

Identification of Potential Diagnostic and Vaccine Candidates of *Helicobacter pylori* by Two-Dimensional Gel Electrophoresis, Sequence Analysis, and Serum Profiling

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There is great interest in characterizing the proteins of the gastric pathogen *Helicobacter pylori*, especially those to which humans respond immunologically, because of the potential importance of such proteins in diagnosis and vaccine development. Two-dimensional gel electrophoresis was used to separate and identify potential antigens of *H. pylori* ATCC 43504. Over 30 proteins were reactive in Western blots with pooled sera from 14 infected patients. These proteins were analyzed by N-terminal sequence analysis. Fourteen proteins were determined to be distinct from any proteins previously described from *H. pylori*; the others were previously isolated and characterized proteins. Analysis of eight distinct *H. pylori* strains showed that most of these antigens were produced by all of the strains. We propose that collection of new antigens such as those recognized here will be useful in serologic tests for detecting and monitoring *H. pylori* infection and may also serve as potential targets for antimicrobial agent or vaccine development.

Helicobacter pylori is a gram-negative bacterium that chronically infects the gastric mucosa of more than half of all humans worldwide and is a major cause of gastritis and peptic ulcer disease and an early risk factor for gastric cancer (6). Only some 10 to 20% of infections, however, result in overt disease. DNA typing has established that *H. pylori* is extremely diverse as a species, and it is likely that the varied outcomes of infection reflect differences in bacterial genotype, human host genotype, and physiologic, immunologic, and environmental factors (25). These considerations make it valuable to thoroughly characterize the proteins and other antigens that *H. pylori* produces and the human responses to them.

Factors important for *H. pylori* colonization or virulence are just beginning to be identified. Some of the more prominent factors include (i) flagellae, which allow the organism to move in the mucous layer (15); (ii) urease complex, which may help maintain a neutral micro pH environment in the face of gastric acidity (11); (iii) the VacA protein, which generates vacuoles in eukaryotic epithelial cells (2); and (iv) the *cag* pathogenicity island, some of whose encoded proteins help trigger severe inflammatory responses and which, like VacA toxigenicity, is disease associated (1). Several other *H. pylori* proteins with known activities, or which are related to similar proteins of known function in other organisms, have been isolated. Most recently, the complete genomic DNA sequence of *H. pylori* 26695 has been reported (28). However, many of the proteins inferred from this DNA sequence have no known function, and this DNA sequence clone does not always predict which open reading frames are likely to encode virulence factors or antigens suitable for diagnostic or vaccine studies.

A number of studies have begun to address associations of specific *H. pylori* antigens to antibodies in patients with particular gastroduodenal pathologies and of possible autoimmune

components to *H. pylori*-associated disease. There is very little information, however, regarding the long-term evolution and clinical implications of these human responses before and after the eradication of *H. pylori* by antibiotic treatment regimens.

Here we have identified 30 well-conserved proteins that are strongly recognized by sera of infected individuals. Fourteen of these 30 proteins had not been identified previously.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions for *H. pylori*. Clinical isolates were from the Berg laboratory collection. Initial two-dimensional (2D) characterization and isolation of *H. pylori* antigens were performed with strain ATCC 43504 (type strain, NCTC 11637), which was isolated from a peptic ulcer patient at Royal Perth Hospital, Perth, Australia. Strains used for comparative purposes were as follows: 26695, the strain whose sequence was fully determined (28), originally from an English gastritis patient; Chico, from a symptomatic male patient from Feather River Hospital, Chico, Calif.; J170, from a gastric ulcer patient in Tennessee and used by DuBois et al. (3a) for monkey colonization experiments; 4655/1, from a symptomatic Gambian child; Rus-95, from a Russian citizen in the United States; Peru #9, from a symptomatic patient in Lima, Peru; C-3c, from a symptomatic Lithuanian patient, and A-1c, an unrelated strain from a Lithuanian gastric cancer patient; and 96-212, from an Aleut (native Alaskan) male with gastric cancer. All *H. pylori* strains were cultured on campylobacter agar Skirrow (Difco) plates supplemented with 10% defibrinated sheep's blood (Quad 5, Helena, Mont.) in chambers that had been made microaerobic by the CampyPak system (BBL). Cells harvested from Skirrow blood agar plates were washed with phosphate-buffered saline (PBS) and lysed according to the procedure of Panini et al. (23).

2D gel electrophoresis (pH 4 to 8). 2D electrophoresis was performed according to the method of O'Farrell (20), as follows. Isoelectric focusing was carried out in glass tubes of inner diameter 2.0 mm with 2% ampholines (BDH; Hofer Scientific Instruments, San Francisco, Calif.), pH 4 to 8, for 9,600 V · h. The final tube gel pH gradient as measured by a surface pH electrode is shown in the figure. After equilibration for 10 min in buffer O (10% glycerol, 50 mM dithiothreitol, 2.3% sodium dodecyl sulfate (SDS), and 62.5 mM Tris [pH 6.8]), the tube gel was sealed to the top of the stacking gel, which was placed on top of a 10% acrylamide slab gel (0.75 mm thick), and SDS slab gel electrophoresis was carried out for 4 h at 12.5 mA/gel. The slab gels were fixed in a solution of 10% acetic acid-50% methanol overnight. The following proteins were added as molecular size standards (Sigma) to the agarose which sealed the tube gel to the slab gel: myosin (220 kDa), phosphorylase A (94 kDa), catalase (60 kDa), actin (43 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa). These standards appear as horizontal lines on the silver-stained 10% acrylamide slab. The silver-

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stained gel was dried between sheets of cellophane paper with the acid edge to the left.

2D gel electrophoresis (pH 8 to 13). 2D electrophoresis adapted for resolution of basic proteins was performed according to the method of O'Farrell et al. (21), as follows. Nonequilibrium pH gradient electrophoresis with 1.5% pH 3.5 to 10 and 0.25% pH 9 to 11 ampholines (Pharmacia Biotechnology, Piscataway, N.J.) was carried out at 140 V for 12 h. Purified tropomyosin, lower spot (33 kDa and pI 5.2), and purified lysozyme (14 kDa and pI 10.5 to 11) (Sigma) were added to the samples as internal pI markers. After equilibration for 10 min in buffer O, the tube gel was sealed to the top of the stacking gel, which was placed on top of a 10% acrylamide slab gel (0.75 mm thick), and SDS slab gel electrophoresis was carried out for 4 h at 12.5 mA/gel. The slab gels were fixed in a solution of 10% acetic acid–50% methanol overnight. As with the low-pH 2D gel, the following proteins were added as molecular size standards to the agarose which sealed the tube gel to the slab gel: myosin (220 kDa), phosphorylase A (94 kDa), catalase (60 kDa), actin (43 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa). These standards appear as horizontal lines on the silver-stained 10% acrylamide slab. The silver-stained gel was dried between sheets of cellophane paper with the acid edge to the left.

Western blotting. Following slab gel electrophoresis, the gel was placed in transfer buffer (12.5 mM Tris [pH 8.8], 86 mM glycine, 10% methanol) and proteins were transblotted onto polyvinylidene difluoride (PVDF) paper overnight at 200 mA (approximately 50 V/gel). The blot was blocked for 2 h in 2% bovine serum albumin (BSA) in 1% Tween–Tris-buffered saline (vol/vol) (TTBS), rinsed in TTBS, incubated with primary antibody diluted 1:2,500 in 1% BSA–TTBS for 2 h, rinsed in TTBS, and incubated with a secondary antibody (anti-human immunoglobulin G–horseradish peroxidase [Zymed] diluted 1:5,000 in TTBS) for 1 h. The blot was rinsed with TTBS, treated with ECL (Amersham), and exposed to X-ray film.

N-terminal sequencing. The PVDF blot was stained with Coomassie brilliant blue. Spots corresponding to Western blot-positive spots were excised by scalpel and sequenced directly with a Hewlett-Packard G1005A N-terminal sequencer. The instrument gave a high repetitive yield (typically 93 to 98%), with a detection limit of approximately 100 to 200 fmol. All sequences were compared to data available on 13 September 1997 in the PIR, NRDB, GenBank, EMBL, and Swiss Protein databases.

Serum pools. The positive serum pool was derived from pooled sera obtained from 14 patients identified by endoscopy as *H. pylori* positive. The negative serum pool was derived from 14 volunteers whose sera were negative by Helico Blot 2.0 (Genelabs Diagnostics, Ltd., Singapore, Singapore).

RESULTS

2D SDS-polyacrylamide gel electrophoresis (PAGE) (pH 4 to 8). The proteins from lysed cell pellets of *H. pylori* ATCC 43504 were separated on a series of 2D gels run in parallel with an initial pH gradient of pH 4 to pH 8. The silver-stained gel (Fig. 1A) revealed prominent individual proteins, with several protein “families”—most notably as clusters of bands at approximately 89, (pI 6.8), 66, and 58 kDa (pI 6.5). The proteins from these 2D gels were transferred to PVDF membranes and incubated with a positive serum pool (Fig. 1B) or a negative serum pool (Fig. 1C). Western blot data revealed at least 17 spots or groups of spots which were recognized by antibodies in the infected patient serum pool. Transblotted 2D spots from the pH 4 to 8 gel were sequenced by Edman-type amino acid analysis, with the protein within selected spots evaluated further for internal sequence information. The sequences from these spots were compared with sequences in available databases (Table 1). Briefly, spots 1 and 2 corresponded to the *H. pylori* urease b subunit (4) and the urease b-associated chaperonin GroEL (5), respectively. Spot 3 consisted of two proteins: the major species was pyruvate flavodoxin oxidoreductase (13), and the minor protein species corresponded to the previously described *H. pylori* hypothetical protein 2, or HP0154 (26, 28). Spot 4 corresponded to HP0537, from the *cag* region (28). Spots 5, 6, and 8 corresponded to flagellin proteins (15). Spot 7 consisted of two proteins which did not match any previously reported sequences from *H. pylori*. The major component, however, has 90% homology with the *Escherichia coli* TufB protein (possibly HP1205), and the minor component has some sequence homology with various ATPase proton pumps (10, 14). Spot 9 was homologous to monomine oxidase from various species (27). Spot 10 corresponded to the neutrophil-activating

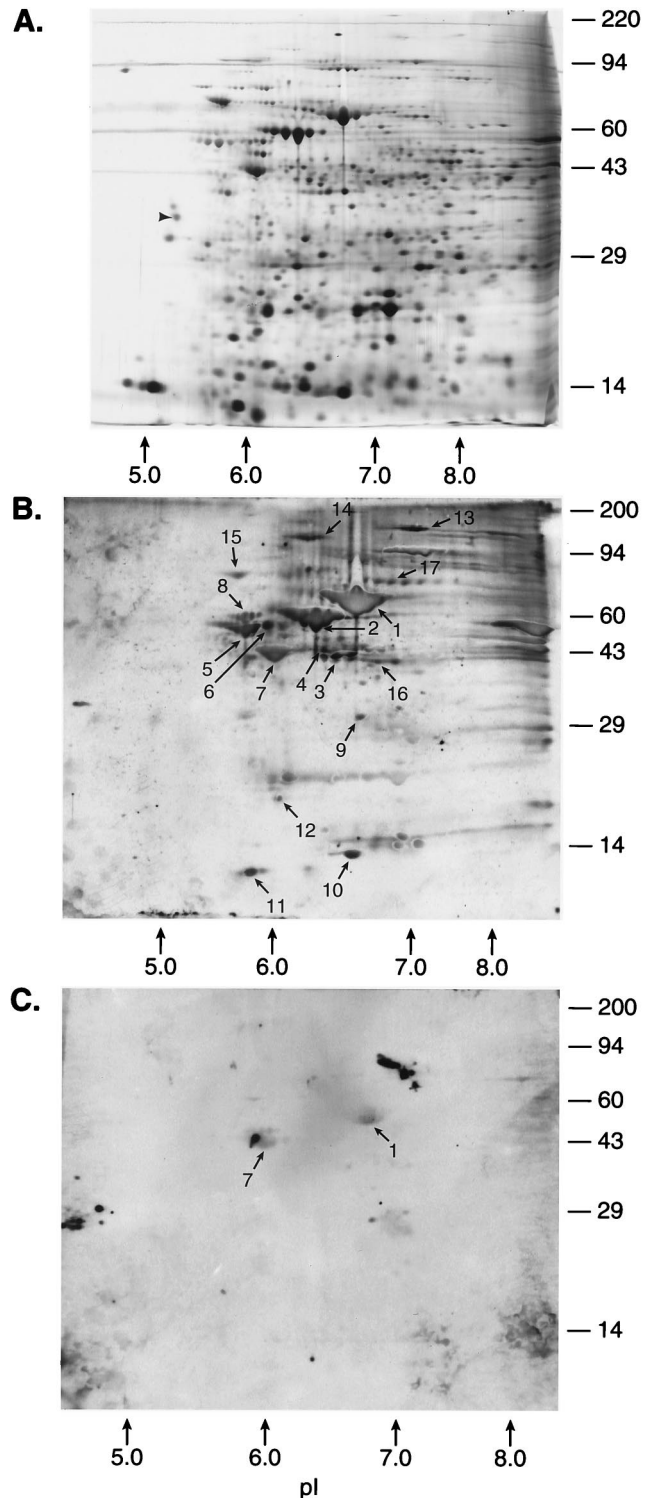


FIG. 1. *H. pylori* 2D map (pH gradient electrophoresis, pH 4 to 8) with identified proteins (listed in Table 1). Strain 43504 was grown as described in Materials and Methods, and 200 μ g of protein extract was loaded in the first dimension. Identified proteins are indicated by spot numbers in Table 1. Molecular size markers are indicated on the right (in kilodaltons). (A) Silver-stained 2D gel. Fifty nanograms of tropomyosin was added as an internal IEF standard. This protein migrates as a doublet with a polypeptide spot of 33 kDa and pI 5.2. (B) Western blot of a duplicate 2D gel with an *H. pylori*-positive serum pool. (C) Western blot of a duplicate 2D gel with an *H. pylori*-negative (control) serum pool.

TABLE 1. Identification of Western blot-positive proteins by N-terminal sequence analysis (pH 4 to 8)

Spot no.	Observed molecular size (kDa)	pI	N-terminal sequence	Antigen, if known (reference)
1	62	6.7	MKKIS	Urease b subunit (4)
2	58	6.3	AKEIK	Urease b-associated chaperonin (5)
3 (major)	42	6.5	AKSIELQEIE	Pyruvate flavodoxin reductase (13)
3 (minor)	42	6.5	MLTXKDIHAL	HP0154 (enolase) (26, 28)
4	41	6.4	XTKIVF	HP0537 (Cag 16) (28)
5	56	5.8	AFQVN	Flagellin a protein (15)
6	58	6.0	AFOVN	Flagellin a protein (15)
7 (major)	42	6.1	XKEKFNRTKP	<i>E. coli</i> TufB protein (10)
7 (minor)	42	6.1	MXGXIIQVLG	ATPase proton pump (14)
8	60	5.8	SFRINTNIAA	Flagellin b precursor (15)
9	31	6.7	MIDXAIIGGG	Monamine oxidase (27)
10	12	6.8	MKTFEILKHL	Neutrophil-activating protein (8)
11	6	5.7	AISKEEVLEY	HP1199 (ribosomal protein L7/L12) (28)
12	20	6.0	MYIPYVIEN	ClpP protein (19)
13	159	7.1	MKLI	(Signal too low)
14			No sequence	
15	79	5.7	GKVGIDLGT	HP0109 (heat shock protein 70) (28)
16 (major)	42	6.8	MREIIXDGNE	HP0589 (ferredoxin oxidoreductase) (28)
16 (minor)			MKLLLE	O'Toole protein (22)
17	78	6.9	MKLLLE	O'Toole protein (22)

protein (8). Spot 11 corresponded to HP1199, a ribosomal protein (28). Spot 12 had homology with the ClpP protease from various bacteria (19). The sequencing signals of spots 13 and 14 were too low to be read with confidence. Spots 15 and 16 (major) corresponded to HP0109 (Hsp 70) and HP0589 (ferredoxin oxidoreductase), respectively (28). Spots 16 (minor) and 17 corresponded to a protein previously isolated by O'Toole et al. (22). In the control blot with sera from *H. pylori*-negative persons, only the urease b subunit (spot 1), likely due to cross-reaction with ureases of intestinal bacteria, and the spot 7 proteins showed cross-reactivity.

2D SDS-PAGE (pH 8 to 13). Additional unique proteins were found by SDS-PAGE with a nonequilibrium gel, even though fewer proteins, overall, were resolved (Fig. 2). Spots 1 through 4 were present in very low quantities; therefore, a clear N-terminal sequence could not be determined with confidence (Table 2). Spot 5 was the urease b subunit also seen in the pH 4 to 8 2D gels. Likewise, spots 6, 7, and 8 corresponded to urease b-associated chaperonin, flagellin b precursor, and flagellin a protein, respectively, which were also separated on the pH 4 to 8 2D gel. Spot 9 (major) corresponded to HP0027 (isocitrate dehydrogenase) (28), with spot 9 (minor) representing a possible contaminant in the sequencing sample. Spot 10 corresponded to an open reading frame from HP1018, an open reading frame with no known database homologs. Spot 11 corresponded to *H. pylori* catalase (12). Spot 12 contained an N-terminal sequence which has been found in several Omp's (Omp 5, 8, 9, 19, and 27) (see reference 28). Spot 13 corresponded to HP1350, a putative protease (28). Spot 14 was the previously reported HopC protein, and spot 16 was the urease a subunit (7, 9). The sequence yields from transblotted spot 15 were low (in the mid-femtomole range), suggesting that the protein was blocked. The sequence information derived from spot 15 gave an N-terminal amino acid sequence which did not

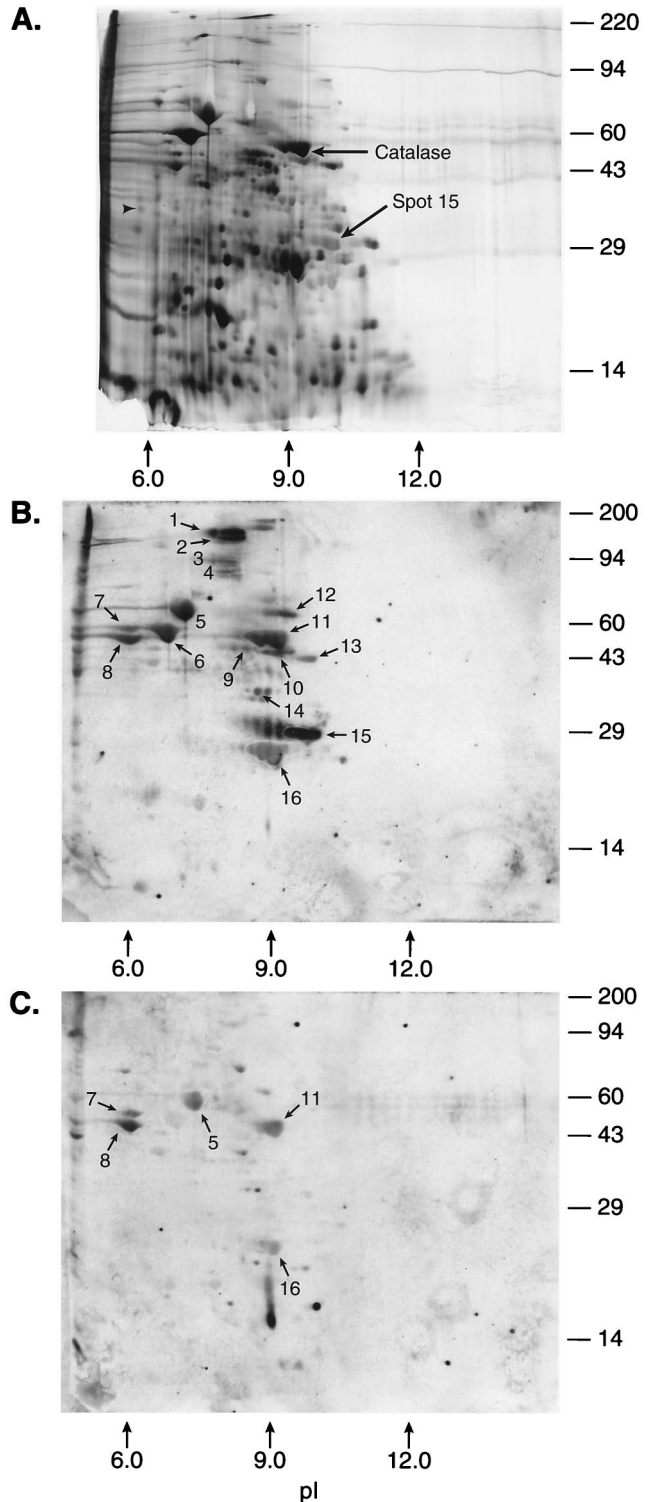


FIG. 2. *H. pylori* 2D map (nonequilibrium pH gradient electrophoresis, pH 8 to 13) with identified proteins (listed in Table 2). Strain 43504 was grown as described in Materials and Methods, and 200 µg of protein extract was loaded in the first dimension. Identified proteins are indicated by spot numbers in Table 2. Molecular size markers are indicated on the right (in kilodaltons). (A) Silver-stained 2D gel. Fifty nanograms of tropomyosin was added as an internal IEF standard. This protein migrates as a doublet with a polypeptide spot of 33 kDa and pI 5.2. Purified lysozyme (14 kDa, pI 10.5 to 11.0) was also added as an internal pI standard. (B) Western blot of a duplicate 2D gel with an *H. pylori*-positive serum pool. (C) Western blot of a duplicate 2D gel with an *H. pylori*-negative (control) serum pool.

TABLE 2. Identification of Western blot-positive proteins by N-terminal sequence analysis (pH 8 to 13)

Spot no.	Observed molecular size (kDa)	pI	N-terminal sequence	Antigen, if known (reference)
1	180	8.2	NPP	(Signal too low)
2	180	8.3	MDXY	(Signal too low)
3	88	8.3	NKITY	(Signal too low)
4	82	8.3	ALXTY	(Signal too low)
5	62	6.7	MKKIS	Urease b subunit (4)
6	58	6.3	AKEIK	Urease b-associated chaperonin (5)
7	60	6.0	SFRIN	Flagellin b precursor (15)
8	58	6.0	AFQVN	Flagellin a precursor (15)
9 (major)	46	8.5	AYNPK	HP0027 (isocitrate dehydrogenase) (28)
9 (minor)			AVTLI	(Possible contaminant)
10	45	9.1	XNIQIQNMPK	HP1018 (28)
11	58	9.1	MVNKD	Catalase (12)
12	65	9.4	EDDGFYTSVG	Omp 5, 8, 9, 19, 27 (28)
13	43	9.7	KEVKEKKA	HP1350 (28)
14	38	8.8	XDDGGFFTVG	HopC protein (9)
15 (major)	28.5	9.8	HECNAAFVAI	Novel (possibly blocked)
15 (minor)			GPKHNXEAGD	Novel (possibly blocked)
16	28	9.0	MKLTP	Urease a subunit (7)

match any known protein sequences. This suggested that the protein(s) in this spot might be modified at the amino terminus, as sequencing yields were low despite the protein(s) being clearly visible on a silver-stained gel.

Comparisons of strains. While the protein profiles of various strains obtained by using 2D gels with the initial focusing gel from pH 4 to 8 were similar by silver stain analysis of whole-cell lysates, the Western blot profiles showed subtle differences (Table 3). 2D spots from ATCC 43504 which were reactive with the positive serum pool were isolated and sequenced. These spots were compared in eight other strains: Chico, 26695, 96-212, 4655/1, J170, A-1c, Rus-95, and Peru #9. Most notably, the presence of flagellins was not apparent in the 26695 2D West-

TABLE 3. Comparison of *H. pylori* strains by 2D PAGE (pH 4 to 8)

Spot from ATCC 43504	<i>H. pylori</i> strain								
	Chico	26695	96-212	4655/1	J170	A-1c	Rus-95	Peru #9	C-3c
1	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+
3 (major)	+	+	+	+	++	+	+	+	+
3 (minor)	+	+	+	+	++	+	+	+	+
4	+	+	+	-	+	-	+	+	+
5	+	+/-	+	+	+	+	+	+	+
6	+	+/-	+	+	+	+	+	+	+
7 (major)	+	+	+	+	+	-	+	+	+
7 (minor)	+	+	+	+	+	-	+	+	+
8	+	-	+	+	+	+	+	+	+
9	+	+	+	-	+	-	+	+	+
10	+	+	+	+	+	-	+/-	+	+
11	+	+	+	+	+	+	+/-	+	+
12	-	+	+	+	++	-	+	+	+
13	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	-	+	+	+
15	+	+	+	+	+	+	+	+	+
16 (major)	+	+	+	+	+	+	+	+	+
16 (minor)	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	++	+	+	+

TABLE 4. Comparison of *H. pylori* strains by 2D PAGE (pH 8 to 13)

Spot from ATCC 43504	<i>H. pylori</i> strain									
	Chico	26695	96-212	4655/1	J170	A-1c	Rus-95	Peru #9	C-3c	
1	+	+	+	+	+	+	+	+	+	
2	+	+	+	+	+	+	+	+	+	
3	+	+	+	-	+	-	+	-	+	
4	+	+	+	-	+	+	-	-	+	
5	+	+	+	+	+	+	+	+	+	
6	+	+	+	+	+	+	+	+	+	
7	+	+	+	+	+	+	+	+	+	
8	+	+	+	+	+	+	+	+	+	
9 (major)	+	+	+	+	+	-	+	+	+	
9 (minor)	+	+	+	+	+	-	+	+	+	
10	+	+	+	+	+	+	+	+	+	
11	+	-	+	+	+	+	+	+	+	
12	+	+	+	+	+	-	+	+	+	
13	+	+	+	+	+	+	+	+	+	
14	+	+	+	+	+	+	+	+	+	
15 (major)	++	++	++	++	++	++	++	++	++	
15 (minor)	++	++	++	++	++	++	++	++	++	
16	+	+	+	+	+	+	+	+	+	

ern blot profile. Several of the identified spots from ATCC 43504 were also missing in the A-1c lysate.

When the isoelectric focusing (IEF) gel was from pH 8 to 13, the most obvious difference in Western blot profiles was in the lack of reactivity of the 26695 strain catalase with the disease-positive serum pool (Table 4). The silver-stained gel also showed a noticeable lack of catalase compared to the silver-stained gels of other strains. It is possible that this gene has been down regulated, or mutated, during laboratory passage, although we have not tested this explicitly. The 26695 strain was evaluated for catalase activity by smearing in 3% H₂O₂. The 26695 strain showed noticeably less activity than in a control (43504) sample (data not shown). Loss of catalase can be fairly common. Westblom et al. (29) investigated catalase-negative mutants of *H. pylori* and found that growth characteristics in vitro were unaffected by the mutations, showing that catalase was not essential for growth of *H. pylori*. It was concluded that catalase-negative mutants of *H. pylori* occurred spontaneously in vitro but had not yet been observed in vivo. The paucity of such catalase-negative strains in clinical specimens may mean that catalase is a virulence factor in vivo that puts mutants at a selective disadvantage.

The only other observed differences between strains involved spots which present in quantities too small to be sequenced.

DISCUSSION

The demonstration that *H. pylori* is a major gastroduodenal pathogen and the realization that strains differ in virulence has created a continuing need for new and improved methods of diagnosis and treatment of infection. Five types of test are in general use to detect *H. pylori* infection: three are invasive, requiring endoscopy (culture, histologic detection, and gastric urease in biopsy [CLO test]), and two are noninvasive (detection of antibodies against *H. pylori* antigens in sera and detection of CO₂ in breath generated from ingested urea by gastric urease). The noninvasive and invasive tests can be of similar accuracy, and noninvasive tests are particularly important for preliminary diagnosis of any possible *H. pylori* infection and in large-scale population surveys, because they are much less costly and disruptive than invasive tests. Many serologic tests

have been developed, most based on pooled *H. pylori* antigen; their performance varies, however, with the antigens chosen, the population from which reference sera are drawn, and age, ethnicity, and the risk of infection by other organisms with cross-reacting antigens in the population studied. Most standardization of serologic tests has been done with adults in Western (industrialized) countries; for children, in particular, there is still considerable uncertainty concerning standards and cutoff values. *H. pylori* strains from different geographic areas may differ greatly in genotype; hence, antigen selection is particularly important in comparisons of immigrant and native populations in a single area or of societies in different regions of the world. While several rapid serological tests have been marketed, none are based upon purified recombinant antigens; the present identification of major, highly conserved *H. pylori* antigens should lead to the development of diagnostic tests that are of much greater sensitivity and specificity than any currently available. A start has been made with Helico Blot 2.0 and also with an assay for detection of CagA (3), which is important because CagA is linked to virulence. However, CagA proteins may differ in strains from different human populations, and so use of this antigen from one strain may well result in underreporting of Cag⁺ frequencies from other regions of the world. This may explain why Cag⁺ phenotypes are relatively infrequent in China (24), although direct tests indicate that all *H. pylori* strains are Cag⁺.

Our experiments were motivated by the great need for an effective anti-*H. pylori* vaccine, especially in Third World, high-risk populations where *H. pylori* eradication by standard antimicrobial therapies is often followed by reinfection. Much attention has been focused on urease-based vaccines because of the essentiality of urease and some encouraging results with mouse *Helicobacter felis* models. VacA has also been considered a candidate based upon results with a mouse *H. pylori* model (17, 18); these mouse models, however, may not adequately mimic the human condition. Clinical trials of urease have been only marginally encouraging (17a). This reinforces the sense that other or additional antigens may be needed for a truly effective vaccine.

Our experiments illustrate that 2D gel electrophoresis can give a global view of the abundant proteins of *H. pylori*. The identification of large numbers of proteins and their characterization with defined serum pools raises the possibility of rapid screening for potential vaccine, as well as diagnostic, candidates. Amino-terminal sequencing and/or proteolytic mass spectral mapping on isolated spots allows for efficient characterization of these potential antigens. Peptidomimetic analysis in parallel with libraries of cloned DNA fragments can provide additional information for the construction of specific vaccine clones or diagnostic recombinant "mosaic" antigens. This is especially important in the case of pathogens whose genomes have not yet been sequenced. One of the many advantages in using "proteome"-type technologies, as here, as opposed to traditional molecular biology (DNA) library approaches, stems from information about likely functionality and utility that comes from initial screening and that is refined as candidate antigens are discovered.

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