcellular distribution of *Hp*'s azide-labeled glycoproteins.** Western blot analysis of subcellular fractions from *Hp* treated with 1 mM  $Ac_4GlcNAz$  (Az) or with the azide-free control sugar  $Ac_4GlcNAc$  (Ac) for 4 days. After subcellular fractionation, samples were reacted with Phos-FLAG and visualized by Western blot with anti-FLAG antibody. A, Glycoprotein profiles of four subcellular fractions are shown: secreted (sec), inner membrane (memb), periplasmic (peri), cytoplasmic (cyto). B, Glycoprotein profile of *Hp*'s surface-associated proteins. C, Glycoprotein profile of *Hp*'s outer membrane fraction.

supplemented with  $Ac_4GlcNAc$ , with sodium azidoacetate (Fig. 2B), or with azidoacetyl ethyl ester (Fig. 2C). SDS-PAGE analysis confirmed the presence of equivalent protein levels in all lanes (supplemental Fig. S1). These data suggest that azide-dependent labeling of *Hp*'s proteins is not because of catabolic incorporation of the azidoacetyl moiety into proteins. The remaining azide-dependent signal could be glycosidase resistant because of the presence of glycans that are not substrates of the enzymes, because similar glycosidase-resistance, and even resistance to chemical cleavage, has been observed for glycoproteins produced by *C. jejuni* (24). With the confidence that the vast majority of *Hp*'s azide-labeled proteins are glycosylated, we undertook experiments to study and identify these labeled glycoproteins.

**Assessment of the Subcellular Distribution of *Hp*'s Azide-Labeled Glycoproteins Reveals Glycoproteins Throughout *Hp*, Including on the Cell Surface**—To study *Hp*'s glycoproteins, we began by assessing the subcellular distribution of these species. In particular, we were curious to see whether any of *Hp*'s secreted and cell surface proteins are glycosylated, as these proteins are likely to directly modulate interactions with the host and could be novel therapeutic targets. *Hp* cells supplemented with the azidosugar  $Ac_4GlcNAz$  or with the azide-free control  $Ac_4GlcNAc$  (31) were fractionated to acquire secreted (36), periplasmic, inner membrane-associated, cytoplasmic (35), and surface associated proteins (37). Further, fractions containing outer membrane proteins were also obtained (15, 38). Western blot visualization of azide-labeled glycoproteins (Fig. 3) and SDS-PAGE analysis of total protein (see supplemental Fig. S2A–C) demonstrated that protein composition varied between fractions, consistent with successful subcellular separation of proteins. Further, malate dehydrogenase assays (50) of fractions revealed activities con-



**FIG. 4. Enrichment of *Hp*'s azide-labeled glycoproteins.** *A*, Targeted isolation of *Hp*'s azide-labeled glycoproteins was facilitated by the Staudinger ligation. First, *Hp*'s azide-labeled glycoproteins were reacted with Phos-FLAG-His<sub>6</sub> to yield glycoproteins containing a tandem affinity tag. Labeled glycoproteins were then enriched by anti-FLAG chromatography followed by Ni<sup>2+</sup> affinity chromatography. Finally, enriched glycoproteins were trypsinized and analyzed by multidimensional protein identification technology (mudPIT). These experiments were performed along side an azide-free control. *B*, SDS-PAGE analysis of proteins from *Hp* treated with Ac<sub>4</sub>GlcNAz (Az) or with the azide-free control sugar Ac<sub>4</sub>GlcNAc (Ac) before enrichment (input) and after elution from the nickel column (eluent) indicates successful isolation of azide-labeled glycoproteins. Proteins were visualized with zinc stain. *C*, Two-dimensional gel analysis of *Hp*'s enriched, azide-modified glycoproteins. Proteins were visualized with zinc stain.

sistent with successful separation of cytoplasmic and periplasmic proteins (35) (supplemental Fig. S2D). No azide-labeled proteins were observed in fractions from cells supplemented with Ac<sub>4</sub>GlcNAc, whereas Ac<sub>4</sub>GlcNAz treatment resulted in robust azide-dependent signal in all subcellular fractions (Fig. 3). Our data demonstrate the remarkable selectivity of the Staudinger ligation between phosphines and azides, as these abiotic functional groups are nonreactive with endogenous functional groups yet display exquisitely selective reactivity with each other to form a covalent adduct (51, 52). Moreover, these data indicate that glycoproteins are present throughout *Hp* and may serve diverse functions. For example, modification of internal glycoproteins may be important for the physiology of *Hp*, whereas glycosylation of secreted and surface proteins may be crucial for *Hp* to survive and cause harm within the gastric mucosa.

The prominent display of glycans on a subset of *Hp*'s cell surface proteins might be harnessed for novel anti-*Hp* therapeutic strategies. For example, these glycosylated proteins might provide the basis of a carbohydrate-based vaccine; analogous glycoconjugate vaccines have been remarkably successful in the clinic (14, 53). Moreover, if glycan modifications are important for the interactions of *Hp* with its host, small molecule inhibitors could target glycan synthesis to inactivate *Hp*. Finally, *Hp*'s glycans could be selectively labeled with azides and targeted with phosphines to disrupt their function (14, 54, 55). Realizing these strategies would first require identification of *Hp*'s glycoproteins.

*Targeted Isolation of *Hp*'s Azide-Labeled Glycoproteins was Facilitated by the Staudinger Ligation*—To identify *Hp*'s glycoproteins, we began by taking advantage of the metaboli-

cally incorporated azide, in conjunction with the Staudinger ligation, to enrich these species. Lysates from *Hp* supplemented with Ac<sub>4</sub>GlcNAz or Ac<sub>4</sub>GlcNAc were subjected to Staudinger ligation with a phosphine comprising a tandem FLAG-hexahistidine peptide (Phos-FLAG-His<sub>6</sub>, Fig. 4A) (29, 56). Tagged glycoproteins containing both the FLAG and His<sub>6</sub> epitopes were enriched by purification via anti-FLAG chromatography followed by Ni<sup>2+</sup> affinity chromatography (29, 56) (Fig. 4A). SDS-PAGE analysis enabled comparison of protein profiles from samples before purification (input) versus after enrichment of azide-labeled species (eluent). A large number of proteins were present in both the azide-labeled and acetyl-labeled samples before purification (Fig. 4B). In contrast, after purification, proteins were present in substantially higher abundance in the enriched, azide-labeled sample relative to the mock-enriched control (Fig. 4B). These data highlight the selectivity of the Staudinger ligation between phosphines and azides. The ladder of proteins observed in the enriched, azide-labeled fraction is consistent with the large number of azide-labeled proteins observed by Western blot analysis of lysate from Ac<sub>4</sub>GlcNAz-treated cells (Figs. 2B, 2C). These data indicate a dramatic enrichment of *Hp*'s azide-labeled glycoproteins using Phos-FLAG-His<sub>6</sub>. Analysis of the enriched, azide-labeled glycoprotein sample by two-dimensional gel electrophoresis yielded greater than 50 detectable species (Fig. 4C), indicating that *Hp* synthesizes myriad glycoproteins. The large number of *Hp* glycoproteins detected is similar in magnitude to the number of glycoproteins identified in large-scale studies of the related species *C. jejuni* (57, 58) and in more distantly related bacteria, including *Mycobacterium tuberculosis* (26), *Neisseria* sp. (20), and *Bacteroides* sp.

(27). *Hp* appears to dedicate considerable metabolic energy to glycoprotein synthesis, suggesting glycoproteins play an important role in *Hp*'s physiology.

**Identification of *Hp*'s Azide-Labeled Glycoproteins by Mass Spectrometry Indicates Glycosylation of a Number of Virulence Factors**—Motivated by these results, we next sought to identify *Hp*'s glycoproteins by mass spectrometry analysis. *Hp*'s enriched, azide-labeled glycoproteins and a mock-enriched control (which was labeled with Ac<sub>4</sub>GlcNAc, reacted with Phos-FLAG-His<sub>6</sub>, and enriched via anti-FLAG and Ni-NTA affinity chromatography, as described above) were digested with trypsin, then analyzed by multi-dimensional protein identification technology (mudPIT) (Fig. 4A) (40). Experimental detection of unique peptides enabled the unambiguous identification of 127 proteins in the azide-labeled sample. In contrast, the mock-enriched control that lacked the azide contained only two *Hp* proteins (HP0721, HP0570), both of which were also present in the azide-labeled sample. These proteins were subtracted from the list of putative glycoproteins identified in the azide-labeled sample, narrowing the pool of candidate glycoproteins to 125 (Table I and Table II). All proteins were identified based on two or more peptides meeting acceptance criteria. Detailed information on all of the proteins identified in the azide-labeled sample and the mock-enriched control, including detected peptides that made assignments possible, can be found in [supplemental Table S1](#). These results demonstrate efficient enrichment and identification of azide-labeled proteins, suggesting this metabolic labeling strategy is a robust method to profile bacterial glycoproteins.

Of the putative glycoproteins identified, 70% have housekeeping functions, 21% are linked to *Hp*'s pathogenesis, and 9% have unknown function. From a basic biology perspective, the identification of a number of putative glycoproteins that have housekeeping functions suggests that glycosylation could play a role in proper folding and function (59), or perhaps that glycosylation provides a selective advantage for survival in the harsh conditions of the gastric tract. From a therapeutic perspective, it is intriguing that 26 of *Hp*'s glycoproteins have known roles in pathogenesis (Table I) that include enabling survival in the host stomach via iron acquisition (*pfr*) (60), pH resistance (*ureA*, *ureB*, *tufB*) (61), oxidative protection (*tsaA*, *sodB*, *katA*, *tagD*, *msrA*) (62, 63), motility (*flgH*) (64), adhesion (*babC*, *babA*, *tlpA*, *dnaK*, *htrA*) (65), eliciting an immune response (*napA*, *msrA*, *icd*, *frdA*) (66, 67), and causing harm to the host (*cag7*, *groES*, *groEL*, *hopQ*, *fusA*) (68, 69). The large percentage of virulence factors in *Hp*'s glycoproteome, relative to the low percentage of virulence factors in *Hp*'s proteome (70, 71), implicates *Hp*'s glycans in pathogenesis. This observation suggests that the known link between protein glycosylation and virulence in *Hp*, which Logan firmly established with her studies of *Hp*'s flagellin glycosylation (7), may be a widespread phenomenon within this bacterium.

We next considered the subcellular distribution of *Hp*'s putative glycoproteins. Based on literature reports and *in silico* predictions, candidate glycoproteins appear to be expressed throughout *Hp* (Fig. 5), including intracellular locations such as the cytoplasm, inner membrane, and periplasm, and extracellular locations such as the outer membrane, cell surface (surface-associated), and the secreted milieu. Moreover, the predicted subcellular distribution of *Hp*'s putative glycoproteins (Fig. 5) appears to reflect the subcellular distribution that we detected experimentally (Fig. 3). Follow-up experiments need to be conducted to establish whether these localization predictions are correct and to confirm that *Hp* synthesizes glycoproteins throughout the cell, as our data suggest.

We next compared the results of our analyses of *Hp*'s glycoproteins to a recent study by Creuzenet and coworkers. In their study, they employed a periodic acid/hydrazide-based screen to identify nine candidate *Hp* glycoproteins (15). Five of the putative glycoprotein hits identified here (*katA*, *gltA*, *tsf*, *atpA*, *atpD*) were also identified as candidate glycoproteins in that screen, further validating our hit list. Moreover, their analyses indicated the presence of glycoproteins in soluble and membrane fractions of *Hp* (15), consistent with the subcellular distribution observed here. These findings underscore the value of our targeted strategy to detect and discover bacterial glycoproteins in a manner that is complementary to alternative techniques.

We note the absence of the flagellin proteins FlaA and FlaB, glycoproteins confirmed to be modified with pseudaminic acid, from our list of identified azide-labeled glycoproteins. This result is consistent with our previous studies, which demonstrated that Ac<sub>4</sub>GlcNAz treatment does not lead to detectable incorporation of azide into *Hp*'s flagellin proteins (31). Our previously reported data indicate that Ac<sub>4</sub>GlcNAz is not converted to the complex sugar pseudaminic acid at detectable levels. Thus, though the studies described here identify a large number of putative *Hp* glycoproteins, they do not exhaustively identify all *Hp* glycoproteins.

**Mass Spectrometry Analyses Reveal Staudinger Ligation-Glycan Adducts**—We sought to characterize the nature of *Hp*'s azide-modified glycans. A sample of *Hp*'s enriched, azide-labeled glycoproteins was analyzed by HPLC-Chip/Q-TOFMS before and after being subjected to beta-elimination to cleave O-linked glycans. For these preliminary experiments, we focused on identifying the simplest Staudinger ligation-glycan adduct that would be formed as a result of metabolic labeling with Ac<sub>4</sub>GlcNAz - addition of a single GlcNAz residue or, if the stereochemistry is changed, an *N*-azidoacetylhexosamine (HexNAz) residue onto a glycosylated protein. Thus, we directed our analysis at the identification of the Staudinger ligation-glycan adduct that would be formed by protein glycosylation with a single HexNAz residue followed by release via beta-elimination - HexNAz-Phos-FLAG-His<sub>6</sub> (**1**) (Fig. 6). Because this adduct has not been



TABLE I  
Putative glycoproteins identified by mudPIT with links to pathogenesis

| Gene   | Acc. #    | Protein name | Predicted MW (kDa) | Function/sequence features                  | Link to pathogenesis                              | Subcellular localization <sup>a</sup> | Spect-counts | Uni-pep | Seq-cov% |
|--------|-----------|--------------|--------------------|---|---|---------------------------------------|--------------|---------|----------|
| HP0073 | P14916    | ureA         | 27.8               | Urease alpha subunit                        | Enables survival in acid                          | Cytoplasm, surface                    | 96           | 4       | 34.4     |
| HP0072 | P69996    | ureB         | 61.6               | Urease beta subunit                         | Enables survival in acid                          | Cytoplasm, surface                    | 188          | 7       | 22.7     |
| HP0527 | NP_207323 | cag7         | 230.1              | cag pathogenicity island, type IV secretion | Oncogene, gastric cancer                          | Outer membrane                        | 2            | 2       | 1.4      |
| HP1563 | P21762    | tsaA         | 23.5               | Alkyl hydroperoxide reductase               | Resists oxidative damage                          | Cytoplasm                             | 428          | 12      | 65.7     |
| HP0325 | O25092    | flgH         | 27.8               | Flagellar export                            | Critical for motility                             | Outer membrane                        | 4            | 2       | 7.2      |
| HP0010 | P42383    | groEL        | 61.5               | Chaperone                                   | Gastric cancer-associated antigen, immunogen      | Cytoplasm, surface                    | 576          | 18      | 37.3     |
| HP0011 | P0A0R3    | groES        | 13.7               | Chaperone                                   | Gastric cancer-associated antigen, immunogen      | Cytoplasm, surface                    | 546          | 7       | 41.9     |
| HP1161 | O25776    | fldA         | 18.5               | Flavodoxin                                  | Critical metabolic gene                           | Unknown                               | 2            | 2       | 20.8     |
| HP0653 | P52093    | pfr          | 20.3               | Nonheme iron-containing ferritin            | Crucial for iron acquisition                      | Cytoplasm                             | 35           | 3       | 25.6     |
| HP0389 | AAD07454  | sodB         | 25.9               | Superoxide dismutase                        | Resists oxidative damage                          | Periplasm                             | 10           | 4       | 28.9     |
| HP0875 | P77872    | katA         | 61.5               | Catalase                                    | Combats oxidative stress                          | Periplasm, surface                    | 91           | 6       | 18.0     |
| HP0390 | O25151    | tagD         | 19.3               | Adhesin thiol peroxidase                    | Combats oxidative stress                          | Periplasm                             | 3            | 3       | 32.6     |
| HP0243 | P43313    | napA         | 17.9               | Neutrophil activation                       | Immunomodulator, biofilm formation                | Cytoplasm, surface                    | 9            | 3       | 25.5     |
| HP0224 | O25011    | msrA         | 43.2               | Methionine sulfoxide reductase              | Antioxidant, immunogen                            | Cytoplasm                             | 42           | 4       | 18.6     |
| HP0317 | AAD07380  | babC         | 85.4               | Outer membrane protein                      | Adhesion, glycan binding                          | Outer membrane                        | 4            | 2       | 5.4      |
| HP1243 | AAD08288  | babA         | 83.3               | Outer membrane protein                      | Adhesion, glycan binding                          | Outer membrane                        | 2            | 1       | 2.8      |
| HP1177 | AAD08221  | hopQ         | 73.3               | Outer membrane protein                      | Present on disease-related strains                | Outer membrane                        | 14           | 2       | 5.5      |
| HP1205 | P56003    | tufB         | 45.9               | Translation elongation factor               | Resistance to prolonged acid exposure             | Cytoplasm                             | 10           | 4       | 17.1     |
| HP1123 | O25748    | slyD         | 21.3               | Peptidyl-prolyl cis-trans isomerase         | Important for urease assembly                     | Cytoplasm                             | 2            | 2       | 16.2     |
| HP1195 | P56002    | fusA         | 80.9               | Translation elongation factor               | Duodenal ulcer-related antigen                    | Cytoplasm, surface                    | 36           | 8       | 21.0     |
| HP0027 | P56063    | icd          | 50.1               | Isocitrate dehydrogenase                    | Immunogen, induces humoral immune system          | Cytoplasm                             | 21           | 4       | 15.6     |
| HP0192 | O06913    | frdA         | 84.0               | Fumarate reductase                          | Immunogen, crucial for survival in gastric mucosa | Inner membrane                        | 28           | 5       | 13.2     |
| HP0109 | P55994    | dnaK         | 70.6               | Chaperone and heat shock protein 70         | Stress induced surface adhesion, immunogen        | Cytoplasm, surface                    | 6            | 3       | 8.7      |
| HP0082 | AAD07152  | tlpC         | 79.1               | Methyl-accepting chemotaxis transducer      | Assist colonization of the stomach                | Inner membrane                        | 10           | 3       | 7.6      |
| HP1019 | AAD08063  | htrA         | 50.6               | Serine protease, chaperone                  | Disrupts intracellular adhesion                   | Periplasm, secreted                   | 5            | 2       | 6.6      |
| HP0099 | AAD07167  | tlpA         | 78.4               | Methyl-accepting chemotaxis protein         | Chemotaxis receptor                               | Outer membrane                        | 3            | 1       | 3.2      |

<sup>a</sup> Subcellular localization was based on literature reports or predicted using PSORTb v.3.0.

## Identification of *Helicobacter pylori*'s Glycoproteins

TABLE II  
Additional putative glycoproteins identified by mudPIT

| Gene   | Acc. #   | Protein name | Predicted MW (kDa) | Function/sequence features              | Subcellular localization <sup>a</sup> | Spect-counts | Uni-pep | Seq-cov% |
|--------|----------|--------------|--------------------|---|---------------------------------------|--------------|---------|----------|
| HP0025 | AAD07106 | omp2         | 81.6               | Outer membrane protein                  | Outer membrane                        | 5            | 3       | 7.9      |
| HP0191 | O06914   | frdB         | 29.3               | Fumarate reductase beta                 | Inner membrane                        | 15           | 4       | 26.2     |
| HP0599 | AAD07662 | hylB         | 51.0               | Hemolysin secretion                     | Outer membrane                        | 31           | 8       | 29.5     |
| HP0371 | AAD07435 | fabE         | 18.1               | Biotin carboxyl carrier                 | Unknown                               | 35           | 4       | 26.7     |
| HP0110 | P55970   | grpE         | 23.3               | Heat shock protein                      | Cytoplasm                             | 14           | 5       | 29.9     |
| HP0631 | AAD07691 | hydA         | 44.6               | Quinine reactive hydrogenase            | Inner membrane                        | 4            | 3       | 9.1      |
| HP1198 | O25806   | rpoBC        | 339.5              | RNA polymerase                          | Cytoplasm                             | 9            | 6       | 3.6      |
| HP1496 | P56078   | ctc          | 20.9               | General stress protein                  | Cytoplasm                             | 6            | 3       | 33.7     |
| HP0824 | P66928   | trxA         | 12.5               | Thioredoxin A                           | Cytoplasm                             | 20           | 2       | 26.8     |
| HP0825 | 3ISH_B   | trxB         | 35.5               | Thioredoxin reductase                   | Cytoplasm                             | 22           | 6       | 33.1     |
| HP1458 | AAD08500 | —            | 12.4               | Thioredoxin                             | Cytoplasm                             | 47           | 2       | 26.4     |
| HP1325 | O25883   | fumC         | 53.6               | Fumarase                                | Cytoplasm                             | 23           | 5       | 25.5     |
| HP1555 | P55975   | tsf          | 41.6               | Translation elongation factor           | Cytoplasm                             | 17           | 3       | 9.1      |
| HP0266 | O25045   | pyrC         | 44.6               | Dihydroorotase                          | Unknown                               | 19           | 5       | 25.1     |
| HP1135 | AAD08177 | atpH         | 21.3               | ATP synthase F1 delta                   | Membrane                              | 10           | 2       | 18.0     |
| HP1134 | P55987   | atpA         | 58.1               | ATP synthase F1 alpha                   | Membrane                              | 28           | 5       | 13.2     |
| HP1132 | P55988   | atpD         | 54.1               | ATP synthase F1 beta                    | Membrane                              | 26           | 6       | 22.3     |
| HP1099 | AAD08142 | eda          | 23.9               | Aldolase                                | Cytoplasm                             | 5            | 3       | 23.2     |
| HP0154 | P48285   | eno          | 49.1               | Enolase                                 | Cytoplasm                             | 12           | 4       | 21.6     |
| HP0512 | P94845   | glnA         | 57.4               | Glutamate synthase                      | Cytoplasm                             | 32           | 4       | 15.4     |
| HP0779 | AAD07828 | acnB         | 97.7               | Aconitase                               | Cytoplasm                             | 72           | 10      | 19.9     |
| HP0163 | P56074   | hemB         | 38.1               | Delta-aminolevulinic acid dehydratase   | Cytoplasm                             | 5            | 3       | 15.2     |
| HP0974 | P56196   | pgm          | 57.7               | Phosphoglycerate mutase                 | Cytoplasm                             | 5            | 4       | 18.1     |
| HP0294 | O25067   | aimE         | 39.7               | Aliphatic amidase                       | Cytoplasm                             | 31           | 3       | 17.9     |
| HP0649 | P56149   | aspA         | 54.6               | Aspartate ammonia lyase                 | Cytoplasm                             | 17           | 5       | 17.5     |
| HP0865 | O25536   | dut          | 16.8               | Nucleotidohydrolase                     | Unknown                               | 3            | 1       | 17.5     |
| HP0020 | AAD07088 | nspC         | 48.2               | Carboxynorperimidine decarboxylase      | Unknown                               | 5            | 4       | 16.9     |
| HP1540 | AAD08580 | fbcF         | 19.2               | Ubiquinol cytochrome c oxidase          | Inner membrane                        | 18           | 2       | 15.9     |
| HP0480 | O25225   | yihK         | 69.9               | GTP-binding                             | Inner membrane                        | 10           | 5       | 14.1     |
| HP0954 | O25608   | —            | 25.4               | NAD(P)H reductase                       | Cytoplasm                             | 3            | 2       | 14.0     |
| HP0176 | P56109   | tsr          | 35.7               | Fructose-bisphosphate aldolase          | Cytoplasm                             | 3            | 2       | 13.8     |
| HP1110 | AAD08154 | porA         | 47.0               | Pyruvate ferredoxin oxidoreductase      | Unknown                               | 47           | 4       | 18.9     |
| HP1111 | AAD08155 | pfor         | 36.9               | Pyruvate ferredoxin oxidoreductase beta | Unknown                               | 37           | 3       | 16.9     |
| HP0632 | AAD07692 | hydB         | 67.6               | Quinone-reactive Ni-Fe hydrogenase      | Inner membrane                        | 7            | 4       | 13.5     |
| HP0026 | P56062   | gltA         | 50.9               | Citrate synthase                        | Cytoplasm                             | 71           | 6       | 13.2     |
| HP0757 | AAD07805 | —            | 34.9               | Beta-alanine synthase                   | Cytoplasm                             | 6            | 2       | 12.4     |
| HP0485 | AAD07551 | —            | 37.7               | Catalase-like protein                   | Periplasm                             | 4            | 3       | 12.3     |
| HP0201 | AAD07269 | plsX         | 38.4               | Fatty acid phospholipid synthesis       | Cytoplasm                             | 5            | 2       | 12.1     |
| HP0183 | P56089   | glyA         | 48.0               | Serine hydroxyl-methyltransferase       | Cytoplasm                             | 17           | 3       | 11.9     |
| HP0690 | AAD07742 | fadA         | 43.4               | Acetyl coenzyme acetyltransferase       | Cytoplasm                             | 5            | 2       | 11.9     |

TABLE II—continued

| Gene   | Acc. #    | Protein name | Predicted MW (kDa) | Function/sequence features                  | Subcellular localization <sup>a</sup> | Spect-counts | Uni-pep | Seq-cov% |
|--------|-----------|--------------|--------------------|---|---------------------------------------|--------------|---------|----------|
| HP0402 | P56145    | pheT         | 89.4               | phenylalanyl-tRNA synthase                  | cytoplasm                             | 20           | 5       | 11.7     |
| HP0096 | AAD07165  | —            | 36.7               | phosphoglycerate dehydrogenase              | cytoplasm                             | 6            | 2       | 11.7     |
| HP0576 | O25300    | lepB         | 35.3               | signal peptidase I                          | inner membrane                        | 11           | 3       | 11.1     |
| HP1013 | O25657    | dapA         | 34.8               | dihydrodipicolinate synthetase              | cytoplasm                             | 8            | 2       | 10.8     |
| HP0397 | AAD07461  | serA         | 60.8               | phosphoglycerate dehydrogenase              | cytoplasm                             | 12           | 3       | 10.7     |
| HP1576 | O26096    | abc          | 38.6               | ABC transporter                             | inner membrane                        | 4            | 2       | 10.7     |
| HP0006 | P56061    | panC         | 32.8               | pantoate-beta-alanine ligase                | cytoplasm                             | 2            | 2       | 10.7     |
| HP0558 | NP_207353 | fabF         | 45.8               | beta ketoacyl-acyl carrier protein synthase | inner membrane                        | 8            | 2       | 10.6     |
| HP0330 | O25097    | ilvC         | 38.5               | ketol-acid reductoisomerase                 | cytoplasm                             | 2            | 2       | 10.3     |
| HP0672 | AAD07733  | aspB         | 45.1               | mitochondrial signature protein             | cytoplasm                             | 2            | 2       | 10.0     |
| HP1103 | O25731    | glk          | 38.6               | glucokinase                                 | cytoplasm                             | 11           | 2       | 9.9      |
| HP0221 | AAD07289  | —            | 38.3               | nifU-like                                   | cytoplasm                             | 2            | 2       | 9.9      |
| HP0604 | AAD07669  | hemE         | 40.4               | uroporphyrinogen decarboxylase              | cytoplasm                             | 3            | 2       | 9.8      |
| HP0306 | P56115    | hemL         | 49.3               | aminomutase                                 | cytoplasm                             | 3            | 2       | 9.7      |
| HP1266 | AAD08310  | nqo3         | 99.1               | NADH-ubiquinone oxidoreductase              | unknown                               | 8            | 4       | 9.3      |
| HP1366 | AAD08410  | mbolIR       | 52.6               | type IIS restriction enzyme R               | unknown                               | 4            | 2       | 8.5      |
| HP0490 | AAD07558  | —            | 45.2               | putative potassium channel                  | unknown                               | 2            | 2       | 8.5      |
| HP1181 | AAD08227  | mfs          | 51.4               | multidrug-efflux transporter                | inner membrane                        | 4            | 2       | 8.4      |
| HP0777 | P56106    | pyrH         | 27.4               | uridine 5' monophosphate kinase             | cytoplasm                             | 13           | 2       | 8.3      |
| HP1422 | P56456    | ileS         | 111.3              | Isoleucyl-tRNA synthetase                   | cytoplasm                             | 5            | 4       | 8.1      |
| HP0322 | AAD07390  | —            | 61.4               | poly E-rich protein                         | cytoplasm                             | 5            | 3       | 7.9      |
| HP0382 | AAD07451  | —            | 48.5               | zinc metallo protease                       | unknown                               | 3            | 2       | 7.9      |
| HP0859 | AAD07905  | rfaD         | 39.4               | mannoheptose epimerase                      | cytoplasm                             | 5            | 2       | 7.8      |
| HP1100 | P56111    | —            | 70.2               | 6-phosphogluconate dehydratase              | cytoplasm                             | 55           | 2       | 6.4      |
| HP0210 | P56116    | htpG         | 74.8               | chaperone and heat shock protein            | cytoplasm                             | 2            | 2       | 7.2      |
| HP0056 | AAD07126  | —            | 141.6              | pyrroline-5-carboxylate dehydrogenase       | cytoplasm                             | 14           | 5       | 7.1      |
| HP0728 | O25428    | tilS         | 41.7               | tRNA(Ile)-lysine synthetase                 | unknown                               | 3            | 2       | 7.1      |
| HP1045 | O25686    | acoE         | 78.8               | acetyl-CoA synthetase                       | cytoplasm                             | 7            | 3       | 6.7      |
| HP0169 | P56113    | prtC         | 49.9               | collagenase                                 | unknown                               | 2            | 2       | 6.7      |
| HP1104 | AAD08150  | cad          | 40.6               | cinnamyl-alcohol dehydrogenase              | cytoplasm                             | 7            | 2       | 6.6      |
| HP0470 | AAD07532  | pepF         | 70.9               | oligoendopeptidase F                        | cytoplasm                             | 5            | 3       | 6.4      |
| HP0422 | AAD07486  | speA         | 74.0               | arginine decarboxylase                      | periplasm                             | 7            | 3       | 6.3      |
| HP0510 | P94844    | dapB         | 29.4               | dihydrodipicolinate reductase               | cytoplasm                             | 4            | 2       | 6.3      |

## Identification of *Helicobacter pylori*'s Glycoproteins

TABLE II—continued

| Gene   | Acc. #    | Protein name | Predicted MW (kDa) | Function/sequence features                 | Subcellular localization <sup>a</sup> | Spect-counts | Uni-pep | Seq-cov% |
|--------|-----------|--------------|--------------------|--|---------------------------------------|--------------|---------|----------|
| HP0680 | P55982    | nrdA         | 94.7               | Ribonucleoside-diphosphate reductase       | Cytoplasm                             | 2            | 2       | 5.5      |
| HP1430 | P56185    | —            | 81.3               | Conserved hypothetical ATP-binding protein | Unknown                               | 4            | 2       | 5.5      |
| HP1012 | AAD08056  | pqqE         | 52.9               | Protease                                   | Unknown                               | 2            | 2       | 5.3      |
| HP1213 | O25812    | pnp          | 80.8               | Polynucleotide phosphorylase               | Cytoplasm                             | 2            | 2       | 4.8      |
| HP1278 | P56142    | trpB         | 45.0               | Tryptophan synthase beta                   | Cytoplasm                             | 16           | 1       | 4.6      |
| HP1072 | P55989    | copA         | 86.1               | Copper-transporting ATPase                 | Inner membrane                        | 3            | 2       | 4.3      |
| HP1450 | O25989    | yidC         | 65.8               | Protein translocase                        | Inner membrane                        | 6            | 2       | 4.3      |
| HP0121 | P56070    | ppsA         | 95.8               | Phosphoenolpyruvate synthase               | Cytoplasm                             | 3            | 2       | 4.1      |
| HP0607 | NP_207402 | acrB         | 119.6              | Acriflavine resistance                     | Inner membrane                        | 2            | 2       | 4.0      |
| HP1402 | AAD08445  | hsdR         | 121.7              | Type I restriction enzyme                  | Unknown                               | 2            | 2       | 3.4      |
| HP1112 | P56468    | purB         | 52.6               | Adenylosuccinate lyase                     | Cytoplasm                             | 5            | 1       | 3.4      |
| HP1478 | AAD08516  | uvrD         | 81.7               | DNA helicase II                            | Cytoplasm                             | 2            | 2       | 3.3      |
| HP1547 | P56457    | leuS         | 97.7               | Leucyl-tRNA synthase                       | Cytoplasm                             | 3            | 2       | 2.9      |
| HP0791 | Q59465    | cadA         | 78.9               | Cadmium-transporting ATPase                | Inner membrane                        | 2            | 2       | 2.9      |
| HP0600 | AAD07663  | spaB         | 71.8               | Multidrug resistance                       | Inner membrane                        | 3            | 1       | 2.7      |
| HP0696 | AAD07747  | —            | 90.7               | N-methylhydantoinase                       | Unknown                               | 3            | 2       | 2.6      |
| HP0958 | —         | —            | 31.3               | Hypothetical protein                       | Unknown                               | 2            | 2       | 12.3     |
| HP0787 | —         | —            | 48.2               | Conserved integral membrane protein        | Inner membrane                        | 21           | 3       | 9.3      |
| HP0231 | —         | —            | 31.1               | Hypothetical protein                       | Unknown                               | 10           | 4       | 22.9     |
| HP0318 | —         | —            | 30.1               | Hypothetical protein                       | Unknown                               | 35           | 3       | 18.4     |
| HP0086 | —         | —            | 53.3               | Hypothetical protein                       | Unknown                               | 35           | 3       | 9.1      |
| HP0773 | —         | —            | 41.9               | Hypothetical protein                       | Unknown                               | 32           | 2       | 8.9      |
| HP0754 | —         | —            | 9.7                | Hypothetical protein                       | Unknown                               | 7            | 2       | 34.1     |
| HP0396 | —         | —            | 74.4               | Hypothetical protein                       | Unknown                               | 3            | 3       | 8.8      |
| HP1079 | —         | —            | 45.2               | Hypothetical protein                       | Unknown                               | 34           | 2       | 6.9      |
| HP1143 | —         | —            | 53.2               | Hypothetical protein                       | Unknown                               | 4            | 1       | 5.0      |

<sup>a</sup> Subcellular localization was based on literature reports or predicted using PSORTb v.3.0.

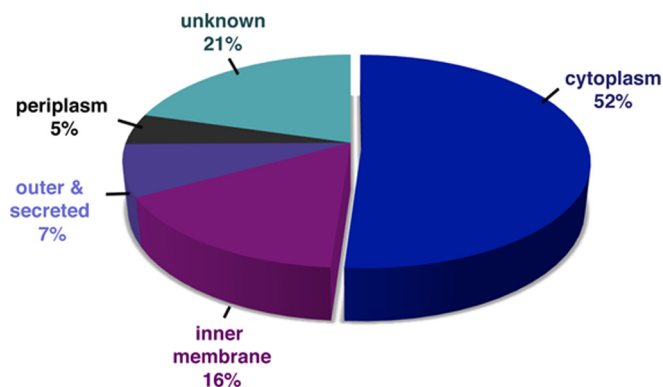
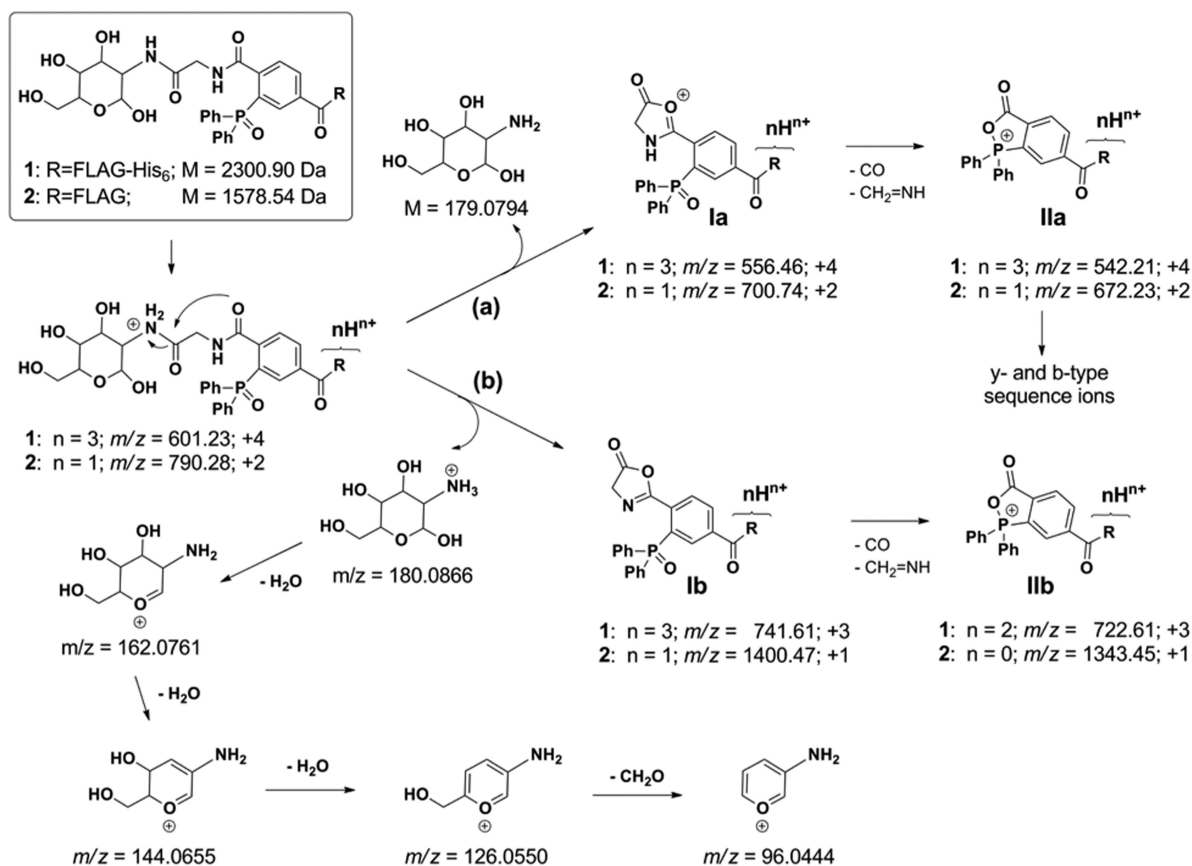


FIG. 5. Predicted subcellular location of candidate glycoproteins. The cellular locations of each identified protein was determined by a combination of literature reports and predictions by PSORTb v.3.0. Secondary surface-associated localization ( $n = 8$ ) was not included.

previously characterized by MS, we first synthesized GlcNAz-Phos-FLAG-His<sub>6</sub> and analyzed its chromatographic and mass spectrometric properties using LC-nanoESI and collision-induced dissociation (CID). The nanoESI mass spectrum of the synthetic standard GlcNAz-Phos-FLAG-His<sub>6</sub> ( $M = 2400.90$  Da) is dominated by  $[M+3H]^{3+}$ ,  $[M+4H]^{4+}$ , and  $[M+5H]^{5+}$  ions (supplemental Fig. S3); additionally, we observed metastable losses of water that were most apparent for the  $[M+5H]^{5+}$  charge state. The intensities of these peaks were highly dependent on voltages controlling ion transmission, so we attempted to minimize these contributions through careful instrument tuning. CID-MS/MS analysis of the  $[M+4H]^{4+}$  ion at  $m/z$  601.24 revealed fragmentation rationalized by initial loss of the neutral glycan via pathway (a) to form product ion  $la^{4+}$  ( $m/z$  556.46) (Figs. 6 and 7A), which proceeds, via net loss of CO and CH<sub>2</sub>NH, to form the base peak in the spectrum



**FIG. 6. Proposed mass spectrometric fragmentation pathways for Staudinger ligation-glycan adducts 1 (HexNAz-Phos-FLAG-His<sub>6</sub>) and 2 (HexNAz-Phos-FLAG).** CID-MS/MS of nanoESI-generated [M+4H]<sup>4+</sup> (adduct 1) and [M+2H]<sup>2+</sup> (adduct 2) ions yield fragment ions Ia and IIa, formed via pathway (a), through which loss of the neutral glycan is hypothesized to generate cyclized Ia, which is followed by sequential losses of CO and CH<sub>2</sub>NH to generate IIa. Charge-retention on the glycan and subsequent fragmentation via pathway (b) rationalizes formation of glycan-derived putative product ions at *m/z* 162.08, *m/z* 144.07, *m/z* 126.06, and *m/z* 96.04. Production of y- and b-type fragment ions is attributed to subsequent fragmentation of IIa yielding C- and N-(Phos-containing)-terminus fragments, respectively (see Figs. 7E and 8E).

(product ion IIa<sup>4+</sup> at *m/z* 542.21) (Figs. 6 and 7A; supplemental Table S3). We also detect triply charged versions of product ions at *m/z* 741.63 and 722.61 (ions Ib<sup>3+</sup> and IIb<sup>3+</sup>, respectively) (Figs. 6 and 7A), which may be formed by loss of the positively charged glycan via pathway (b) (Fig. 6). This pathway may also be responsible for formation of the glycan-derived product ions that appear in the spectrum at *m/z* 162.08, *m/z* 144.07, *m/z* 126.06, and *m/z* 96.04 (see proposed structures in Figs. 6 and 7B). We also detected FLAG-His<sub>6</sub>-derived product ions. These include abundant immonium ions at *m/z* 110.07 (His) and *m/z* 129.10 (Lys), as well as y-type (72) fragments containing the FLAG-His<sub>6</sub> C terminus (see Fig. 7E) and b-type (72) fragments; the latter are denoted by b<sub>n</sub><sup>+</sup> and contain the glycan-eliminated N terminus (see Fig. 7E) that is attributed to subsequent fragmentations from ion IIa<sup>4+</sup>. To determine if the Staudinger ligation-glycan adduct HexNAz-Phos-FLAG-His<sub>6</sub> (1) is released from *Hp*'s enriched, azide-labeled glycoproteins on beta-elimination, a sample was analyzed by HPLC-Chip/Q-TOFMS before and after being subjected to beta-elimination to cleave O-linked glycans.

Although analysis of the pre-beta-eliminated sample showed no evidence for product 1, LC-nanoESI analysis of the beta-eliminated sample showed a chromatographic peak appearing at the retention time characteristic of synthetic 1, and producing a mass spectrum with *m/z* values consistent with the mass of product 1 (M = 2,300.90 Da, mass measurement error < 5 ppm) (supplemental Fig. S4 and supplemental Table S2). Furthermore, when the [M+4H]<sup>4+</sup> ion at *m/z* 601.24 from putative product 1 was subjected to MS/MS analysis, the measured mass spectrum showed excellent agreement with the synthetic standard (Figs. 7C, 7D; supplemental Table S4). These results suggest that one metabolic fate of Ac<sub>4</sub>GlcNAz in *Hp* is conversion to HexNAz (presumably GlcNAz) and incorporation into *Hp*'s O-linked glycoproteins.

Although the above data indicate the presence of a Staudinger ligation-glycan adduct in an azide-labeled glycoprotein sample, we sought to confirm that these adducts are absent from acetyl-labeled controls. Because of the metastable water losses observed with the Phos-FLAG-His<sub>6</sub> tag and the multiplicity of charge states, we chose to conduct fol-

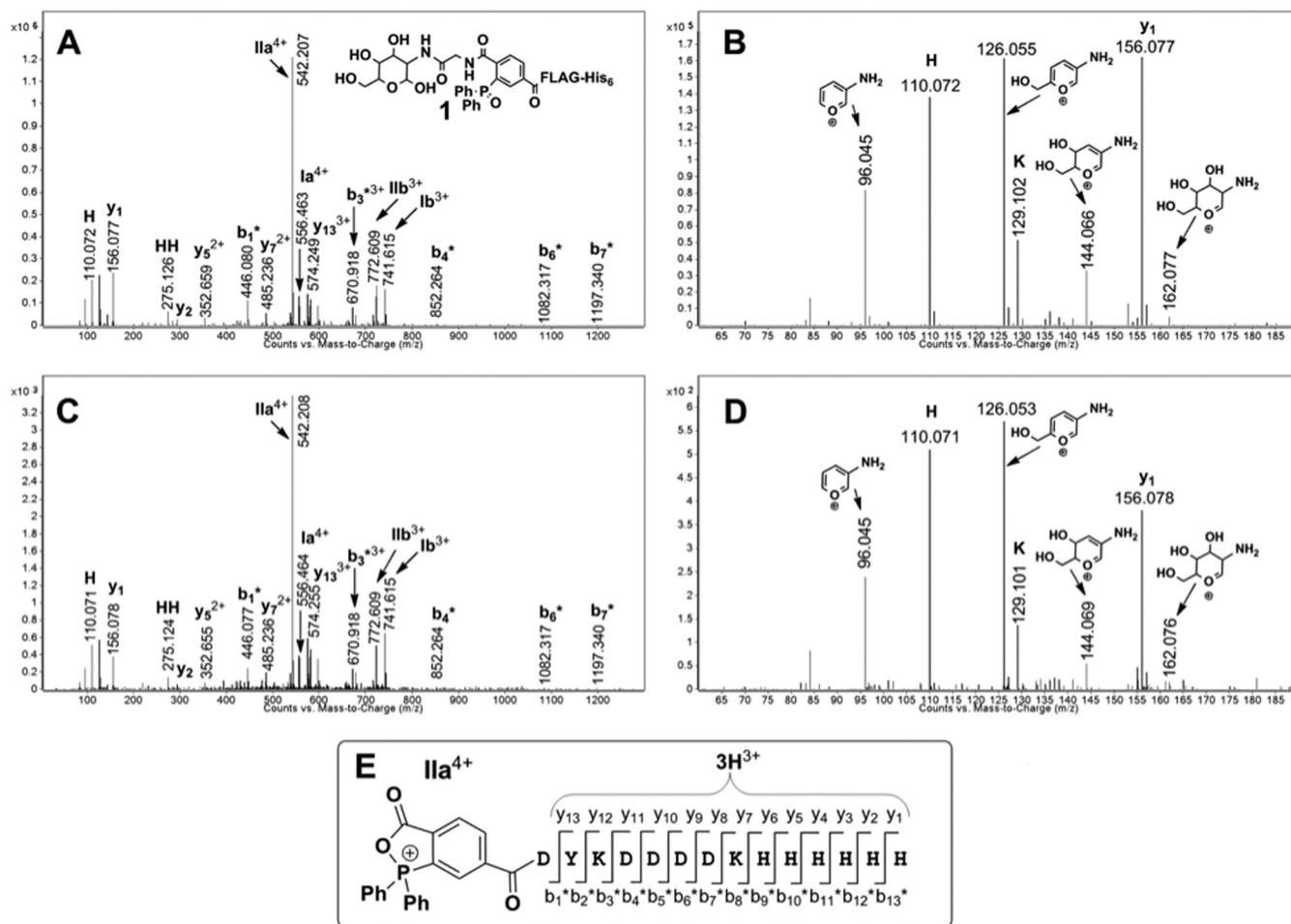


FIG. 7. LC-nanoESI-CID-MS/MS spectra of (A, B) synthetic standard GlcNAz-Phos-FLAG-His<sub>6</sub> and (C, D) beta-eliminated glycans from enriched azide-labeled glycoprotein sample. A, CID-MS/MS spectrum of GlcNAz-Phos-FLAG-His<sub>6</sub> synthetic standard **1** showing ions Ia<sup>4+</sup> and Ib<sup>3+</sup> (see Fig. 6) resulting from glycan loss, and ions Ila<sup>4+</sup> and Ilb<sup>3+</sup> following net loss of CO, NH = CH<sub>2</sub> (Fig. 6). As summarized in (E), subsequent fragmentation of ion Ila<sup>4+</sup> is proposed to yield y-type fragment ions that include the C terminus of the FLAG-His<sub>6</sub> peptide and b-type ions, labeled with an asterisk, that include the Phos-modified N terminus. B, Expansion (magnified 5x along the y axis) of the low-mass region from (A) displays a variety of peaks that include glycan-derived fragments with proposed structures represented. Immonium ions (H and K) are also detected in this mass range. C, CID-MS/MS spectra of beta-eliminated glycans from enriched azide-labeled glycoprotein sample showing mass spectral features that agree with those of the synthetic standard (A). D, Expansion (magnified 5x along the y axis) of the low-mass region from (C), showing the production of glycan-derived peaks from the beta-eliminated putative HexNAz-Phos-FLAG-His<sub>6</sub> (**1**). E, Schematic representation of ions (y- and b-type) derived from further Ila<sup>4+</sup> fragmentation. The asterisk associated with the b-type ions denotes a Phos-modified N terminus. Charge states are labeled when greater than +1. All CID-MS/MS spectra were measured using nitrogen as the collision gas, a 4 Da window for ion isolation, and collision energy of 20 V.

low-up experiments with the less complex Phos-FLAG tag. We first characterized synthetic GlcNAz-Phos-FLAG (**2**) (M = 1578.54 Da) by LC-nanoESI and CID. With the basic His<sub>6</sub> chain eliminated, **2** yielded a nanoESI mass spectrum dominated by the lower charge state [M+2H]<sup>2+</sup> ion at m/z 790.28 (supplemental Fig. S5; supplemental Table S5). As was observed for **1**, the CID-MS/MS spectrum for GlcNAz-Phos-FLAG (**2**) (Fig. 8A and 8B) was dominated by peaks resulting from neutral glycan loss via pathway (a) to form product ions Ia<sup>2+</sup> and Ila<sup>2+</sup> (Figs. 6 and 8A); however, glycan-derived fragments (m/z = 96.04, 126.05, 144.07 and 162.08) were weaker, whereas y- and b-type sequence ions (Figs. 8A and 8E) were

more abundant in the spectrum of GlcNAz-Phos-FLAG (**2**), a change that may result from the lower charge state of this precursor.

With characterization of the targeted analyte in hand, lysates from *Hp* supplemented with Ac<sub>4</sub>GlcNAz or Ac<sub>4</sub>GlcNAc were rinsed extensively to remove residual free sugar, and then subjected to Staudinger ligation with Phos-FLAG. After extensive washing to remove unreacted Phos-FLAG from labeled lysates via ultrafiltration, samples were analyzed by HPLC-Chip/Q-TOFMS before and after being subjected to beta-eliminations to cleave O-linked glycans. LC-nanoESI analysis of the beta-eliminated glycans from the azide-labeled

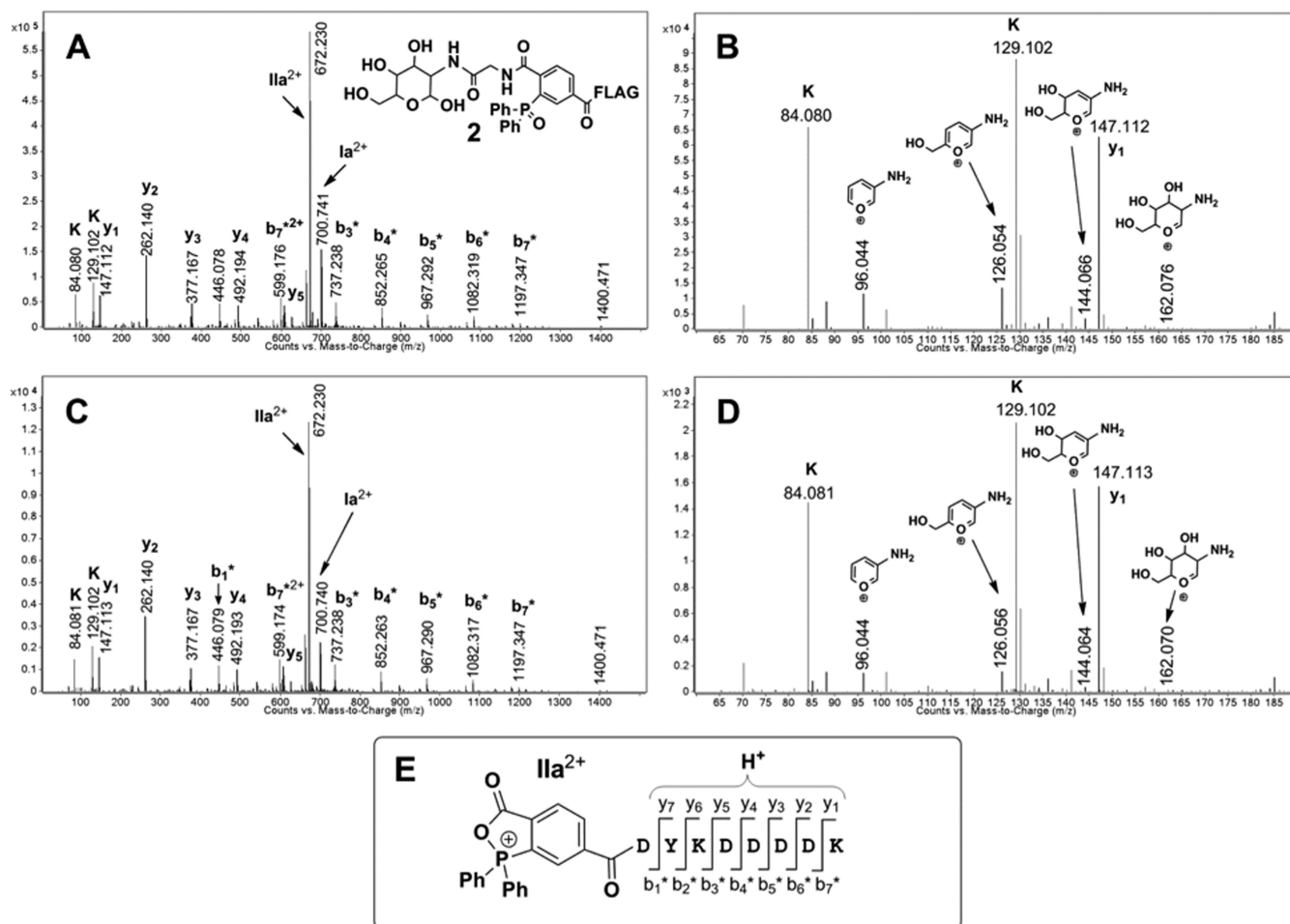
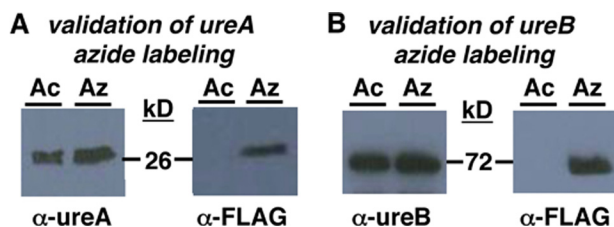


FIG. 8. LC-nanoESI-CID-MS/MS spectra of (A, B) synthetic standard GlcNAz-Phos-FLAG (**2**) and (C, D) beta-eliminated glycans from an azide-labeled, Phos-FLAG-ligated, *Hp* cell lysate. A, CID-MS/MS spectrum of GlcNAz-Phos-FLAG synthetic standard **2** showing ions  $Ia^{2+}$  (see Fig. 6) resulting from glycan loss, and ions  $Ia^{2+}$  following net loss of CO, NH = CH<sub>2</sub> (Fig. 6). As summarized in (E), subsequent fragmentation of ion  $Ia^{2+}$  is proposed to yield y-type fragment ions that include the C terminus of the FLAG peptide and b-type ions, labeled with an asterisk, that include the Phos-modified N terminus. B, Expansion of the low-mass region from (A) displays a variety of peaks that include glycan-derived fragments with proposed structures represented. Immonium ions (K) are also detected in this mass range. C, CID-MS/MS spectra of beta-eliminated glycans from an azide-labeled, Phos-FLAG-ligated, *Hp* cell lysate showing mass spectral features that agree with those of the synthetic standard (A). D, Expansion of the low-mass region from (C), showing the production of glycan-derived peaks from the beta-eliminated putative HexNAz-Phos-FLAG (**2**). E, Schematic representation of ions (y- and b-type) derived from further  $Ia^{4+}$  fragmentation. The asterisk associated with the b-type ions denotes a Phos-modified N terminus. Charge states are labeled when greater than +1. All CID-MS/MS spectra were measured using nitrogen as the collision gas, a 4 Da window for ion isolation, and collision energy of 35 V.

sample revealed a doubly-charged peak at  $m/z$  790.28 that corresponds to the mass of HexNAz-Phos-FLAG (**2**) ( $M = 1578.54$  Da, mass measurement error  $< 5$  ppm; supplemental Fig. 6E and supplemental Table S2). The CID-MS/MS spectrum of the peak at  $m/z$  790.28 (Fig. 8C and 8D; supplemental Table S6) is consistent with the Staudinger ligation product between GlcNAz and Phos-FLAG - HexNAz-Phos-FLAG (**2**). This HexNAz-Phos-FLAG adduct is not detectable in the beta-eliminated sample from the corresponding acetyl-labeled control (supplemental Fig. S6C), nor is it present at detectable levels in tagged azide- or acetyl-labeled lysates before beta-elimination (supplemental Fig. S6A and S6B). The exclusive detection of this adduct in beta-eliminated azide-

labeled samples indicates that it is derived from glycosylated *Hp* proteins and that beta-elimination conditions released this glycan adduct. The absence of this adduct in samples before beta-elimination further supports the conclusion that this adduct is derived from *Hp* glycoprotein conjugates and is released by the beta-elimination reaction. These data provide strong evidence that some *Hp* glycoproteins are covalently modified with O-linked HexNAz.

N-acetylhexosamine (HexNAc) residues are found in a number of characterized bacterial glycoproteins, including those synthesized by *Campylobacter jejuni* (73) and *Helicobacter pullorum* (74). Indeed, *H. pullorum* has an N-linked protein glycosylation system that makes use of a pentasaccharide



**FIG. 9. Secondary screens to assess the glycosylation status of urease alpha (ureA) and urease beta (ureB) subunits.** A, Western blot analysis of immunopurified ureA from *Hp* treated with Ac<sub>4</sub>GlcNAz (Az) or Ac<sub>4</sub>GlcNAc (Ac). These samples were reacted with Phos-FLAG, electrophoresed, and then probed with anti-ureA or anti-FLAG antibody. B, Western blot analysis of immunopurified ureB from *Hp* treated with Ac<sub>4</sub>GlcNAz (Az) or Ac<sub>4</sub>GlcNAc (Ac). These samples were reacted with Phos-FLAG, electrophoresed, and then probed with anti-ureB or anti-FLAG antibody.

with a core HexNAz. Structural studies are underway in our laboratory to assess whether *Hp*'s O-linked HexNAz is elaborated on to form a higher order glycan.

In our experiments, detection of HexNAz-Phos-FLAG-His<sub>6</sub> and HexNAz-Phos-FLAG adducts by HPLC-Chip/Q-TOFMS analysis was greatly aided by the presence of the peptide moiety, which has favorable chromatographic and ionization characteristics. Thus, MOE facilitated detection of *Hp*'s labeled glycans in unanticipated ways. If chromatographic resolution and signal enhancement are generalizable, metabolic glycan labeling coupled to reaction with a readily ionizable tag has the potential to amplify glycan detection in myriad bacteria and overcome challenges associated with the poor resolution and ionizability of glycans.

*ureA and ureB are Biochemically Validated Glycoproteins*—Of the putative glycoproteins identified, two of them - urease alpha (ureA) and urease beta (ureB) - are surface-associated colonization and persistence factors (75). We sought to evaluate their glycosylation status, because this has important implications for host-pathogen interactions and the development of novel anti-*Hp* therapeutics. To assess the glycosylation status of ureA and ureB, these proteins were immunopurified from *Hp* treated with 1 mM Ac<sub>4</sub>GlcNAz or Ac<sub>4</sub>GlcNAc, reacted with Phos-FLAG, and then analyzed by Western blot. Probing immunopurified ureA with anti-ureA revealed the presence of a band at ~27 kDa (Fig. 9A), indicating successful enrichment of ureA. Further, probing with anti-FLAG antibody revealed the presence of azide-dependent signal in ureA (Fig. 9A). Similar analyses of immunopurified ureB indicated successful enrichment of ureB (Fig. 9B) and the presence of azide-dependent signal in ureB (Fig. 9B). These results suggest that ureA and ureB are glycosylated with azide-modified glycans.

Based on our glycan mass spectrometry results, we were curious to see whether urease (composed of alpha and beta subunits) is among the glycoproteins modified with O-linked HexNAz in *Hp*. To assess this possibility, urease was biochemically purified from Ac<sub>4</sub>GlcNAz-labeled *Hp*. Purified ure-

ase (supplemental Fig. S7) was reacted with Phos-FLAG and rinsed extensively to remove residual Phos-FLAG. *Hp*'s labeled urease was analyzed by HPLC-Chip/Q-TOFMS before and after being subjected to beta-elimination to cleave O-linked glycans. Although analysis of the pre-beta-eliminated sample showed no evidence for product 2 (supplemental Fig. S8A), LC-nanoESI analysis of the beta-eliminated sample showed a chromatographic peak appearing at the retention time characteristic of synthetic HexNAz-Phos-FLAG (2) (supplemental Fig. S8B) and producing a mass spectrum (supplemental Fig. S8C) with *m/z* values consistent with the mass of product 2 ( $M = 1578.54$  Da, mass measurement error < 5 ppm; supplemental Table S2). Furthermore, when the  $[M+2H]^{2+}$  ion at *m/z* 790.28 from putative product 2 was subjected to MS/MS analysis, the measured mass spectrum showed excellent agreement with the synthetic standard (supplemental Figs. S8D and S8E; supplemental Table S7). These results suggest that urease is glycosylated with O-linked HexNAz and have implications for the development of urease-based therapeutic strategies to prevent and treat *Hp* infection. Taken together, these data validate urease as a glycosylated protein and indicate that MOE is a robust approach to profiling bacterial glycoproteins.

#### CONCLUDING REMARKS

Glycosylation of *Hp*'s flagellin proteins has been directly linked to *Hp*'s pathogenesis, suggesting that *Hp*'s glycoproteins are potential targets of therapeutic intervention. Despite their potential therapeutic importance, only the two flagellin glycoproteins have been firmly characterized in *Hp* (7). Mounting evidence from our lab (76) and others (15) suggests protein glycosylation is common in *Hp*. Employing experimental approaches to selectively study and identify *Hp*'s glycoproteins should facilitate the discovery of new therapeutic targets and shed light on the role of protein glycosylation in *Hp*'s physiology. In this article, we investigated the production of glycosylated proteins in *Hp* using a combination of metabolic glycan labeling and mass spectrometry analysis. Metabolic labeling of glycans with unnatural sugars and subsequent enrichment has been applied to identify glycoproteins in eukaryotic systems (29, 56, 77). Though metabolic glycan labeling has been used to study bacterial glycolipids (78), to our knowledge this study is the first to demonstrate the applicability of this chemical approach to bacterial glycoproteomics (79).

Metabolic incorporation of azide-labeled sugars, followed by reaction with phosphine probes via Staudinger ligation, facilitates detection, visualization, and enrichment of cellular glycoproteins (29). We began our studies by assessing the subcellular distribution of *Hp*'s azide-labeled glycoproteins to gain insight into their potential functions. Western blot data indicated that glycoproteins are present throughout *Hp*, including on the cell surface, embedded in both the inner and outer membranes, and within the periplasm and cytoplasm.



The observed subcellular distribution suggests that protein glycosylation is abundant, widespread, and critical for *Hp*'s physiology. Broadly, glycans modulate protein function in one of two ways: by providing epitopes for binding and recognition by other proteins (80, 81), and by stabilizing proteins (e.g. folding, solubility, protease susceptibility) (82–84). *Hp*'s intracellular glycans are likely involved in stabilizing proteins, whereas those exposed to the extracellular milieu (surface and secreted glycoproteins) may stabilize proteins as well as modulate interactions with the host.

Based on our results, the subcellular distribution of glycoproteins in *Hp* appears to be broader than that observed in other bacteria to date. For example, in *C. jejuni* (85, 86), *Neisseria sp.* (20, 87), and *Bacteroides sp.* (27), general protein glycosylation systems are present within the periplasm and modify proteins that traffic through this compartment (e.g. periplasmic, membrane-bound, and secreted proteins). In *Actinobacillus*, in contrast, there is a general protein glycosylation system within the cytosol that modifies cytosolic proteins (88). Finally, in *Haemophilus influenzae*, monosaccharides are added to proteins in the cytoplasm and then transported to the cell envelope (89). Future research will have to be conducted to confirm the localization of our candidate glycoproteins. If the observed, broad distribution is confirmed by follow-up experiments, this result suggests that *Hp* may have two general protein glycosylation systems (one within the cytosol and one within the periplasm) or an exclusively cytoplasmic glycosylation pathway that modifies proteins before sorting. Based on predictions by the carbohydrate-active enzymes (CAZy) database (90), there are 22 glycosyltransferases within *Hp* strain 26695, and half of these have unknown functions. Therefore, there are numerous glycosyltransferases within *Hp* that may be responsible for the synthesis of the observed glycoproteins. Characterizing which of these genes are responsible for carbohydrate assembly pathways is challenging because, in contrast to most bacteria, the genes for *Hp* carbohydrate biosynthesis pathways (e.g. O-antigen biosynthesis) are spread throughout the chromosome (91). Efforts are underway in our laboratory to identify the glycosyltransferases responsible for protein glycosylation in *Hp*.

We were excited to observe that a subset of *Hp*'s azide-modified glycoproteins are present on the cell surface, as these surface-exposed glycoproteins and the glycans that modify them are potential therapeutic targets. For example, these glycosylated proteins could provide the basis for a carbohydrate-based vaccine (14). Unusual bacterial monosaccharides, such as di-*N*-acetylbacillosamine produced by *Neisseria sp.* (92), can themselves be immunogenic. These glycans, in the context of a glycosylated protein, may serve as particularly effective conjugate vaccines. Alternatively, glycosylation pathways could be targeted with small molecule inhibitors to interrupt interactions between *Hp* and its host. Finally, our demonstration that azide-labeled glycoproteins

are present on the cell surface suggests that azide-covered *Hp* could be targeted with therapeutic phosphines. Covalent modification with properly designed phosphines could disrupt glycan function or render *Hp* innocuous within the host (93). Indeed, Kaewsapsak *et al.* designed phosphine therapeutics conjugated to the immune stimulant 2,4-dinitrophenyl to selectively kill *Hp* covered with azide-labeled glycans (55).

Staudinger ligation with Phos-FLAG-His<sub>6</sub> allowed for selective and efficient isolation of *Hp*'s azide-labeled glycoproteins. MudPIT analyses of enriched azide-labeled proteins and a mock-enriched control enabled the unambiguous identification of 125 putative glycoproteins. We identified a large number of glycoprotein hits in this study relative to the small number (nine) identified in Creuzenet and coworkers' recent periodic acid/hydrazide-based screen of *Hp*'s glycoproteins (15). We attribute much of this difference to the high incidence of background signal associated with periodic acid/hydrazide chemistry (15), rendering the identification of glycoprotein signal relative to nonglycoprotein noise difficult. In contrast, metabolic glycan labeling has a very high signal-to-noise ratio, enabling the facile detection and selective enrichment of glycoproteins. Thus, metabolic glycan labeling offers a complementary and efficient alternative to existing approaches for bacterial glycoprotein detection and identification.

In our studies, we detected Staudinger ligation-HexNAz adducts in beta-eliminated glycan samples from enriched azide-labeled glycoproteins, azide-labeled *Hp*, and purified azide-labeled urease. The exclusive presence of these adducts in samples from azide-labeled *Hp* provides evidence that the Staudinger ligation is exquisitely selective for azide-bearing molecules. Moreover, these results indicate that one metabolic fate of Ac<sub>4</sub>GlcNAz in *Hp* is conversion to HexNAz. The addition of the Phos-FLAG moiety onto the HexNAz epitope greatly enhanced the chromatographic resolution, ionization, and detection of this glycan species. Optimization of the phosphine probe to contain isotopic tags or characteristic fragment ions will further facilitate glycoprotein identification and glycan characterization. Thus, MOE is a robust approach for glycoprotein discovery.

A large number of proteins with known links to colonization, persistence and virulence were identified as glycoprotein hits (Table I), and two of these colonization factors, ureA and ureB, were biochemically validated as glycoproteins in our studies. This finding is consistent both with previous studies indicating that flagellin glycosylation is important to *Hp*'s ability to survive within the host (94), and more broadly, with the known links between protein glycosylation and virulence in a number of medically significant bacterial pathogens (13, 14). Further, this observed link underscores the importance of *Hp*'s glycoproteins as potential therapeutic targets.

The discovery that the urease subunits are glycosylated has potential implications for the development of urease-based therapeutic strategies to prevent and treat *Hp* infection. Vaccines based on recombinant urease have had some success

in preclinical (95, 96) and clinical studies (97, 98). However, these vaccines are based on recombinant proteins produced in *Escherichia coli* or *Salmonella* in their nonglycosylated state. New vaccines based on *Hp*'s natively glycosylated urease have the potential to stimulate a robust immune response and thus protect patients more effectively than those based on recombinant urease.

In summary, we were able to use metabolic glycan labeling to enrich and identify 125 putative *Hp* glycoproteins. Further, we validated the glycosylation status of two of these hits. Finally, we characterized the resulting glycan adducts. These results reveal that glycosylated proteins are abundant in *Hp*, indicate that *Hp*'s glycoproteins have a wide range of functions, and reveal new links between *Hp*'s pathogenesis and glycosylation. Armed with this information, the stage is set for the development of glycosylation-based therapeutic strategies to eradicate *Hp* infection. In particular, this work opens the door to phosphine-based therapeutics that target *Hp*'s azide-labeled glycans.

Broadly, this work validates MOE as a viable approach to discover and characterize bacterial glycoproteins. In addition to enabling the discovery of *Hp*'s glycoproteins, MOE has the potential to facilitate the study of glycoproteins in other bacteria. Indeed, reports of metabolic labeling of glycoproteins with azide-modified pseudaminic acid in *C. jejuni* (54) and with alkyne-bearing fucosylated sugars in *Bacteroidales sp.* (99) suggest that this glycoproteomic approach should transfer readily to these organisms. The ability to use a general metabolic precursor rather than a specific glycan-binding reagent will facilitate the detection and discovery of glycoproteins in a broad range of bacteria. Thus, MOE will propel bacterial glycoproteomics forward and, in the process, unveil novel therapeutic targets.

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☐ This article contains supplemental Figs. S1 to S8 and Tables S1 to S7.

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