INFECTION AND IMMUNITY, July 2002, p. 3396–3403 Vol. 70, No. 7
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.7.3396–3403.2002

Proteome Analysis of Secreted Proteins of the Gastric Pathogen *Helicobacter pylori*

Dirk Bumann,[1] Sevil Aksu,[2] Meike Wendland,[1] Katharina Janek,[3] Uschi Zimny-Arndt,[2] Nicolas Sabarth,[1] Thomas F. Meyer,[1]* and Peter R. Jungblut[2]

Department of Molecular Biology[1] and Central Core Facility for Protein Analysis,[2] Max-Planck-Institute for Infection Biology, and Charité, Institute for Biochemistry,[3] Berlin, Germany

Received 5 December 2001/Returned for modification 14 January 2002/Accepted 21 March 2002

Secreted proteins (the secretome) of the human pathogen *Helicobacter pylori* may mediate important pathogen-host interactions, but such proteins are technically difficult to analyze. Here, we report on a comprehensive secretome analysis that uses protein-free culture conditions to minimize autolysis, an efficient recovery method for extracellular proteins, and two-dimensional gel electrophoresis followed by peptide mass fingerprinting for protein resolution and identification. Twenty-six of the 33 separated secreted proteins were identified. Among them were six putative oxidoreductases that may be involved in the modification of protein-disulfide bonds, three flagellar proteins, three defined fragments of the vacuolating toxin VacA, the serine protease HtrA, and eight proteins of unknown function. A cleavage site for the amino-terminal passenger domain of VacA between amino acids 991 and 992 was determined by collision-induced dissociation mass spectrometry. Several of the secreted proteins are interesting targets for antimicrobial chemotherapy and vaccine development.

The widespread human pathogen *Helicobacter pylori* is a major cause of gastric and duodenal ulcers and gastric cancer (48, 53). To identify factors of *H. pylori* that are potentially involved in pathogen-host interactions (11), proteome analysis has been successfully used by numerous groups (4, 7, 14, 17, 21, 22, 24, 29, 37, 50). Secreted proteins (the secretome) may be of special importance, since these proteins come into direct contact with host compartments; however, technical difficulties have led to somewhat contradictory results and have prevented a comprehensive analysis (8, 19, 25, 34, 42, 51). *H. pylori* is commonly cultivated in rich media complemented with various additions of serum containing numerous foreign proteins that are difficult to resolve from extracellular *H. pylori* proteins. Only a few studies have used protein-free media (1, 19, 26, 38, 49, 51), and growth is usually much slower in such media. Moreover, *H. pylori* is particularly prone to spontaneous autolysis (34), resulting in the nonspecific release of numerous proteins; the latter makes the interpretation of protein patterns obtained from culture supernatants difficult.

In this study, we optimized culture conditions for minimal autolysis, adapted a precipitation method for the optimal recovery of extracellular proteins, resolved the various secreted proteins by two-dimensional gel electrophoresis, and identified 26 protein species. Based on a comparison of the intensities of staining of specific protein species in supernatants and whole-cell samples, we obtained a semiquantitative estimate for secretion selectivity. Among the secreted proteins were several redox-active enzymes, various components of the flagellar apparatus, three fragments of the vacuolating cytotoxin VacA, the serine protease and chaperone HtrA, and several previously uncharacterized proteins that are potential targets for therapy and vaccine development. To our knowledge, this is the first comprehensive analysis of the *H. pylori* secretome.

* Corresponding author. Mailing address: Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie, Schumannstraβe 21/22, D-10117 Berlin, Germany. Phone: 49 30 28460 400. Fax: 49 30 28460 401. E-mail: meyer@mpiib-berlin.mpg.de.

MATERIALS AND METHODS

Culture conditions. We tested a number of different protein-free media for *H. pylori* broth growth and obtained the best results for strains 26695 and J99 with brain heart infusion (BHI) broth supplemented with β-cyclodextrin as described previously (1, 19, 51); however, under our conditions, 1% cyclodextrin allowed for better growth than the previously used lower concentration (0.1%).

H. pylori was first cultured at 37°C on serum agar plates containing vancomycin, nystatin, and trimethoprim (31) in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) for 3 days. Plate-grown bacteria were resuspended and washed in BHI. Fifteen milliliters of BHI containing vancomycin, nystatin, trimethoprim, and 1% β-cyclodextrin was inoculated with *H. pylori* cells to obtain an optical density at 600 nm (OD_{600}) of 0.02. This culture was grown overnight at 37°C and 150 rpm in a microaerobic atmosphere to reach an OD_{600} of 0.5 to 1. The bacteria were recovered by centrifugation, washed with BHI, and used to inoculate a second liquid culture (seven cultures, 60 ml each) to obtain an OD_{600} of 0.01. After 20 h of growth at 37°C and 150 rpm, the cultures typically reached the midexponential growth phase with an OD_{600} of 0.3 to 0.5, equivalent to about 4×10^8 CFU ml^{-1}.

Precipitation of extracellular proteins. The exponential cultures were centrifuged for 15 min at 20,000 × *g* and 4°C, and the supernatant was filtered through a 0.45-μm-pore-size membrane filter to remove residual bacteria. Extracellular proteins were precipitated by using a recently described modified trichloroacetic acid (TCA) method (16). The filtrate (380 ml) was mixed with 120 ml of prechilled 25% TCA and incubated on ice-water for 15 min. The mixture was centrifuged for 10 min at 10,000 × *g* and 4°C, the pellet was resuspended in 10 ml of acetone and dissolved by using an ultrasonic water bath, and the mixture was centrifuged. Acetone washing was repeated twice, and the final pellet was air dried.

Two-dimensional gel electrophoresis. Protein samples were solubilized for 30 min at an ambient temperature in 9 M urea–1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)–70 mM dithiothreitol (DTT)–2% Servalyte (pI 2 to 4) (Serva). For the resolution of protein samples, a two-dimensional gel electrophoresis system (23 by 30 cm) was used (14). For the identification of proteins and the comparative quantification of Coomassie brilliant blue G-250 staining intensities, 200 to 300 μg (whole-cell samples) or 50 to 70 μg (extracellular proteins) was applied to the anodic side of the isoelectric focusing gel. Spots were identified and quantified by using the gel analysis program TOPSPOT.

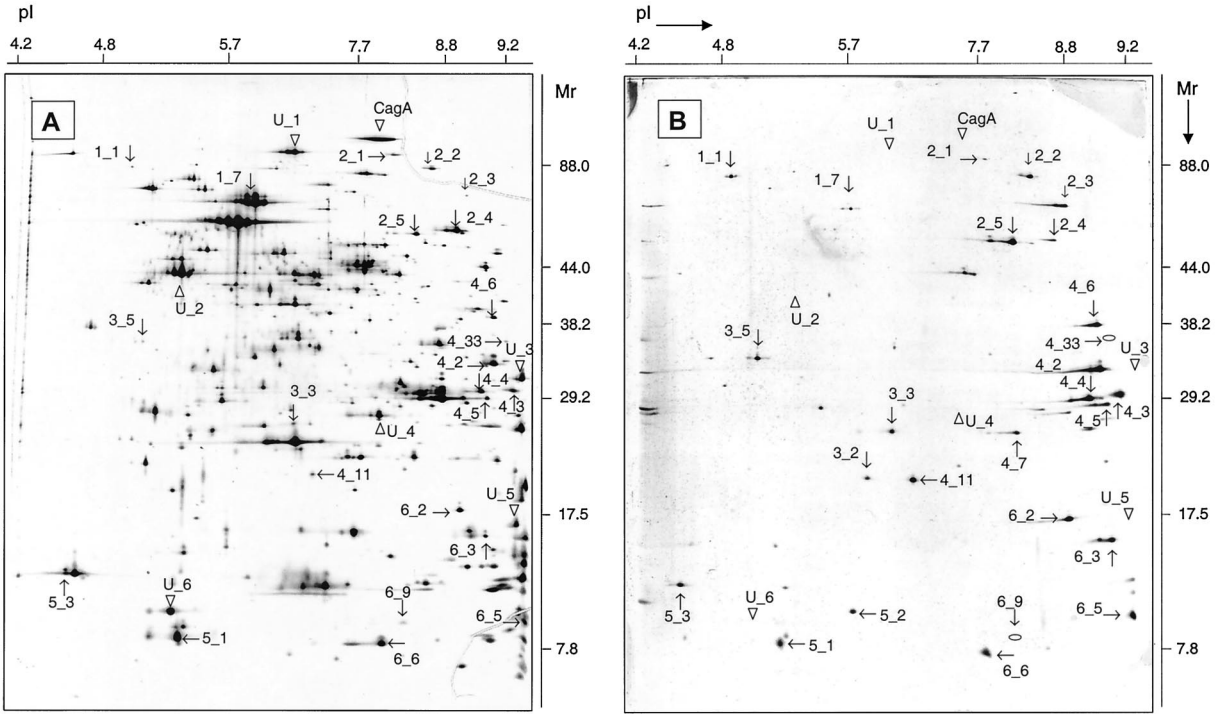

FIG. 1. Protein compositions of whole-cell proteins (A) and secreted proteins (B) of *H. pylori* 26695. Two-dimensional gel electrophoresis was done with *H. pylori* harvested during exponential growth in liquid cultures with a protein-free medium. The numbers correspond to those in Table 1. Proteins were detected by silver staining. Spots 4_33 and 6_9 of the secretome were visualized only in Coomassie brilliant blue G-250-stained patterns. Their positions are marked by ellipses on the silver-stained standard patterns. Six abundant protein species that had putative cytosolic localizations and functions but that were not detected in the supernatants are marked with open arrowheads and labeled U_1 to U_6.

Peptide mass fingerprinting. Proteins were identified by peptide mass fingerprinting after in-gel tryptic digestion of excised spots (14). The peptide mixture was mixed (1:1) with a saturated α-cyano-4-hydroxycinnamic acid solution in 50% acetonitrile–0.3% trifluoroacetic acid (TFA), and 2 μl was applied to the sample template of a matrix-assisted laser desorption ionization mass spectrometer (Voyager Elite; Perseptive). Peptide mass fingerprints were searched with the program MS-FIT (http://prospector.ucsf.edu/ucsfhtml/msfit.htm) by using all *H. pylori* proteins in the National Center for Biotechnology Information database, allowing an accuracy of 100 ppm for the peptide masses. Partial enzymatic cleavages leaving two cleavage sites, oxidation of methionine, pyroglutamic acid formation at N-terminal glutamine, and modification of cysteine by acrylamide were considered in these searches.

To determine the sequence of a putative carboxy-terminal peptide from a VacA protein fragment (spot 5_2), a tryptic peptide mixture was separated by reversed-phase capillary chromatography on a PepMap C_{18} column (0.3 by 150 mm, 5 μm, 100 Å; LC Packings, Amsterdam, The Netherlands) with linear gradient elution (eluent A, 0.05% TFA in water; eluent B, 0.045% TFA in 70% acetonitrile) at a flow rate of 4 μl/min. Identification of the peptide from amino acids (aa) 982 to 991 of the vacuolating cytoxin precursor was performed online with an electrospray ion trap mass spectrometer (LCQ; Finnigan, San Jose, Calif.) by collision-induced dissociation (CID). The mass spectrometer was operated in the constant CID mode, and CID spectra were obtained for the ion m/z = 587.8 during the entire liquid chromatography run. A mass spectrometry ion search was carried out with the program Mascot (http://www.matrixscience.com) by using the National Center for Biotechnology Information database.

RESULTS

Secreted proteins of the human pathogen *H. pylori* are of special interest because they come in direct contact with host tissues and may mediate important pathogen-host interactions. To identify secreted proteins of *H. pylori* by using proteome analysis, a protein-free culture medium is required. Both tested *H. pylori* strains, 26695 and J99, grow well in BHI supplemented with 1% β-cyclodextrin, with division times of 3 to 4 h; this growth is comparable to the growth of *H. pylori* in standard liquid media containing fetal calf serum. Starting from plate cultures, two short consecutive liquid cultures were used, with a washing step in between to minimize any contamination with material released from dead bacteria during the initial plate culture.

Extracellular proteins of bacterial cultures are usually recovered by precipitation of filtered culture supernatants with TCA. Standard TCA precipitation procedures give a poor yield for *H. pylori* supernatants, and solubilized precipitates are difficult to resolve by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). We therefore used a recently developed alternative TCA precipitation method (16) that results in high yields and well-resolved gel electrophoresis patterns.

Spontaneous lysis of *H. pylori* has been repeatedly found to obscure selective protein secretion (8, 19, 25, 34, 42, 51). While lysis would release a majority of the protein species, selective secretion should result in only a few extracellular proteins. To assess the relative roles of lysis and secretion in our cultures, we compared the protein compositions of culture supernatants and whole *H. pylori* cells by using two-dimensional gel electrophoresis. *H. pylori* 26695 and J99 supernatants contain only a few protein species, and most species that are present in the corresponding whole *H. pylori* lysates are lacking in the supernatants (Fig. 1A versus B). Among previously identified proteins (14) that are abundant in lysates but absent in supernatants, there are three ribosomal proteins (RplA, spot U_3; RplJ, spot U_5; and Rpl7/l12, spot U_6), the transcription factor EF-Tu (spot U_2), an enzyme of the fatty acid biosyn-

TABLE 1. Identified extracellular proteins of *H. pylori*

TOPSPOT no.	Selectivity[a]	Protein		Signal peptide[b]	% Sequence coverage[c]	Theor./pract.[d]		Protein class[e]
		Designation	Name			M_r	pI	
2_2	++	HP0887	Vacuolating cytotoxin (fragment) VacA	+	43	139,312.8/87,970.0	9.02/8.35	D3
1_1	++	HP0870	Flagellar hook protein FlgE	−	39	76,207.0/87,900.0	5.04/4.82	C4
5_3	+	HP1161	Flavodoxin FldA	−	57	17,492.5/12,380.0	4.45/4.46	F5
6_2	+	HP1286	Conserved hypothetical secreted protein	+	46	20,614.9/17,500.0	9.21/8.80	O1
4_11	++	HP1118	γ-Glutamyltranspeptidase (fragment)	+	19	61,151.7/20,980.0	9.27/6.27	B10
2_5	++	HP1019	Serine protease HtrA	−	45	47,983.8/58,440.0	8.96/8.22	L3
1_7	Reference	HP0072	Urease B (UreB)	−	28	61,671.0/74,000.0	5.64/5.69	E5
3_5	++	HP0907	Hook assembly protein (flagella) FlgD	−	43	33,710.3/34,000.0	5.14/5.02	C4
5_2	++	HP0887	Vacuolating cytotoxin (fragment) VacA	+	6	139,313.3/10,536.0	9.02/5.70	D3
4_4	++	HP0231	Hypothetical protein	+	33	29,459.3/29,863.0	9.09/8.91	—
4_2	+	HP0175	Cell binding factor 2	+	30	34,031.2/33,170.0	9.29/8.95	C1
4_6	++	HP1118	γ-Glutamyltranspeptidase (fragment)	+	35	61,151.7/38,276.0	9.27/8.94	B10
2_1	+	HP0887	Vacuolating cytotoxin (fragment) VacA	+	47	139,313.3/95,741.0	9.02/8.01	D3
2_3	++	HP0906	Hypothetical protein	−	44	58,161.2/74,870.0	9.14/8.76	—
2_4	±	HP1118	γ-Glutamyltranspeptidase	+	45	61,151.9/59,328.0	9.27/8.62	B10
4_33	++	HP1454	Hypothetical protein	+	67	33,871.1/35,666.0	9.31/8.98	—
4_3	++	HP1098	Conserved hypothetical secreted protein	+	43	31,594.4/30,220.0	8.77/9.07	O1
4_5	+	HP1186	Carbonic anhydrase	+	57	23,215.8/29,200.0	9.23/8.96	E1
4_7	++	HP0367	Hypothetical protein	−	62	23,211.0/26,084.0	7.85/8.20	—
6_3	+	HP1173	Hypothetical protein	+	34	20,586.0/15,140.0	8.95/8.97	—
6_5	±	HP1118	γ-Glutamyltranspeptidase (fragment)	+	21	61,151.9/10,236.0	9.27/9.20	B10
6_9	++	HP1557	Flagellar hook-basal body complex protein FliE	−	38	12,195.9/9,082.0	7.97/8.00	C4
6_6	+	HP1458	Thioredoxin TrxC	−	67	11,744.8/7,800.0	7.72/7.70	B10
3_3	+	HP0377	Thiol-disulfide interchange protein DsbC	+	31	25,340.0/26,100.0	8.18/5.95	P1
3_2	++	HP1118	γ-Glutamyltranspeptidase (fragment)	+	17	61,151.7/21,100.0	9.27/5.79	B10
5_1	+	HP0824	Thioredoxin TrxA	−	52	11,854.8/8,390.0	5.16/5.18	B10

[a] Semiquantitative classification based on Coomassie brilliant blue G-250-stained gels; see the text for definitions. Reference, reference spot for nonsecreted species.
[b] +, yes; −, no.
[c] Based on complete protein sequences; protein fragments yield underestimated values.
[d] Theor./pract., theoretical and experimental molecular weights and isoelectric points.
[e] According to reference 48: D3, cellular processes—cell killing; C4, cell envelope—surface structures; F5, energy metabolism—electron transport; O1, hypothetical—general; B10, biosynthesis of cofactors, prosthetic groups, and carriers—thioredoxin, glutaredoxin, and glutathione; L3, translation—degradation of proteins, peptides, and glycopeptides; E5, central intermediary metabolism—other; C1, cell envelope—membranes, lipoproteins, and porins; E1, central intermediary metabolism—general; P1, unknown—general; —, not classified.

thesis pathway (FabG, spot U_4), and an enzyme of the TCA cycle (aconitate hydratase 2, spot U_1). All these proteins have a cytosolic localization and function in other bacteria, indicating that they might be used as tracers for the release of intracellular contents. Their absence in the *H. pylori* supernatants thus suggests that under our culture conditions, little if any cell lysis occurs.

On the other hand, differential protein stability could also result in the appearance of just a few proteins in the supernatants. Among many nonspecifically released proteins, most could be degraded, leaving only a few very stable proteins that could then be recovered by TCA precipitation. To test this possibility, *H. pylori* lysates were obtained by sonication and incubated for 2 days under our normal culture conditions. There was no obvious change in protein composition during this incubation period (data not shown), in agreement with earlier studies that failed to detect nonspecific protease activity in *H. pylori* (28). These results suggest that specific secretion instead of differential stability is the most likely cause for the selective recovery of a limited set of *H. pylori* proteins from the culture supernatants.

In total, there were 33 protein species that were reproducibly detected in supernatants of three independent *H. pylori* 26695 cultures by using Coomassie brilliant blue G-250 staining, and 26 of these were identified by using peptide mass fingerprinting of tryptic digests and comparison with protein data from the complete genome sequence (Table 1). The remaining seven spots contained too little material for reliable identification. Among the identified species, there was a weak spot (1_7) containing urease B. Urease B is the most abundant

protein in whole cells, and the low abundance in supernatants is consistent with the recent finding that this protein is not secreted by rapidly growing *H. pylori* cells (19). The very small amounts in supernatants might result from the lysis of a small proportion of cells. We used this spot as a reference to calculate a semiquantitative estimate for the selectivity of secretion.

For this purpose, staining intensities of all species were obtained from both the supernatants and the whole-cell samples by using an automated analysis program for two-dimensional gels, TOPSPOT. Proteins that are released together with urease B from the small fraction of lysed cells should have a similar abundance relative to that of urease B in both samples. On the other hand, species that are strongly overrepresented relative to urease B in supernatants are probably selectively secreted. For example, spot 2_2 has a 7-fold lower staining intensity than UreB in whole-cell lysates but an 11-fold higher staining intensity in supernatants, indicating an 80-fold selective enrichment; these results are in agreement with the well-known secretion by autotransport of the corresponding protein, VacA (41).

Since technical limitations make it difficult to obtain accurate data for protein abundance from staining intensities, we used a semiquantitative scoring system (±, 3- to 10-fold; +, 10- to 50-fold; ++, more than 50-fold) to represent the calculated enrichment factors (Table 1). Among the 26 identified species, 1 was considered to be nonsecreted (UreB; reference spot), 2 were only weakly overrepresented in the supernatants (±), 9 were moderately overrepresented (+), and 14 were strongly enriched (++). Both spots with apparently weak selectivity partially overlapped unrelated abundant proteins in whole-cell samples (spot 2_4 overlapped catalase; spot 6_5 overlapped ribosomal protein S10) (14), so that the comparative quantification probably yielded underestimated selectivity values. This problem might also exist for some other spots containing only one dominant protein species in supernatant samples but also some unrelated protein species in whole-cell samples; however, this situation would not interfere with interpretation, since the true selectivity of secretion would always be actually higher than estimated.

Sixteen of the identified species had putative signal peptide sequences for *sec*-dependent transport across the plasma membrane (Table 1). Among the 10 species lacking obvious signal sequences, three were homologous to flagellum-associated proteins that are known to be transported by the type III secretion apparatus of the flagellum (16), and one was nonsecreted UreB.

Most of the species have apparent molecular masses that are consistent with the values predicted from the genome sequence, although small changes, such as cleavage of signal peptides, would not be resolved. However, for vacuolating toxin VacA and γ-glutamyltranspeptidase, fragments of considerably smaller sizes are present in the supernatants. The most dominant VacA species have apparent molecular masses of about 90 kDa (spot 2_2) and about 12 kDa (spot 5_2), which are similar to those of the previously reported mature exotoxin and a hypothetical small fragment that was postulated because of an inconsistency between the summed molecular masses of detected fragments and the predicted value for the complete autotransporter protein (27). The detection of mass peaks in tryptic digests that match the fragments predicted for VacA allows the localization of these fragments within the sequence. For spot 2_2, matching masses were detected for peptides from aa 74 to 851 (Fig. 2), confirming the identification of this fragment as the 88.2-kDa mature exotoxin (aa 34 to 854) (27). For spot 5_2, matching masses were detected for peptides from aa 894 to 981 (Fig. 2 and 3A), indicating that this fragment is indeed a carboxy-terminal portion of the passenger domain of this autotransporter that is analogous to the α-protein of the homologous neisserial immunoglobulin A (IgA) protease (35). Interestingly, the masses of two fragments matched peptides from aa 982 to 991 and from aa 983 to 991. The sequence of the putative peptide from aa 982 to 991 was experimentally verified with CID mass spectrometry (Fig. 3B). The alanine instead of an arginine or a lysine at the carboxy terminus indicates that this peptide cannot have been generated by the tryptic digest but instead appears to constitute the carboxy terminus of protein species 5_2. These data suggest that the VacA passenger domain is released from the membrane by cleavage between aa 991 and 992. For the weaker spot, 2_1, matching masses were detected for peptides from aa 89 to 981, suggesting that this fragment contains the entire yet uncleaved passenger domain.

The most dominant species of γ-glutamyltranspeptidase are fragments with apparent molecular masses of 35 kDa (spot 4_6) and 23 kDa (spot 4_11), which are similar to what has been reported for this protein in *H. pylori* and homologous proteins in other bacteria (10). Mass spectrometric analysis suggests that the larger fragment (spot 4_6) is the N-terminal portion containing at least aa 37 to 342, whereas the smaller fragment (spot 4_11) is the C-terminal portion containing at least aa 448 to 564. This assignment is in good agreement with the previously postulated cleavage site at aa 379 (10). The weaker spot, 2_4, seems to represent an almost intact species containing at least aa 37 to 567.

CagA is a known secretion substrate of the *H. pylori* type IV secretion apparatus encoded on the Cag pathogenicity island. Interestingly, CagA is not detectable in supernatants, although it is abundantly expressed (Fig. 1), suggesting that the type IV secretion apparatus is not active under our culture conditions. This suggestion is in agreement with the results of previous studies, indicating that host cell contact is required for CagA secretion (4, 30).

The two-dimensional gel electrophoresis patterns of the secretome obtained under well-defined conditions and the identities of 26 protein species were introduced into the 2D-PAGE database (http://www.mpiib-berlin.mpg.de/2D-PAGE). Clicking on the spots of the presented two-dimensional gel makes available information on the protein identity, M_r, and pI; peptide mass fingerprinting mass spectra; protein sequence; gene sequence; references; potential posttranslational modifications; and other information.

DISCUSSION

Extracellular components of *H. pylori* are known to mediate multiple pathogen-host interactions during an infection. A few secreted molecules, such as VacA (41) and thioredoxin A (54), have been identified, but a comprehensive characterization of the secretome of *H. pylori* has remained difficult because of technical problems resulting in conflicting data on the secretion of a number of proteins (8, 19, 25, 34, 42, 51). Here, we established methods for culturing *H. pylori* with minimal lysis,

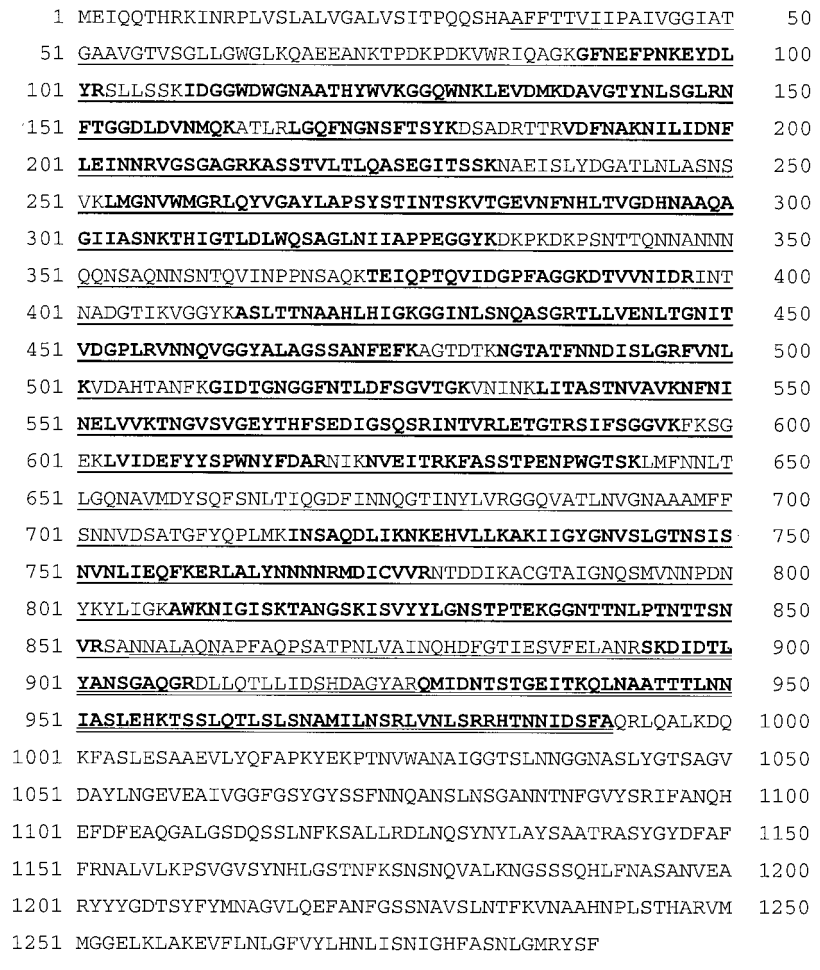

FIG. 2. Complete sequence of vacuolating cytotoxin VacA (HP0887). Peptides with matching masses in tryptic digests of spot 2_2 or spot 5_2 are shown in bold. The mature exotoxin (27) (underlining) and the carboxy-terminal fragment of the passenger domain (double underlining) are also indicated.

for efficiently recovering extracellular proteins, and for obtaining a semiquantitative estimate of the selectivity of extracellular secretion. The protein composition of extracellular proteins dramatically differed from that of whole-cell samples, as revealed by two-dimensional gel electrophoresis; many highly abundant *H. pylori* proteins, including several with a likely cytosolic localization, were undetectable in the extracellular samples, indicating that lysis was indeed minimal. Only 3 of the investigated 26 species in the supernatants had a borderline overrepresentation in the supernatants (less than 10-fold compared to nonsecreted urease B), and these specific instances might actually have been due to other, nonresolved proteins with strong staining in the whole-cell samples. Many of the residual species were overrepresented in the supernatants by more than 50-fold. This approach was successful for both of the completely sequenced strains tested, 26695 and J99, and additional strains are currently being studied.

Out of 33 protein species in the supernatants of *H. pylori* strain 26695 cultures, 26 were identified by using peptide mass fingerprinting of tryptic digests. In most instances, the identified species represented proteins with molecular masses compatible with those of the predicted complete proteins. This result indicates that little nonspecific protease activity is present in culture supernatants. On the other hand, both VacA and γ-glutamyltranspeptidase were present in smaller fragments with molecular masses that were similar to those of previously reported fragments of these proteins (10, 27). The mass spectrometric detection of specific tryptic fragments within the various species allowed us to verify previously postulated cleavage patterns of the corresponding intact proteins. A small VacA fragment that is cosecreted with the mature exotoxin was detected, and its carboxy terminus was identified by CID spectrometry. Although this fragment has not yet been functionally characterized, this dominant secreted species is an interesting candidate for host-pathogen interactions. The analogous α-protein of the neisserial IgA protease translocates to the nuclei of primary human cells in vitro (36) and seems to induce strong host immune responses in colonized human patients (13).

For some secreted proteins, putative secretion mechanisms can be predicted from well-characterized homologous proteins in other organisms. Three proteins of the flagellar apparatus are probably transported by a type III secretion mechanisms, as has been reported for homologous proteins in *Salmonella*

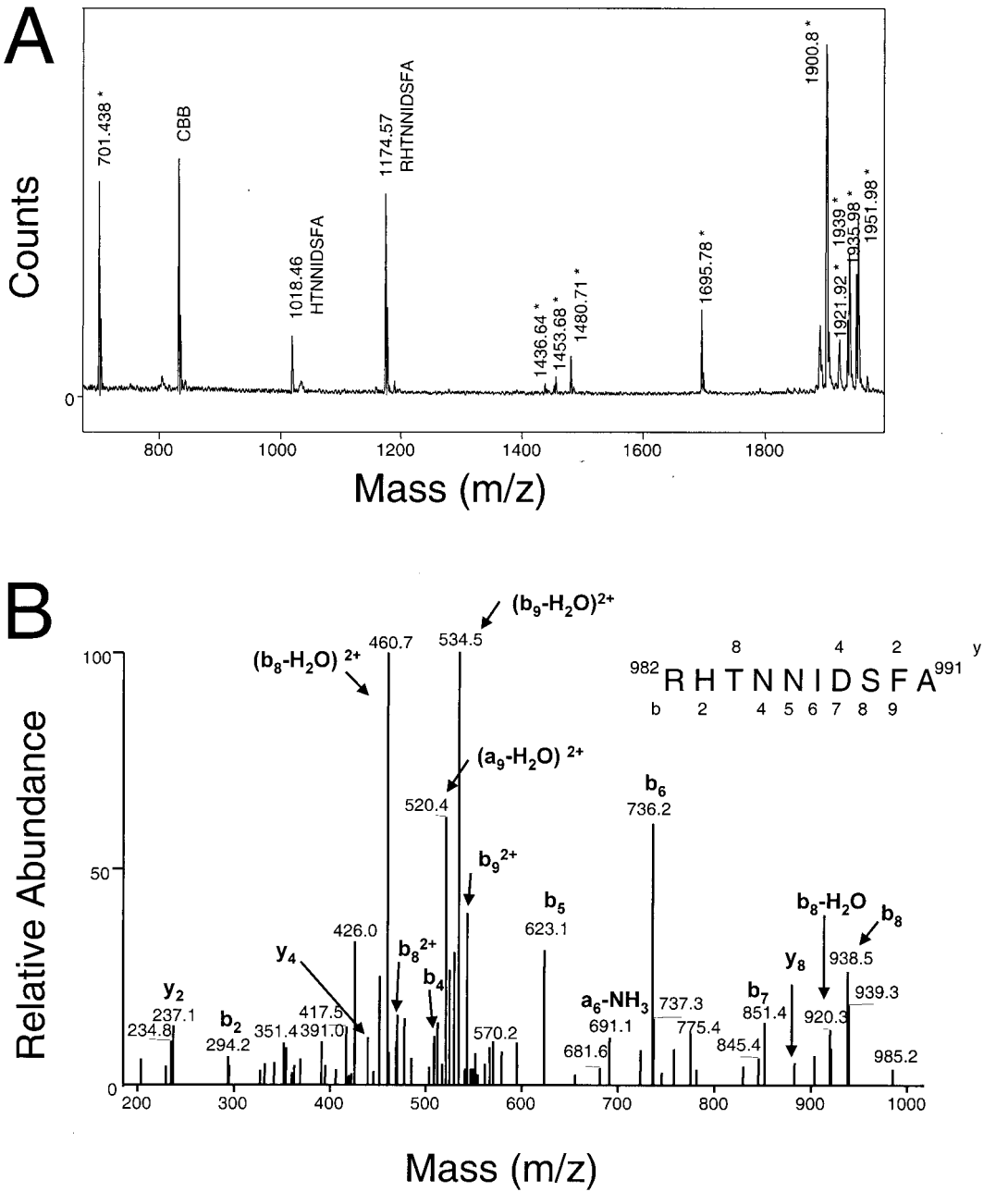

FIG. 3. Mass spectrometric characterization of spot 5_2. (A) Matrix-assisted laser desorption ionization mass spectrometric peptide mass fingerprint of a tryptic digest. All spots marked with an asterisk fit tryptic peptides of vacuolating cytotoxin VacA (HP0887) (Fig. 2). The peak designated CBB corresponds to Coomassie brilliant blue G-250. Peptide masses 1,018.46 and 1,174.57 fit peptides from aa 983 to 991 and from aa 982 to 991, respectively. (B) CID spectrum of a tryptic peptide with m/z = 587.8 (corresponding to a doubly-charged peptide with a mass of 1,174.6).

and other bacteria (16). The vacuolating toxin VacA is an autotransporter similar to the neisserial IgA protease (41).

For the other proteins, it is presently unclear by what mechanism they might be secreted. Six of them (with the exception of nonsecreted urease B) lack an obvious signal peptide, suggesting that they might be secreted by a *sec*-independent mechanism, such as a type I, type III, or type IV secretion system (40). A type IV secretion system that can translocate proteins into host cells is encoded on the *H. pylori* pathogenicity island (4) and could be involved in the secretion of some of the identified proteins (3, 30, 43, 46). However, this scenario is rather unlikely, since its only described secretion substrate, CagA, is absent in the culture supernatants; these data suggest that this secretion apparatus is inactive without contact with host cells, in agreement with previous data (4, 30). The flagella constitute a type III secretion system that might transport other proteins in addition to flagellar components, but this notion remains to be tested. Proteins with obvious homologies

to proteins of type I secretion systems are absent from the *H. pylori* genome.

Sixteen of the identified secreted species are derived from proteins with a potential signal peptide. Such proteins could be secreted by a type II secretion system or the two-partner secretion pathway or might be autotransporters. Proteins with obvious homology to type II secretion systems or the pore-forming protein FhaC of the two-partner secretion pathway are absent from the *H. pylori* genome. Autotransporters contain a C-terminal domain that forms a pore through which the N-terminal portion is transported. Release of this N-terminal portion occurs by proteolytic cleavage, whereas the C-terminal domain remains in the outer membrane. None of the identified secreted proteins, except for VacA, has significant homology to the autotransporter family, and none of them, except for VacA and γ-glutamyltransferase, appears in the supernatants as a considerably smaller N-terminal fragment of a pro-protein. In summary, the secretion pathway for most of the secreted proteins remains unclear, and as-yet-unknown pathways might be involved.

Independent of the actual secretion pathway, extracellular proteins of *H. pylori* might mediate important pathogen-host interactions as they directly contact host compartments. One well-characterized example is the prominent secreted protein, vacuolating cytotoxin (VacA), that induces a multitude of alterations to host cells (39). Besides VacA, other secreted proteins might also participate in pathogen-host interactions. Three of the secreted proteins (FlgE, FlgD, and FliE) are part of the flagellar apparatus of *H. pylori*. The release of flagellar proteins into the extracellular medium is commonly observed in bacterial in vitro cultures (16), although the in vivo relevance of this observation is unclear. Pathogen flagella are frequently recognized by host antibodies and, in *H. pylori*-infected mice, antibodies that recognize the prominent secreted protein FlgE are consistently detected (unpublished data). The secretion of such antigens could interfere with antibody binding to bacterium-bound flagella and might thus improve motility, which is required for successful colonization (32).

Four of the secreted proteins (TrxA, TrxC, DsbC, and FldA) are homologous to oxidoreductases involved in the modification of disulfide bonds. One secreted protein with no assigned function (HP0231) also has significant homology to protein-disulfide isomerases (see below). Furthermore, glutathione, which modulates the redox state of disulfides, is the most prominent substrate of another secreted virulence factor (23), γ-glutamyltranspeptidase, and *H. pylori* infection is associated with decreased levels of glutathione in the gastric mucosa (44, 47, 52). In conclusion, *H. pylori* appears to modulate the disulfide bonds of its microenvironment, as has been postulated on the basis of secretion data for thioredoxin A (TrxA) (54). Alteration of disulfide bonds could participate in altering properties of important host proteins in the gastric microenvironment, such as the viscosity of mucus or the binding ability of immunoglobulins (2, 54). In addition to the modification of disulfide bonds, some of these secreted proteins might mediate additional host-pathogen interactions. The secreted flavodoxin (FldA) is associated with the pathogenesis of mucosa-associated lymphoid tissue lymphoma (9), and the secreted thioredoxins might be potent chemoattractants for neutrophils, monocytes, and T cells (6). Finally, the essential peroxiredoxin system of *H. pylori* depends on thioredoxin (5).

The chaperone and serine protease HtrA (45) is a virulence factor in diverse pathogens (12, 33, 55), but its potential substrates and its role in *H. pylori* remain to be elucidated. Interestingly, this protein is strongly recognized by human and murine antibodies from infected individuals (unpublished data), and a homologue in *Haemophilus influenzae* is a protective antigen (18).

Carbonic anhydrase is a rare component in supernatants but might be more efficiently secreted under specific conditions. It could be involved in the *H. pylori* acid regulation of the microenvironment by consuming hydronium ions to generate volatile carbon dioxide from the urea cleavage product bicarbonate. Alternatively, it might participate in carbon dioxide assimilation, which is essential for *H. pylori* (15).

A group of eight secreted proteins have no homologues with clear function (hypothetical proteins), and two of them (HP0906 and HP0367) have been detected here for the first time on the protein level. Based on the various potentially important functions of the other secreted proteins, this group of proteins might be interesting for further characterization. This suggestion is also supported by the fact that four of these eight proteins (HP0175, HP0231, HP1098, and HP1173) are strongly recognized by murine and/or human sera from infected individuals (20, 22; unpublished data). Moreover, one of these proteins (HP0231) has significant homology to COG1651 (cluster of orthologous groups of proteins), which contains many protein-disulfide isomerases.

In conclusion, a comprehensive analysis of the *H. pylori* secretome revealed several proteins that are known to mediate important pathogen-host interactions and many additional candidates with potentially interesting properties that need to be further characterized. Moreover, *H. pylori* might be able to regulate protein secretion (54), and studies are currently being carried out to investigate this issue. Among the identified proteins are several interesting candidates for innovative approaches to treat or prevent *H. pylori* infections. The oxidreductases and HtrA might be interesting targets for antimicrobial agents that would interfere with the ability of *H. pylori* to modify its microenvironment. Several secreted proteins are recognized by the host immune system, suggesting that they are interesting vaccine antigen candidates.

ACKNOWLEDGMENTS

We thank Stefanie Lamer for excellent assistance in the initial spot identification.

This work was supported in part by grants 031U107C and 031U207C from the Bundesministerium für Bildung und Forschung.

REFERENCES

1. **Albertson, N., I. Wenngren, and J. E. Sjostrom.** 1998. Growth and survival of *Helicobacter pylori* in defined medium and susceptibility to Brij 78. J. Clin. Microbiol. **36:**1232–1235.
2. **Allen, A., J. Newton, L. Oliver, N. Jordan, V. Strugala, J. P. Pearson, and P. W. Dettmar.** 1997. Mucus and *H. pylori*. J. Physiol. Pharmacol. **48:**297–305.
3. **Asahi, M., T. Azuma, S. Ito, Y. Ito, H. Suto, Y. Nagai, M. Tsubokawa, Y. Tohyama, S. Maeda, M. Omata, T. Suzuki, and C. Sasakawa.** 2000. *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. J. Exp. Med. **191:**593–602.
4. **Backert, S., E. Ziska, V. Brinkmann, U. Zimny-Arndt, A. Fauconnier, P. R. Jungblut, M. Naumann, and T. F. Meyer.** 2000. Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. Cell. Microbiol. **2:**155–164.

5. **Baker, L. M., A. Raudonikiene, P. S. Hoffman, and L. B. Poole.** 2001. Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: genetic and kinetic characterization. J. Bacteriol. **183:**1961–1973.
6. **Bertini, R., O. M. Howard, H. F. Dong, J. J. Oppenheim, C. Bizzarri, R. Sergi, G. Caselli, S. Pagliei, B. Romines, J. A. Wilshire, M. Mengozzi, H. Nakamura, J. Yodoi, K. Pekkari, R. Gurunath, A. Holmgren, L. A. Herzenberg, L. A. Herzenberg, and P. Ghezzi.** 1999. Thioredoxin, a redox enzyme released in infection and inflammation, is a unique chemoattractant for neutrophils, monocytes, and T cells. J. Exp. Med. **189:**1783–1789.
7. **Bumann, D., T. F. Meyer, and P. R. Jungblut.** 2001. Proteome analysis of the common human pathogen *Helicobacter pylori*. Proteomics **1:**473–479.
8. **Cao, P., M. S. McClain, M. H. Forsyth, and T. L. Cover.** 1998. Extracellular release of antigenic proteins by *Helicobacter pylori*. Infect. Immun. **66:**2984–2986.
9. **Chang, C. S., L. T. Chen, J. C. Yang, J. T. Lin, K. C. Chang, and J. T. Wang.** 1999. Isolation of a *Helicobacter pylori* protein, FldA, associated with mucosa-associated lymphoid tissue lymphoma of the stomach. Gastroenterology **117:**82–88.
10. **Chevalier, C., J. M. Thiberge, R. L. Ferrero, and A. Labigne.** 1999. Essential role of *Helicobacter pylori* gamma-glutamyltranspeptidase for the colonization of the gastric mucosa of mice. Mol. Microbiol. **31:**1359–1372.
11. **Go, M. F., and S. E. Crowe.** 2000. Virulence and pathogenicity of *Helicobacter pylori*. Gastroenterol. Clin. North Am. **29:**649–670.
12. **Johnson, K., I. Charles, G. Dougan, D. Pickard, P. O'Gaora, G. Costa, T. Ali, I. Miller, and C. Hormaeche.** 1991. The role of a stress-response protein in *Salmonella typhimurium* virulence. Mol. Microbiol. **5:**401–407.
13. **Jose, J., U. Wolk, D. Lorenzen, H. Wenschuh, and T. F. Meyer.** 2000. Human T-cell response to meningococcal immunoglobulin A1 protease associated alpha-proteins. Scand. J. Immunol. **51:**176–185.
14. **Jungblut, P. R., D. Bumann, G. Haas, U. Zimny-Arndt, P. Holland, S. Lamer, F. Siejak, A. Aebischer, and T. F. Meyer.** 2000. Comparative proteome analysis of *Helicobacter pylori*. Mol. Microbiol. **36:**710–725.
15. **Kelly, D. J.** 1998. The physiology and metabolism of the human gastric pathogen *Helicobacter pylori*. Adv. Microb. Physiol. **40:**137–189.
16. **Komoriya, K., N. Shibano, T. Higano, N. Azuma, S. Yamaguchi, and S. I. Aizawa.** 1999. Flagellar proteins and type III-exported virulence factors are the predominant proteins secreted into the culture media of *Salmonella typhimurium*. Mol. Microbiol. **34:**767–779.
17. **Lock, R. A., S. J. Cordwell, G. W. Coombs, B. J. Walsh, and G. M. Forbes.** 2001. Proteome analysis of *Helicobacter pylori*: major proteins of type strain NCTC 11637. Pathology **33:**365–374.
18. **Loosmore, S. M., Y. P. Yang, R. Oomen, J. M. Shortreed, D. C. Coleman, and M. H. Klein.** 1998. The *Haemophilus influenzae* HtrA protein is a protective antigen. Infect. Immun. **66:**899–906.
19. **Marcus, E. A., and D. R. Scott.** 2001. Cell lysis is responsible for the appearance of extracellular urease in *Helicobacter pylori*. Helicobacter **6:**93–99.
20. **McAtee, C. P., K. E. Fry, and D. E. Berg.** 1998. Identification of potential diagnostic and vaccine candidates of *Helicobacter pylori* by "proteome" technologies. Helicobacter **3:**163–169.
21. **McAtee, C. P., P. S. Hoffman, and D. E. Berg.** 2001. Identification of differentially regulated proteins in metronidozole resistant *Helicobacter pylori* by proteome techniques. Proteomics **1:**516–521.
22. **McAtee, C. P., M. Y. Lim, K. Fung, M. Velligan, K. Fry, T. P. Chow, and D. E. Berg.** 1998. Characterization of a *Helicobacter pylori* vaccine candidate by proteome techniques. J. Chromatogr. B Biomed. Appl. **714:**325–333.
23. **McGovern, K. J., T. G. Blanchard, J. A. Gutierrez, S. J. Czinn, S. Krakowka, and P. Youngman.** 2001. Gamma-glutamyltransferase is a *Helicobacter pylori* virulence factor but is not essential for colonization. Infect. Immun. **69:**4168–4173.
24. **Moese, S., M. Selbach, U. Zimny-Arndt, P. R. Jungblut, T. F. Meyer, and S. Backert.** 2001. Identification of a tyrosine-phosphorylated 35 kDa carboxy-terminal fragment (p35CagA) of the *Helicobacter pylori* CagA protein in phagocytic cells: processing or breakage? Proteomics **1:**618–629.
25. **Mori, M., H. Suzuki, M. Suzuki, A. Kai, S. Miura, and H. Ishii.** 1997. Catalase and superoxide dismutase secreted from *Helicobacter pylori*. Helicobacter **2:**100–105.
26. **Nedenskov, P.** 1994. Nutritional requirements for growth of *Helicobacter pylori*. Appl. Environ. Microbiol. **60:**3450–3453.
27. **Nguyen, V. Q., R. M. Caprioli, and T. L. Cover.** 2001. Carboxy-terminal proteolytic processing of *Helicobacter pylori* vacuolating toxin. Infect. Immun. **69:**543–546.
28. **Nilius, M., M. Pugliese, and P. Malfertheiner.** 1996. *Helicobacter pylori* and proteolytic activity. Eur. J. Clin. Investig. **26:**1103–1106.
29. **Nilsson, C. L., T. Larsson, E. Gustafsson, K. A. Karlsson, and P. Davidsson.** 2000. Identification of protein vaccine candidates from *Helicobacter pylori* using a preparative two-dimensional electrophoretic procedure and mass spectrometry. Anal. Chem. **72:**2148–2153.
30. **Odenbreit, S., J. Puls, B. Sedlmaier, E. Gerland, W. Fischer, and R. Haas.** 2000. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. Science **287:**1497–1500.
31. **Odenbreit, S., B. Wieland, and R. Haas.** 1996. Cloning and genetic characterization of *Helicobacter pylori* catalase and construction of a catalase-deficient mutant strain. J. Bacteriol. **178:**6960–6967.
32. **O'Toole, P. W., M. C. Lane, and S. Porwollik.** 2000. *Helicobacter pylori* motility. Microbes Infect. **2:**1207–1214.
33. **Pedersen, L. L., M. Radulic, M. Doric, and K. Y. Abu.** 2001. HtrA homologue of *Legionella pneumophila*: an indispensable element for intracellular infection of mammalian but not protozoan cells. Infect. Immun. **69:**2569–2579.
34. **Phadnis, S. H., M. H. Parlow, M. Levy, D. Ilver, C. M. Caulkins, J. B. Connors, and B. E. Dunn.** 1996. Surface localization of *Helicobacter pylori* urease and a heat shock protein homolog requires bacterial autolysis. Infect. Immun. **64:**905–912.
35. **Pohlner, J., R. Halter, K. Beyreuther, and T. F. Meyer.** 1987. Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. Nature **325:**458–462.
36. **Pohlner, J., U. Langenberg, U. Wolk, S. C. Beck, and T. F. Meyer.** 1995. Uptake and nuclear transport of *Neisseria* IgA1 protease-associated alpha-proteins in human cells. Mol. Microbiol. **17:**1073–1083.
37. **Rain, J. C., L. Selig, H. De Reuse, V. Battaglia, C. Reverdy, S. Simon, G. Lenzen, F. Petel, J. Wojcik, V. Schachter, Y. Chemama, A. Labigne, and P. Legrain.** 2001. The protein-protein interaction map of *Helicobacter pylori*. Nature **409:**211–215.
38. **Reynolds, D. J., and C. W. Penn.** 1994. Characteristics of *Helicobacter pylori* growth in a defined medium and determination of its amino acid requirements. Microbiology **140:**2649–2656.
39. **Reyrat, J. M., R. Rappuoli, and J. L. Telford.** 2000. A structural overview of the *Helicobacter* cytotoxin. Int. J. Med. Microbiol. **290:**375–379.
40. **Sandkvist, M.** 2001. Type II secretion and pathogenesis. Infect. Immun. **69:**3523–3535.
41. **Schmitt, W., and R. Haas.** 1994. Genetic analysis of the *Helicobacter pylori* vacuolating cytotoxin: structural similarities with the IgA protease type of exported protein. Mol. Microbiol. **12:**307–319.
42. **Schraw, W., M. S. McClain, and T. L. Cover.** 1999. Kinetics and mechanisms of extracellular protein release by *Helicobacter pylori*. Infect. Immun. **67:**5247–5252.
43. **Segal, E. D., J. Cha, J. Lo, S. Falkow, and L. S. Tompkins.** 1999. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. Proc. Natl. Acad. Sci. USA **96:**14559–14564.
44. **Shirin, H., J. T. Pinto, L. U. Liu, M. Merzianu, E. M. Sordillo, and S. F. Moss.** 2001. *Helicobacter pylori* decreases gastric mucosal glutathione. Cancer Lett. **164:**127–133.
45. **Spiess, C., A. Beil, and M. Ehrmann.** 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell **97:**339–347.
46. **Stein, M., R. Rappuoli, and A. Covacci.** 2000. Tyrosine phosphorylation of the *Helicobacter pylori* CagA antigen after cag-driven host cell translocation. Proc. Natl. Acad. Sci. USA **97:**1263–1268.
47. **Suzuki, H., M. Mori, K. Seto, A. Kai, C. Kawaguchi, M. Suzuki, M. Suematsu, T. Yoneta, S. Miura, and H. Ishii.** 1999. *Helicobacter pylori*-associated gastric pro- and antioxidant formation in Mongolian gerbils. Free Radic. Biol. Med. **26:**679–684.
48. **Telford, J. L., A. Covacci, R. Rappuoli, and P. Chiara.** 1997. Immunobiology of *Helicobacter pylori* infection. Curr. Opin. Immunol. **9:**498–503.
49. **Testerman, T. L., D. J. McGee, and H. L. Mobley.** 2001. *Helicobacter pylori* growth and urease detection in the chemically defined medium Ham's F-12 nutrient mixture. J. Clin. Microbiol. **39:**3842–3850.
50. **Utt, M., I. Nilsson, A. Ljungh, and T. Wadstrom.** 2002. Identification of novel immunogenic proteins of *Helicobacter pylori* by proteome technology. J. Immunol. Methods **259:**1–10.
51. **Vanet, A., and A. Labigne.** 1998. Evidence for specific secretion rather than autolysis in the release of some *Helicobacter pylori* proteins. Infect. Immun. **66:**1023–1027.
52. **Verhulst, M. L., A. H. van Oijen, H. M. Roelofs, W. H. Peters, and J. B. Jansen.** 2000. Antral glutathione concentration and glutathione S-transferase activity in patients with and without *Helicobacter pylori*. Dig. Dis. Sci. **45:**629–632.
53. **Walker, M. M., and J. E. Crabtree.** 1998. *Helicobacter pylori* infection and the pathogenesis of duodenal ulceration. Ann. N. Y. Acad. Sci. **859:**96–111.
54. **Windle, H. J., A. Fox, E. D. Ni, and D. Kelleher.** 2000. The thioredoxin system of *Helicobacter pylori*. J. Biol. Chem. **275:**5081–5089.
55. **Yamamoto, T., T. Hanawa, S. Ogata, and S. Kamiya.** 1997. The *Yersinia enterocolitica* GsrA stress protein, involved in intracellular survival, is induced by macrophage phagocytosis. Infect. Immun. **65:**2190–2196.

Editor: E. I. Tuomanen