Molecular Microbiology (2000) **36**(3), 710–725

Comparative proteome analysis of *Helicobacter pylori*

P. R. Jungblut,[1]* D. Bumann,[2] G. Haas,[2] U. Zimny-Arndt,[1] P. Holland,[2] S. Lamer,[1] F. Siejak,[2] A. Aebischer[2] and T. F. Meyer[2]

Max-Planck-Institute for Infection Biology, [1]Central Support Unit Biochemistry and [2]Department of Molecular Biology, Berlin, Germany.

Summary

***Helicobacter pylori*, the causative agent of gastritis, ulcer and stomach carcinoma, infects approximately half of the worlds population. After sequencing the complete genome of two strains, 26695 and J99, we have approached the demanding task of investigating the functional part of the genetic information containing macromolecules, the proteome. The proteins of three strains of *H. pylori*, 26695 and J99, and a prominent strain used in animal models SS1, were separated by a high-resolution two-dimensional electrophoresis technique with a resolution power of 5000 protein spots. Up to 1800 protein species were separated from *H. pylori* which had been cultivated for 5 days on agar plates. Using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) peptide mass fingerprinting we have identified 152 proteins, including nine known virulence factors and 28 antigens. The three strains investigated had only a few protein spots in common. We observe that proteins with an amino acid exchange resulting in a net change of only one charge are shifted in the two-dimensional electrophoresis (2-DE) pattern. The expression of 27 predicted conserved hypothetical open reading frames (ORFs) and six unknown ORFs were confirmed. The growth conditions of the bacteria were shown to have an effect on the presence of certain proteins. A preliminary immunoblotting study using human sera revealed that this approach is ideal for identifying proteins of diagnostic or therapeutic value. *H. pylori* 2-DE patterns with their identified protein species were added to the dynamic 2D-PAGE database (http://www.mpiib-berlin.mpg.de/2D-PAGE/). This basic knowledge of the proteome in the public domain will be an effective instrument for the identification of new virulence or pathogenic factors, and antigens of potentially diagnostic or curative value against *H. pylori*.**

Received 7 February, 2000; accepted 23 February, 2000. *For correspondence. E-mail jungblut@mpiib-berlin.mpg.de; Tel. (+49) 30 28460 170; Fax (+49) 30 28460 174.

Introduction

The presence of bacteria in the stomach mucosa was described by Bizzozero as early as 1893 (Bizzozero, 1893). Only 90 years later Warren and Marshall (Warren, 1983; Marshall and Warren, 1984) succeeded in cultivating bacteria, later named *Helicobacter pylori*, which had been isolated from the gastric epithelia of active chronic gastritis patients. With the detection of *H. pylori* in the stomach, a paradigm shift in medical microbiology occurred. Epidemiological studies revealed a statistically significant correlation between the presence of *H. pylori* and stomach carcinomas (Forman *et al*., 1991; Nomura *et al*., 1991; Parsonnet *et al*., 1991). It is now recognized that *H. pylori* is a major cause of inflammation leading to dyspepsia, duodenal or gastric cancer or gastric mucosa-associated lymphoid tissue lymphoma (MALT). In 1994, the WHO declared *H. pylori* to be a definitive carcinogen (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 1994).

Diagnosis of *H. pylori* is performed by invasive and non-invasive methods. Invasive methods include biopsies, urease test, histology, direct microscopy, culture and PCR from biopsy material. Non-invasive tests are the ^{13}C-urea breath test and serological tests like ELISA and immunoblots. PCR, ELISA and immunoblotting require the identification of gene or protein targets characterizing *H. pylori* presence (Megraud, 1997). The genes *cagA* and *ureC* can be detected directly in biopsies by PCR (Lage *et al*., 1995). Blots of one-dimensional SDS–PAGE gels revealed several diagnostically relevant antigens, including CagA, VacA, urease α subunit, heat shock protein B and 35 kDa antigen. Others were only characterized by their apparent molecular mass (Nilsson *et al*., 1997; Aucher *et al*., 1998; Lamarque *et al*., 1999).

H. pylori infection can be successfully treated by 'triple therapy' combining a proton pump inhibitor with two antibiotics (Goddard and Logan, 1995; Labenz and Borsch, 1995; Moayyedi *et al*., 1995). The high cost of antibiotic treatment, the likelihood of the development of antibiotic resistance and potential reinfection have provided impetus for the development of a therapeutic and/or prophylactic vaccine against *H. pylori*. In small animal models such as the mouse, the *H. pylori* urease was the first protein shown to provide protective immunity to a *Helicobacter* infection (Michetti *et al*., 1994). Since then, VacA (Marchetti *et al*., 1995; 1998; Crabtree, 1998;), CagA (Crabtree, 1998; Marchetti *et al*., 1998), catalase (Radcliff *et al*., 1997), a nickel-binding heat shock protein

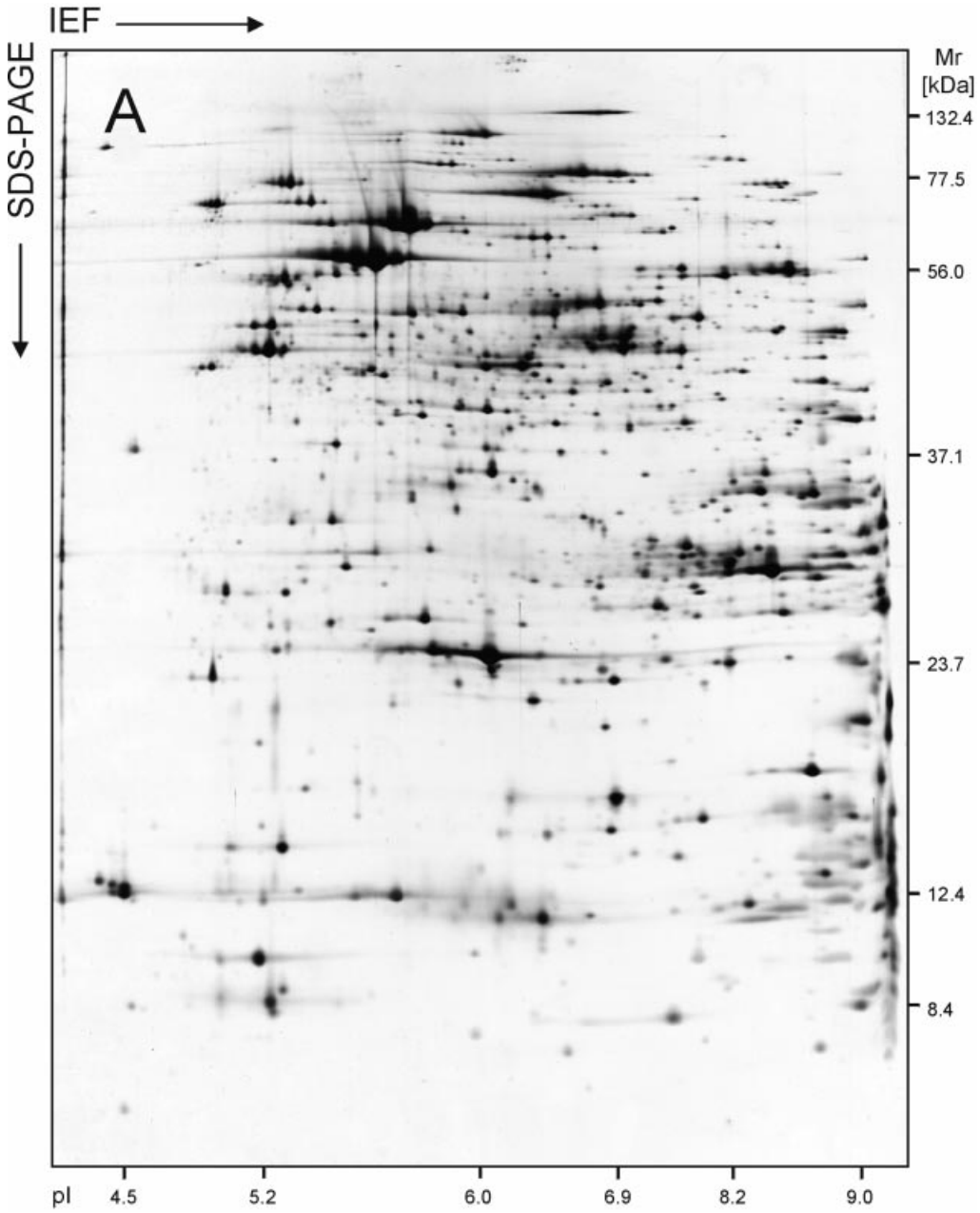

Fig. 1. Two-DE gel of total cell protein of (A) *H. pylori* 26695 (B) *H. pylori J99* and (C) *H. pylori* SS1. The original gel size is 23 × 30 × 0.075 cm. The proteins were detected by silver staining.

(Gilbert *et al*., 1995) and a citrate synthase homologue (Dunkley *et al*., 1999) have also been used successfully as vaccines in mouse models. In addition, the Lewis antigen-binding adhesin BabA (Ilver *et al*., 1998) and HP0175, an open reading frame (ORF) with some homology to *Campylobacter jejuni* cell binding protein 2, were proposed as vaccine candidates (McAtee *et al*., 1998a). Given the enormous heterogeneity of *H. pylori* strains there is a great need for additional vaccine candidates conserved between strains and antigens of diagnostic value.

In animal models, several strategies have been applied to gain protective immunization. In 1992, Chen *et al*. (1992) reported induction of protective immunity in mice after oral vaccination with whole cell sonicates, this was later confirmed by Czinn *et al*. (1993). Workers in our laboratory reported 100% protection after single oral immunization with an attenuated *Salmonella typhimurium*

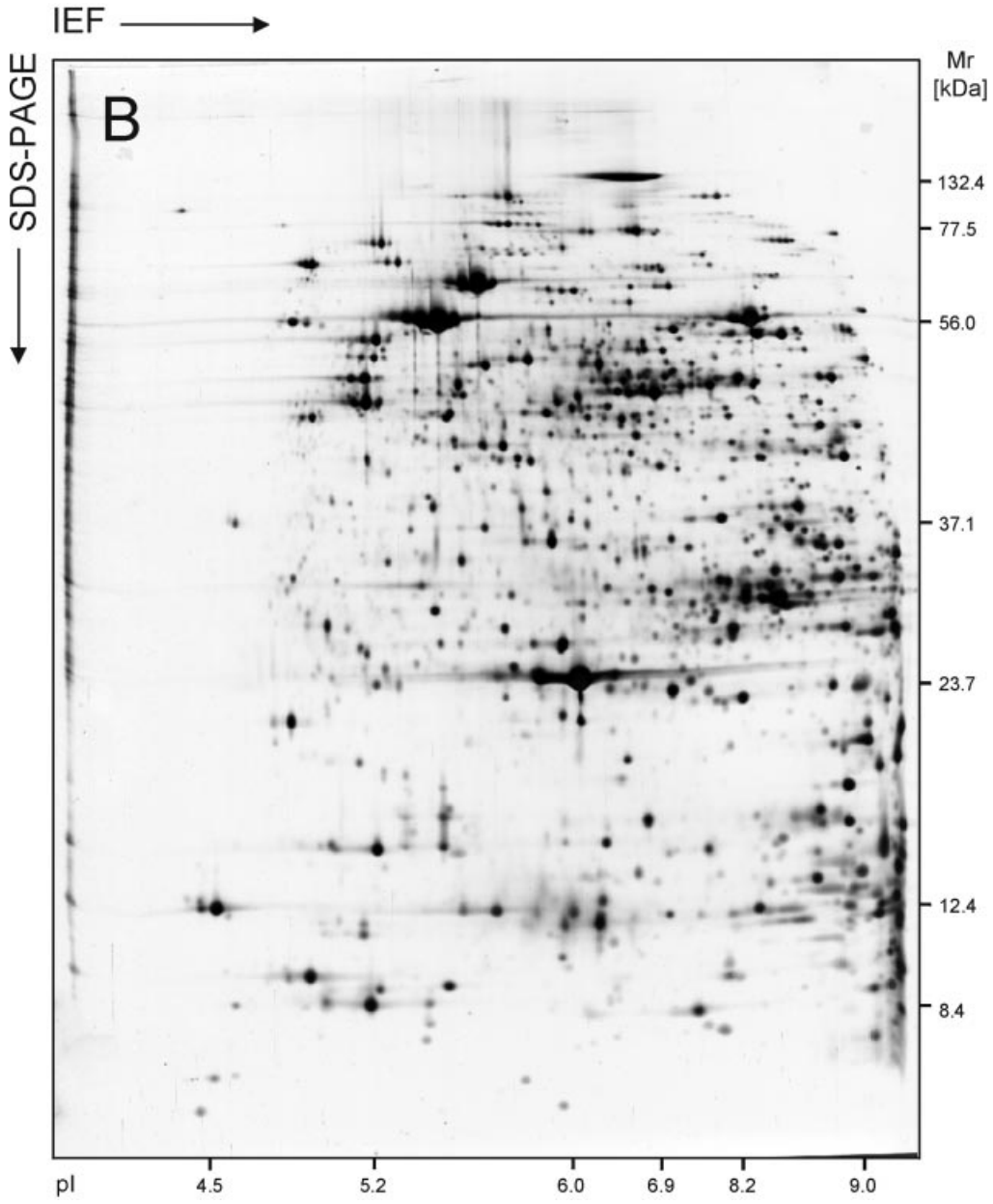

Fig. 1. Continued.

live vaccine expressing UreA and UreB (Gomez-Duarte *et al*., 1998). The high efficacy in the mouse model, combined with remarkable immunogenicity, safety and low-cost production, makes attenuated live recombinant Salmonella a promising vaccine strategy for the control of *H. pylori*-related diseases in humans (Gomez-Duarte *et al*., 1999).

To date, the complete genome of 26 microorganisms has been sequenced, including two strains of *H. pylori* 26695 (Tomb *et al*., 1997) and J99 (Alm *et al*., 1999). Two-dimensional gel electrophoresis (2-DE) of proteins allows the separation of up to 10 000 protein species in one electrophoretic run (Klose and Kobalz, 1995). This resolution is clearly exceeding the number of genes predicted for the two *H. pylori* strains sequenced [26695, 1590 predicted genes (Tomb *et al*., 1997); J99, 1495 predicted genes (Alm *et al*., 1999)] and is therefore sufficient for proteome analysis of this microorganism. The combination of 2-DE with mass spectrometry enables the identification of proteins on 2-DE patterns in a large scale (Müller *et al*., 1996; Shevchenko *et al*., 1996). Comparison of different biological situations may be

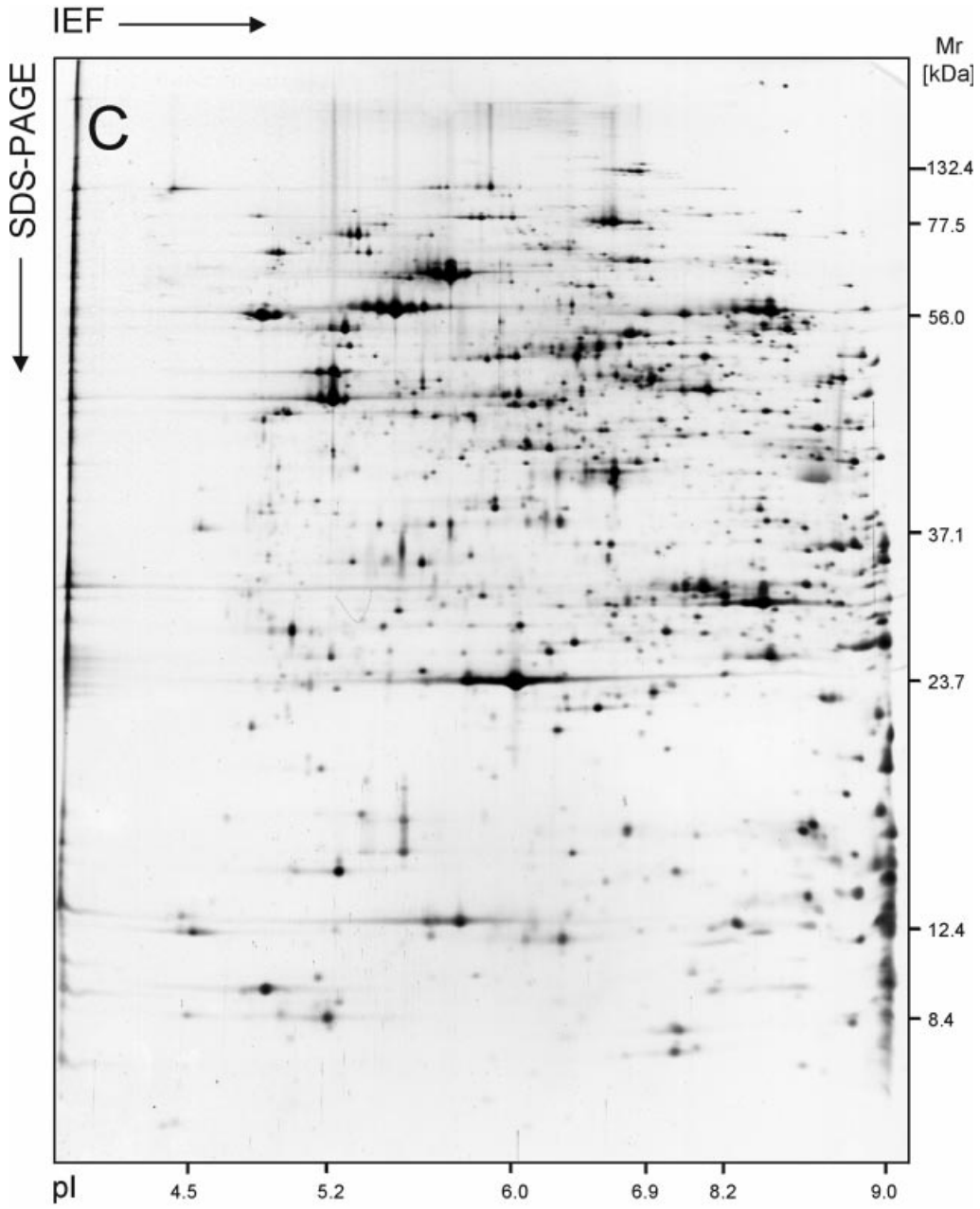

Fig. 1. Continued.

monitored on the protein level by subtractive analyses (Aebersold and Leavitt, 1990). In preliminary attempts to combine 2-DE with immunoblotting (Dunn *et al*., 1989), small gel 2-DE and immunoblotting were applied to detect antigens (McAtee *et al*., 1998b). We have constructed a 2-DE database containing information on pathogenic microorganisms (Mollenkopf *et al*., 1999) (http://www.mpiib-berlin.mpg.de/2D-PAGE/). This database is a federated member of the world 2-DE database (http://www.expasy.ch/ch2d/2d-index.html) and contains data on *Mycobacterium tuberculosis* (Jungblut *et al*., 1999a) and *Borrelia garinii* (Jungblut *et al*., 1999b).

Here we present the first attempt at a systematic analysis of about 1800 *H. pylori* proteins, the identification of 152 proteins by peptide mass fingerprinting matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and the organization of these data in a dynamic 2-DE database which is accessible via the Internet. This comprehensive analysis is the basis for comparative proteome analyses as exemplified by a

Table 1 Comparison of 10 assigned spots in strains 26695 and J99.

26695		J99			Amino acid changes	Shift	
ORF	pI	ORF	pI	Identity	26695 →J99	Predicted	2-DE
HP0824	5.16	jhp763	5.16	Thioredoxin TrxA	Identical	No shift	No shift
HP0011[a]	6.12	jhp9	6.12	Co-chaperone GroES	Identical	No shift	No shift
HP0072[a]	5.64	jhp67	5.64	Urease β subunit UreB	Identical	No shift	No shift
HP1161[a]	4.45	jhp1088	4.45	Flavodoxin FldA	V→I; S→G; T→N; S→A	No shift	No shift
HP1458	7.72	jhp1351	7.72	Thioredoxin	S→L; M→V; I→T	No shift	No shift
HP0480[a]	5.30	jhp432	5.30	GTP-binding protein, fusA-homologue YihK	R→K; I→L; A→T; T→A	No shift	No shift
HP1199[a]	5.22	jhp1122	5.00	Ribosomal protein L7/L12 Rpl7/l12	K→E	J99→left	J99→left
HP0010[a]	5.55	jhp8	5.50	Chaperone and heat shock protein GroEL	H→D; E→Q	J99→left	J99→left
HP0389	5.77	jhp992	6.04	Superoxide dismutase Sod	D→A; G→E; B Q→K; E→G; I→V	J99→right	J99→right
HP1563	5.88	jhp1471	5.98	Alkyl hydroperoxide reductase TsaA	A→T; T→S; Q→H	J99→right	J99→right

Spots with a comparable intensity at the same position or with a horizontal shift of up to 2 cm were assigned to the same protein. The pI given in the table was calculated from the sequence of the TIGR gene sequence database with the help of the pI calculation program of EXPASY. The theoretical pI values and the resulting predicted pH shift were compared with those found on the 2-DE patterns.
a. Detected in this study as antigen.

comparison of the protein composition of three different strains of *H. pylori*, the comparison of different biological situations and identification of antigens.

Results

Protein separation and identification

The protein composition of *H. pylori* 26695, SS1 and J99 was resolved on large 2-DE gels (Fig. 1). In all three strains, the protein spots are spread over the whole pI range of 4–10 and the whole M_r range 5–150 kDa. There is a tendency for an increased number of protein species in the basic range of the gel and several of them are accumulated at the basic end of the gel. In total 1863, 1448 and 1622 spots were detected on the patterns of *H. pylori* 26695, SS1 and J99 respectively. The comparison of the three patterns reveals a high genetic variability. Whereas several main spots are found at the same position, many positional shifts and differentially present or absent spots are observed. Peptide mass fingerprinting using MALDI-MS allowed us to identify 10 spots, which were assigned easily between the two strains 26695 and J99 (Table 1). Three protein species were identical as predicted from the genome sequence and indeed they were at the same position within the 2-DE patterns. Flavodoxin, thioredoxin and FusA have four, three and two amino acid exchanges respectively, without a net charge change and therefore appear at the same position in the 2-DE pattern. Four protein species with amino acid exchanges resulting in a net charge change of at least 1 show the predicted shift. As expected the shift obtained from 1 net charge results in a larger shift for low M_r proteins as compared with high M_r proteins. In the M_r range, up to 60 kDa a net charge shift of 1 discriminates two protein species on large, high-resolution 2-DE gels, as shown for GroEL and TsaA.

Peptide mass fingerprinting using MALDI-MS was used to identify 152 spots of strain 26695. The complete pattern was digitized and subdivided in six sectors. Sector B of strain 26695 is shown in Fig. 2. All of the spots marked with numbers were identified. The web-site of this journal (http://www.blackwell-science.com/mmi) contains all of the sectors with all of the identified proteins (web-site Figure 1) and a systematic protein list, in which the numbers of the spots lead to the protein name and if known to the function of the protein (web-site Table 1). For this web-site table the classification of *H. pylori* proteins of the TIGR database (http://www.tigr.org/tdb/mdb/mdb.html), which was derived from the classification of *Escherichia coli* (Riley, 1993), was used.

The 152 identified protein spots represented 126

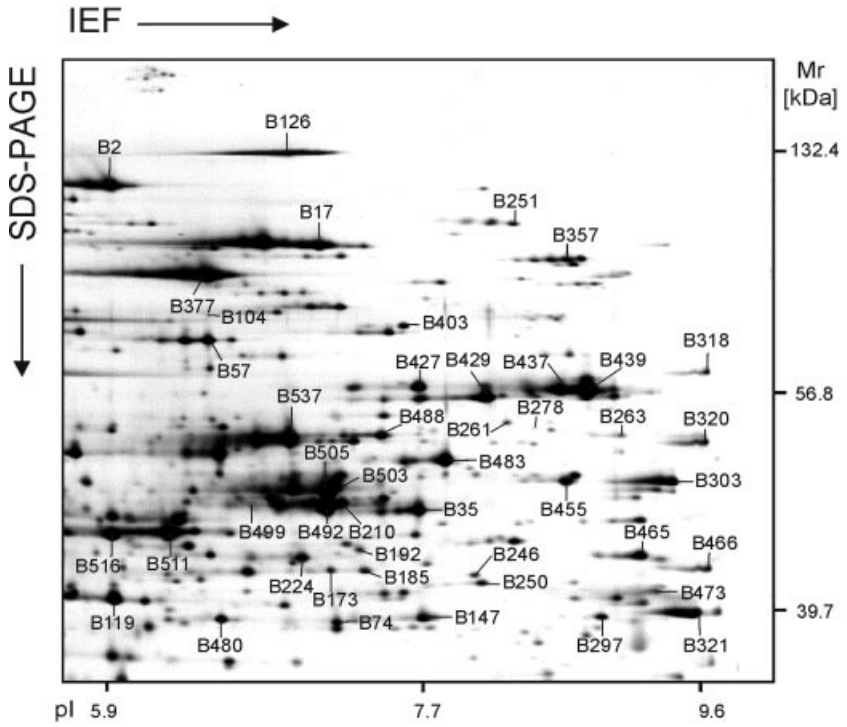

Fig. 2. Sector B of the 2-DE pattern of *H. pylori* 26695 cell proteins. Identified proteins are marked with corresponding accession numbers in Table 1 (web page of this journal).

genes. Several proteins appeared in horizontal spot series resulting from protein species of one protein with differently charged side groups caused by post-translational modifications. One hundred of the identified protein species (67% of all identified proteins) were within the 10% most intense silver-stained spots of the 26695 strain. Except for two, all of the 20 most intense spots were identified (Table 2). The two spots not identified were not stained by Coomassie brilliant blue. The first five most intense spots clearly dominated the pattern and were, in order of decreasing intensity: GroEL, UreB, TsaA, GroEL and CagA. GroEL and UreB contributed four and three spots respectively, which correspond to different protein species, these were all included in the list of the 20 most intense spots.

The 126 identified proteins represent about 8% of the total number of 1590 genes predicted from the genome (Tomb *et al.*, 1997). The identified proteins are dispersed over nearly all the protein classes. One pair of paralogous proteins was identified: CeuE HP1561 and CeuE HP1562. The following protein classes are underrepresented by the identified 126 proteins, with a percentage below 8% of the predicted number of ORFs: biosynthesis of cofactors, prosthetic groups and carriers, transport and binding proteins, DNA metabolism, cell envelope, cellular processes and other categories. More than 20% of the predicted proteins of a certain protein class were found in the following protein classes: central intermediary metabolism, energy metabolism, transcription, protein fate and unknowns. More than 40% of the predicted members of a protein family were found in the following protein families: pyridoxine; glutathione; anaerobic proteins; TCA cycle; chromosome-associated proteins; DNA-dependant RNA polymerase; transcription factors, translation factors; protein folding and stabilization; degradation of proteins, peptides and glycopeptides; surface structures; detoxification. Several well-known virulence factors were identified (Table 3) and contribute to the most intense spots of the 2-DE pattern of *H. pylori* 26695 under the chosen growth conditions.

PH dependent protein composition

H. pylori is a microorganism capable of growing under extreme acidic conditions in the presence of urea (Segal *et al.*, 1992; Solnick *et al.*, 1995). We studied the effect of pH on protein composition by growing *H. pylori* on agar plates with pH values between 5 and 8. Several differences in the protein composition of bacteria grown at these conditions were observed. Five of the differences detected are shown in Fig. 3. The spot intensities were measured after scanning the images, performing spot detection with the evaluation program Topspot and adding the pixel intensities within one spot (Table 4). Patterns were normalized on 10 spots predicted to be constant in intensity. The mean intensity value and variation coefficient were calculated from three experiments each, starting with three independent *H. pylori* cultivations per pH value. Spot 1 decreased in intensity with decreasing the pH value from 8 to 5. It was identified as serine protease HtrA

Table 2. The 20 most abundant protein species of the 2-DE pattern of *H. pylori* 26695.

Spot no.	Intensity	M_r kDa	pI	Identity	Short name	ORF
A390	1899.53	59.4	5.5	Chaperone and heat shock protein	GroEL	HP0010
A343	1432.48	64.7	5.6	Urease β subunit	UreB	HP0072
D341	1355.94	23.7	6.0	Alkyl hydroperoxide reductase	TsaA	HP1563
A194	1209.85	60.0	5.4	Chaperone and heat shock protein	GroEL	HP0010
B126	1193.57	132.4	6.6	Cag pathogenicity island protein	Cag26	HP0547
A192	834.17	60.0	5.4	Chaperone and heat shock protein	(GroEL)	HP0010
D322	791.85	28.9	8.6	Urease, α subunit	UreA	HP0073
D329	765.38	26.7	9.1	Ribosomal protein	Rps4	HP1294
E35	735.28	10.0	5.1	S4 general stress protein	Ctc	HP1496
				Ribosomal protein L7/L12	Rpl7/l12	HP1199
D281	727.92	16.0	6.8	Adhesin-thiol peroxidase	TagD	HP0390
A388	704.27	59.7	5.6	Chaperone and heat shock protein	(GroEL)	HP0010
F16	691.56	11.5	6.4	Co-chaperone	GroES	HP0011
A323	682.03	64.8	5.7	Urease β subunit	UreB	HP0072
E54	673.21	12.3	5.6	Neutrophil activating protein	NapA	HP0243
A325	667.99	65.1	5.6	Urease β subunit	UreB	HP0072
F44	667.53	11.4	8.3	–	–	–
F52	640.92	11.8	8.6	–	–	–
D142	635.50	17.5	8.8	Conserved hypothetical secreted protein	–	HP1286
B537	576.40	52.2	6.7	Aminopeptidase a/i	PepA	HP0570
A477	567.52	46.6	5.2	Translation elongation factor EF-Tu	TufB	HP1205

The intensity was determined by adding the optical densities of all of the pixels within each spot. M_r and pI were estimated from the 2-DE position. The spots were identified by peptide mass fingerprinting MALDI-ms. Protein names in brackets represent proteins in series where the main spot was identified by peptide mass fingerprinting.

(HP1019). Decreasing the pH from 8 to 5 decreased spot 5 in intensity and spots 2–4 were completely absent at pH 5. These proteins were identified as different protein species of the vacuolating cytotoxin (HP0887).

Table 3. Known virulence factors identified on the 2-DE pattern of *H. pylori* 26695.

Spot no.	Short name	ORF
A323, A325, A343	UreB	HP0072
B126	Cag26	HP0547
B251	VacA	HP0887
B318	Cag8	HP0528
B437, B439	Catalase	HP0875
C134, C197	SodB	HP0389
D121, D132, D345	HpaA	HP0410
D200, D316, D318, D322, D323, D326	UreA	HP0073
F6, F9, F16	GroES	HP0011

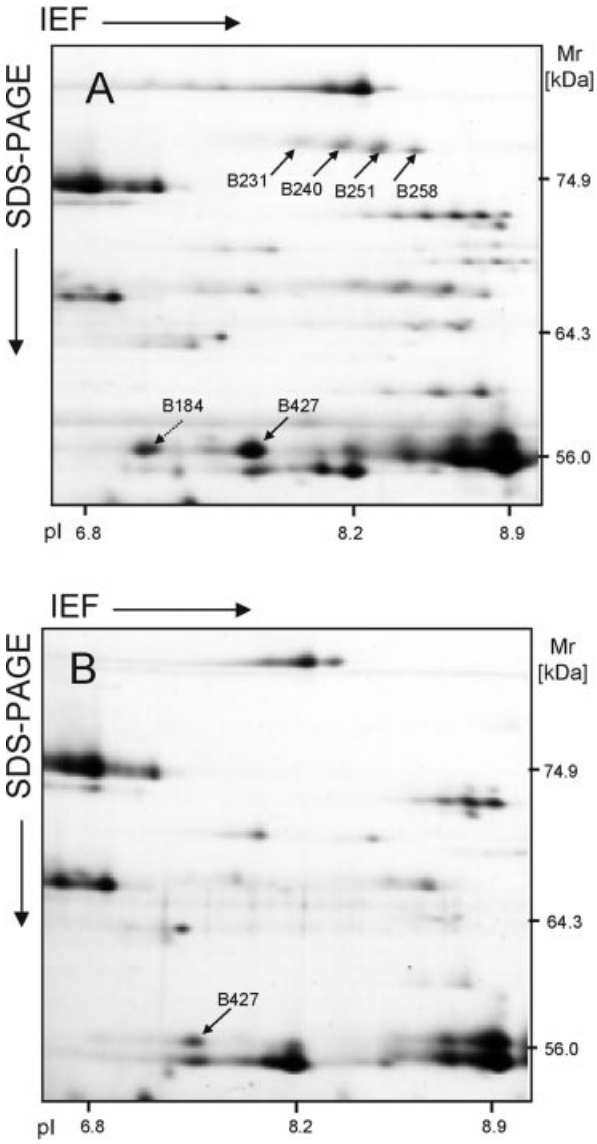

Fig. 3. Part of sector B with protein species differing in spot intensity depending on pH during cultivation. Six spots are marked with database numbers, which showed clearly different intensities. Five of them were identified (Table 4). B184 had not previously been identified. A, *H. pylori* 26695 cultivated at pH 8; B, *H. pylori* 26695 cultivated at pH 5.

Antigens detected by human sera

SDS–PAGE is a common method for the detection of antigens. Unfortunately its resolution power is optimal for protein mixtures of up to only 100 protein species. Therefore a clear assignment to a certain protein species is often not possible if the expected complexity is above 100. *H. pylori* extracts contain at least 1800 protein species (Fig. 1) therefore high-resolution 2-DE is required to detect and identify antigens on the protein species level.

Sera from three patients were used to detect antigens

Table 4. Spots varying in intensity dependent on the pH of the medium.

	Mean value of intensity (sum of pixel OD)				Coefficient of variability (%)					
Spot no.	pH 5	pH 6	pH 7	pH 8	pH 5	pH 6	pH 7	pH 8	Identity	ORF
1 B429	397.5	631.5	777.3	832.7	28.1	6.6	5.8	21.0	Serine protease HtrA	Hp1019
2 B231	–	24.5	123.9	138.9	–	–	49.6	31.8	Vacuolating toxin VacA	Hp0887
3 B240	–	53.2	214.1	216.4	–	53.7	19.7	15.8	Vacuolating toxin VacA	Hp0887
4 B251	–	73.6	294.1	275.9	–	8.5	14.6	18.0	Vacuolating toxin VacA	Hp0887
5 B258	40.5	109.1	242.3	217.3	35.4	19.7	11.8	31.0	Vacuolating toxin VacA	Hp0887

H. pylori 26695 was cultivated for 5 days on agarose plates adjusted to pH values between 5 and 8. Three cultures per pH value and spot no. were analysed by 2-DE and evaluated by the software program Topspot. Spot intensity was normalized on 10 spots with predicted identical intensity. Mean values for five spots evaluated as clearly different were calculated and are presented together with their coefficients of variability. OD, optical density.

Table 5. Identified antigens of *H. pylori* 26695 detected by immunoblotting with human sera.

Spot no.	Identity	ORF	Mpi40	Mpi44	Mpi54
A194	Chaperone and heat shock protein GroEL	HP0010	x	x	x
A323	Urease β subunit UreB	HP0072	–	x	–
A325	Urease β subunit UreB	HP0072	–	x	–
A343	Urease β subunit UreB	HP0072	–	x	–
A349	GTP-binding protein, fusA-homologue YihK	HP0480	–	x	–
A390	Chaperone and heat shock protein GroEL	HP0010	x	x	x
A431	Glutamine synthase GlnA	HP0512	–	–	x
B2	Aconitase B AcnB	HP0779	–	x	–
B17	Fumarate reductase flavoprotein subunit FrdA	HP0192	–	–	x
B126	Cag pathogenicity island protein Cag26	HP0547	–	–	x
B210	Isocitrate dehydrogenase Icd	HP0027	–	x	–
B320	Signal recognition particle protein Ffh	HP1152	–	x	–
B439	Catalase	HP0875	x	x	–
B455	Processing protease YmxG	HP0657	–	x	–
B483	Citrate synthase GltA	HP0026	–	x	–
C109	Hydrogenase expression/formation protein HypB	HP0900	–	x	x
D230	Oxygen insensitive NAD(P)H nitroreductase	HP0954	–	x	–
D249	Cell binding factor 2	HP0175	–	x	–
D265	Penicillin tolerance protein LytB	HP0400	–	x	–
D281	Adhesin-thiol peroxidase TagD	HP0390	–	x	–
D287	Pyruvate ferredoxin oxidoreductase γ unit	HP1108	–	x	–
D295	3-ketoacyl-acyl carrier protein reductase FabG	HP0561	–	x	–
D313	Iron (III)ABC transporter, periplasmic iron-binding protein CeuE	HP1562	–	x	–
D316	Urease, α subunit UreA	HP0073	x	x	–
D322	Urease, α subunit UreA	HP0073	–	x	–
E27	Ribosomal protein L7/L12 Rpl7/l12	HP1199	–	x	x
E35	Ribosomal protein L7/L12 Rpl7/l12	HP1199	–	x	x
E43	Hypothetical protein	HP0697	–	x	–
E62	Flavodoxin FldA	HP1161	–	–	x
F6	Co-chaperone GroeS	HP0011	–	x	x
F9	Co-chaperone GroeS	HP0011	–	x	x
F16	Co-chaperone GroeS	HP0011	–	x	x

After protein separation by 2-DE the resulting protein pattern was blotted onto PVDF. The antigens immobilized on the membrane reacted with antibodies of sera from an adenocarcinoma patient (Mpi44) and from an ulcus ventriculi patient (Mpi54). The serum of a patient with clearly no *H. pylori* history (Mpi40) was analysed to detect potential unspecific immune reactions. x, positive reaction; -, no reaction.

on blots of 2-DE separated *H. pylori* 26695 proteins. The first patient (Mpi54) suffered from ulcus ventriculi and gastritis and was *H. pylori* positive as determined by histology, urease test, culture and a high ELISA titre. The second patient (Mpi44) had a clinical diagnosis of adenocarcinoma of the stomach and stomach cardia, the *Helicobacter* status was unclear because of a positive urea test, negative histology and culture, a non-specific, directed against IgG, high ELISA titre and a specific, directed against *H. pylori* IgG and IgA negative ELISA titre. The third serum was from a patient who was clearly *H. pylori* negative by all of the above criteria and had a clinical diagnosis of chronic antrum gastritis.

Antibodies bound to antigens were detected with a secondary antibody against human Ig and visualized with an ECL system. The serum of the *H. pylori* negative patient reacted only with some of the most abundant *H. pylori* proteins on the 2-DE pattern including GroEL, urease α subunit and catalase (Table 5). The intensity of these spots on the ECL blots was very low. The other two immunoblots had several additional spots in common (Fig. 4A and B). The identified antigens are shown in Table 5. The main antigens GroEL and the ribosomal protein L7/L12, both present with several spots in horizontal series, occurred as high-intensity spots in both immunoblots. Three GroES spots were detected by both sera (Mpi44 and Mpi54), but with a clearly higher intensity on the blot using serum from the ulcer patient. A series of about 60 spots directly below GroEL with high to low intensity common to both immunoblots could only partly be assigned to spots on the silver stained pattern. Only one of them has been identified: spot A431, glutamine synthase. A spot group below the ribososomal protein L7/L12 has the same constellation as in the silver stained pattern but is shifted relatively to the ribosomal protein to the basic side of the pattern. This protein (E41 and E59) is a common antigen of the two sera tested and was identified as thioredoxin. The third component (E45) was not identified. The serum of the patient with adenocarcinoma reacted uniquely with strong signals with GTP binding protein TypA/Bipa, urease α and β subunit, catalase, isocitrate dehydrogenase and the hypothetical protein HP0697. Only flavodoxin (FldA) with middle intensity was unique for the serum of the ulcer patient.

Discussion

Protein separation and identification

In contrast to other microorganisms, e.g. *Mycobacterium tuberculosis* (Jungblut *et al*., 1999a) and *Borrelia garinii* (Jungblut *et al*., 1999b), basic proteins are dominant in all of the 2-DE patterns of *H. pylori* strains investigated. This is in agreement with the high pI values calculated from the protein sequences deduced from the genes of *H. pylori*. More than 70% of the predicted proteins in *H. pylori* have a calculated pI greater than 7.0 compared with about 40% in *Haemophilus influenzae* and *E. coli* (Tomb *et al*., 1997). Despite the fact that only 98 genes of strain 26695 are absent in J99, there are only 41 with perfect identity and only 310 with more than 98% amino acid conservation between these two strains (Alm *et al*., 1999).

Alm *et al*. predicted 1552 and 1495 ORFs for strain 26695 and J99 respectively. The protein patterns revealed 1863 and 1622 protein species, respectively, for these two strains. Within 152 identified spots, 126 (83%) ORFs were represented. If the same percentage is assumed for the total detected spot number a gene number represented on the 2-DE patterns of 1546 and 1346 for 26695 and J99, respectively, may be predicted. This is only slightly below the totally predicted gene numbers. However, one should be aware of the fact that not all of the proteins will be present in the biological situation studied here and that a high dynamic range of protein amount is to be expected for the different protein species, which may not be covered by silver staining. For a middle-sized protein, if 10^7 cells are applied to the gel, about 1000 molecules per cell are necessary for detection using silver staining on a 2-DE gel. It has to be expected that a considerable number of protein species with a copy number below 1000 per cell are present in the cell. Therefore, the estimation of the number of genes to be represented on the 2-DE gels shown above seems to be too high.

The significance of the proteome approach for confirmation of predicted protein species has been emphasized (Humphery-Smith *et al*., 1997). To date our study has revealed expression of 27 conserved hypothetical proteins and six unknowns not described previously at the protein level.

Genetic variability at the proteome level

A comparison of 10 spots with the same or nearly the same position on the 2-DE pattern has shown that one amino acid exchange causing a change of pI of 0.05 units results in a clearly detectable shift in the 2-DE pattern. The influence of the proteins on the pH gradient in ampholyte isoelectric focusing under non-equilibrium conditions is irrelevant. Proteins with identical sequence occur at the same position within the two compared patterns, as was the case for the three proteins thioredoxin, GroES and urease β subunit. Thus, these patterns may serve as references for the assessment of post-translational modifications and virtual patterns of ORFs determined by DNA sequencing from other strains may be established.

The extreme genome plasticity predicted by pulsed-field gel electrophoresis (PFGE) (Jiang *et al*., 1996) was relativated by the overall conservation in genomic

organization and gene order (Alm *et al*., 1999). The proteome analysed by 2-DE shows now at a first glance again a high variability. But considering the high sensitivity of isoelectric focusing against exchanges of single amino acids this variability may reflect the exact chemical structure of the protein species and not the differential presence of the protein as defined by its function. The 2-DE approach analyses the genetic variability at the protein species level. Further identifications of protein species of different *H. pylori* strains will reveal the variability at the protein level.

PH dependent protein composition

H. pylori has the capability to survive under extremely acidic conditions. This survival is mediated by the production of urease (Evans *et al*., 1991; Clyne *et al*., 1995). However, both urease negative mutants survived a 60 min exposure at pH 3.5 (Clyne *et al*., 1995) and urease positive, acid sensitive mutants exist (Bijlsma *et al*., 1998) showing the existence of additional mechanisms for acid resistance. Proteome analysis will help to reveal factors at the protein level, which contribute to the survival of *H. pylori* in the stomach. These factors are *per definitionem* virulence factors. Decreasing the pH of the growth medium from pH 8 to pH 5 decreased the amount of vacuolating cytotoxin VacA (Hp0887) and serine protease HtrA (Hp1019). Whereas VacA is a well-known virulence factor (Cover and Blaser, 1992), HtrA has not been described before to have this role/activity. An increased secretion of these proteins may explain the decrease of protein amount within the cell during acidification. VacA is activated by decreasing the pH to 5.5 (de Bernard *et al*., 1995). This activation may also be accompanied by an increased secretion and therefore decrease of VacA concentration within the cell. Both vacuolization of the surface epithelium and the destruction of the protective mucus layer by proteases are important activities during the pathogenesis of *H. pylori*. These two proteins represent only two obvious differences in the patterns obtained from different pH during cultivation. The detection and identification of further variants will give more detailed information on the molecular mechanisms of the survival of *H. pylori* within an acidic surrounding.

Antigens detected by human sera

The aim to correlate antibody recognition of certain *H. pylori* antigens with clinical manifestations of disease and to identify vaccine candidates has prompted several groups to undertake immunoblot analyses using panels of sera from infected and non-infected patients [Faulde *et al*., 1992; 1993; Klaamas *et al*., 1996; Nilsson *et al*., 1997; Mattsson *et al*., 1998) for original publications and (Zevering *et al*., 1999) for a comprehensive overview]. Given the complexity of the *H. pylori* genome, it is to be expected that conventional SDS–PAGE and immunoblot analyses yielded mostly information on the molecular weight of immunoreactive bacterial proteins and not on sequence information. Methodological differences and the use of antigen preparations of different *H. pylori* strains hamper a comparison between the different studies. Nonetheless, several proteins with the respective M_r recognized by serum antibodies were identified including CagA (110–120 kDa), VacA (87 kDa), urease α and β subunit (67 kDa and 31 kDa, respectively), GroEL (60 kDa), flagellins (50 kDa), p35 (35 kDa) and a 26 kDa antigen. Antibodies against CagA, VacA and the 35 kDa antigen suggested infection with a type I strain (Xiang *et al*., 1995) and were likely correlated with development of ulcers (Telford *et al*., 1994; Weel *et al*., 1996; Atherton *et al*., 1997; Aucher *et al*., 1998; Lamarque *et al*., 1999).

We detected numerous antigenic proteins in Hp26695 with individual sera from patients with a history of *H. pylori* and were able to determine the identity of a subgroup using a proteomics approach (Table 5). Recently, similar 2-DE analyses of *H. pylori* ATCC 43504 (McAtee *et al*., 1998c) and *H. pylori* G27 (Kimmel *et al*., 2000) was employed to identify antigens by immunoscreening using pooled or individual sera from infected patients. Three known antigens, urease β subunit, chaperonin GroEL and isocitrate dehydrogenase Icd were detected in all three studies and 15 of the antigens in at least two of the studies. The fact that some of these proteins were used with success in vaccination studies in animal models of *H. pylori* infection (Kleanthous *et al*., 1998) strongly supports 2-DE as an approach for identification of vaccine candidates. This reasoning led to the identification of a protein with homology to *Campylobacter jejeuni* cell binding protein 2 (McAtee *et al*., 1998a) an ORF that was also present in *H. pylori* 26695, HP0175. The antigenicity of this gene product was confirmed here. However, only one of the sera recognized the spot corresponding to this protein, indicating either that not all patients react to this protein or that *H. pylori* strains exist that lack the respective gene or have an orthologous gene with modified sequence. The enormous genetic variation in *H. pylori* suggests that the latter two explanations are more likely. The sera used in the present study, reacted with a characteristic pattern of proteins with apparent molecular weights in the range of 40–60 kDa and pIs of 4,9–6,4. Proteins with similar relative co-ordinates were also identified by McAtee *et al*. (1998c) and Kimmel *et al*. (2000). Similarly, a set of three antigens is detected displaying a M_r below 10 and a pI of 5.1. We have identified these proteins as candidate vaccine antigens for further study. These results prompted us to perform an extended analysis of antigenic proteins recognized by a large number of individual sera that will lead to the identification of proteins of diagnostic value and also potential vaccine

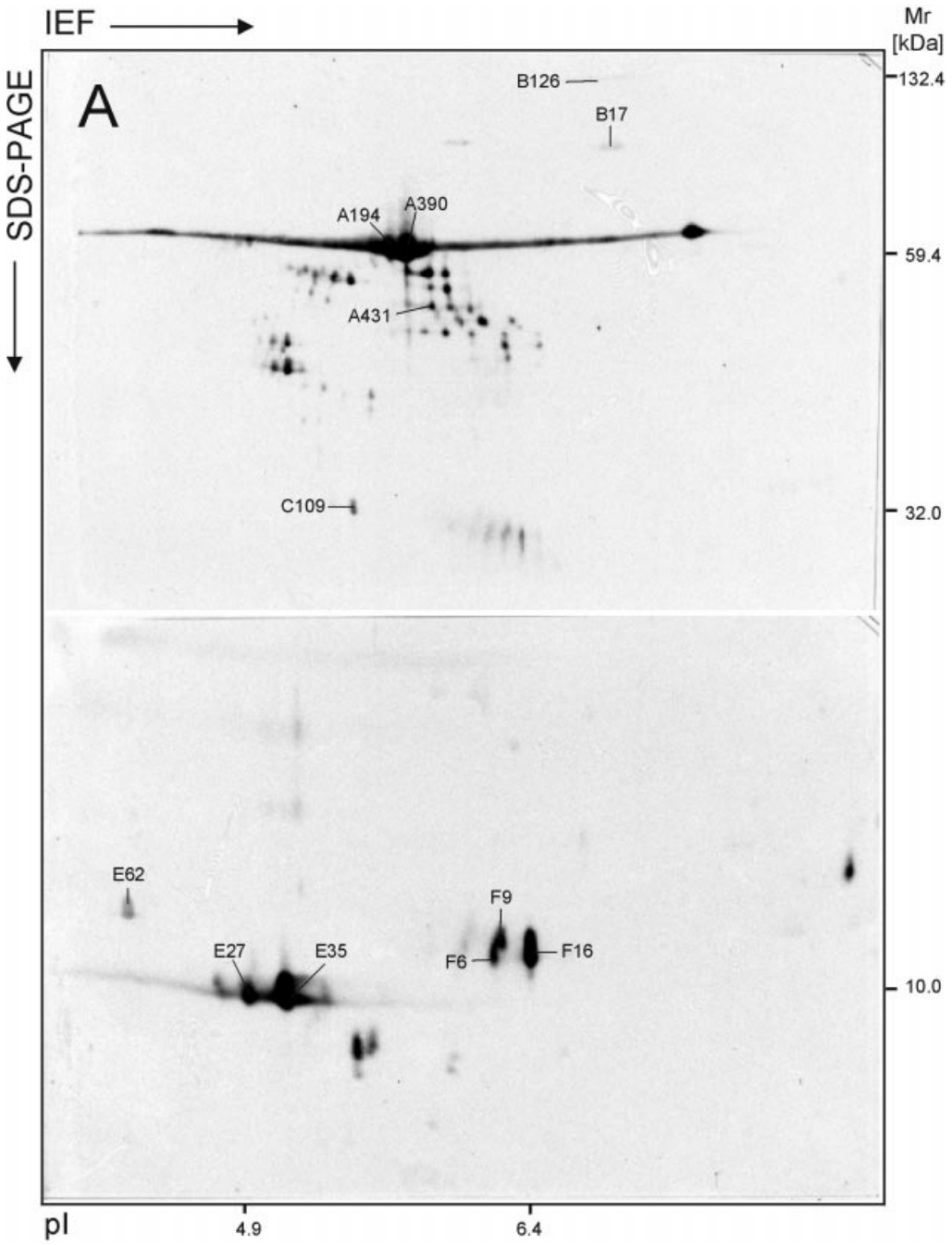

Fig. 4. Antigens of *H. pylori* 26695 detected by immunostaining on 2-DE blots. Spots marked with numbers were identified and the numbers correspond to the 2-DE database numbers. The protein name may be found in the 2-DE database or in Table 5.A. patient serum Mpi54, peptic ulcer. B. patient serum Mpi44, adenocarcinoma.

candidates (G. Haas *et al.*, in preparation). It is of note that all proteins with vaccine efficacy tested in animals to date are abundant proteins that are also detected by immunoblot analyses (compare with Table 2). This suggests that the frequency of a protein is a key criterion to identify it as a vaccine candidate and that despite the fact that antibodies may not be protective they discriminate between bacterial proteins accessible to the immune system and those that are not.

Virulence factors

Virulence factors are defined as gene products that are indispensable for colonization and host to host transmission

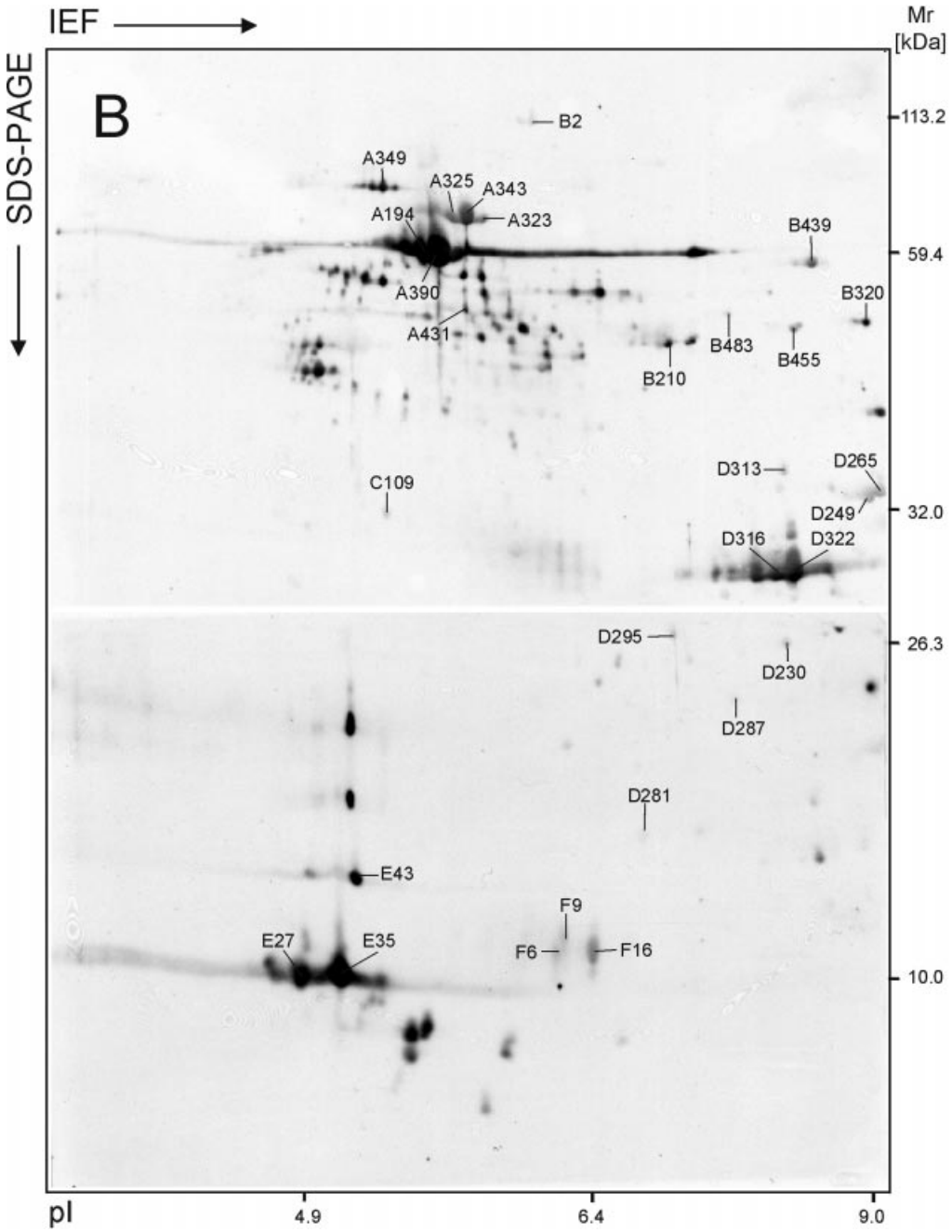

Fig. 4. Continued.

competence of a pathogen, and may include gene products that are important pathogenic factors (for review see McGee and Mobley, 1999). The growing list of those identified for *H. pylori* includes: (i) proteins involved in adhesion, such as BabA, which mediates binding to Lewis^b antigen and might be a key factor in the pathogenesis of duodenal ulcer and adenocarcinoma (Ilver *et al*., 1998; Gerhard *et al*., 1999), AlpA and AlpB (Odenbreit *et al*., 1999), which are members of a large family of related outer membrane proteins (Tomb *et al*., 1997), the sialic acid lectin HpaA (HP0410), a lipoprotein (Evans *et al*., 1993) which may contribute to adherence factors detected by haemagglutination or adherence assays; (ii) proteins required for motility (Bijlsma *et al*., 1999) such as flagellins; (iii) factors involved in acid neutralization such as urease or detoxification of aggressive oxygen metabolites, such as catalase and super oxide dismutases; (vi) proteins involved in iron uptake and storage, such as a lactoferrin-binding protein, siderophores and potential periplasmic iron binding proteins

such as CeuE or ferritin orthologs such as NapA or Pfr (Doig *et al*., 1993; Frazier *et al*., 1993; Evans *et al*., 1995); and (v) proteins involved in pathogenicity such as the cag pathogenicity island encoded proteins or the vacuolating toxin VacA.

The reference strain 26695 is deficient in several of the above mentioned virulence factors: it does not produce functional BabA (Ilver *et al*., 1998), lacks immunoreactive flagellins (McAtee *et al*., 1998c) and we have observed some subclones with very variable levels of catalase activity. Many proteins belonging to the aforementioned classes of virulence factors were easily identified in our 2-DE analysis (Table 3) and the urease subunits, Cag26, catalase and GroES were also recognized by at least one of the sera. Although there is no paucity in the detection of cell envelope proteins, the class of outer membrane proteins both on silver stained gels and on immunoblots is only represented by outer membrane protein HP1564 within the identified proteins. The list of the 20 most abundant proteins (Table 2) contains additionally to known virulence factors the protein TagD, which is described in the NCBI sequence database as an adhesin and NapA, which was mentioned as immunogenic *H. pylori* protein by Kimmel *et al*. (2000).

Genome sequencing is the first step for the understanding of complex molecular events in medicine and biology. Whereas the complete genome of an organism may be determined, a proteome of an organism will never be completely described. Proteomics allows us to view the interaction between the genetic information and the environment by the comparison of different biological situations. The use of standardized 2-DE technology, mass spectrometry and 2-DE databases will enable in comparative proteome analyses of pathogenic microorganisms the detection of functionally interesting proteins as a prerequisite for the elucidation of antigens, virulence and pathogenicity factors.

Experimental procedures

H. pylori *strains and growth conditions*

In this study, the proteomes of three different *H. pylori* strains were compared. Hp26695 and J99 were used because their genome has been entirely sequenced (Tomb *et al*., 1997; Alm *et al*., 1999) and this made it possible to identify individual 2-DE protein spots by mass fingerprinting (see below). A mouse-adapted *H. pylori* strain the 'Sydney strain' SS1 (Lee *et al*., 1997), that has been used for preclinical vaccine testing (Corthesy-Theulaz *et al*., 1997; Radcliff *et al*., 1997; Gomez-Duarte *et al*., 1998) was also analysed.

All *H. pylori* strains were grown on serum plates (Odenbreit *et al*., 1996) at 37°C in a microaerobic atmosphere (5% O_2, 85% N_2 and 10% CO_2) for 2 or 5 days for the pH variations investigated. The bacteria were harvested, washed twice in ice-cold PBS containing proteinase inhibitors (1 mM PMSF, 0.1 μM pepstatin, 2.1 μM leupeptin and 2.9 mM benzamidin) and lysed by resuspension in half a volume of distilled water. The resulting volume in μl was multiplied by (i) 1.08 to obtain the amount of urea in mg to be added and (ii) 0.1 to obtain the volume in μl of 1.4 M DTT and 40% Servalyte (Serva) pI 2–4 to be added. CHAPS was added to obtain an end concentration of 1%. The end concentrations of DTT and urea were 70 mM and 9 M respectively. Solubilization of the proteins occurred within 30 min at room temperature. A protein concentration of 15 μg $\mu l^{-1} \pm 25\%$ was obtained.

Two-dimensional electrophoresis

For the resolution of the *H. pylori* proteome, we used a 23 cm × 30 cm 2-DE gel system (Jungblut *et al*., 1994; Klose and Kobalz, 1995) with a resolution power of about 5000 protein species. For subtractive analyses (Aebersold and Leavitt, 1990) and database construction, we applied 50–100 μg of protein to the anodic side of the IEF gel. In the second dimension we used 0.75 mm thick gels. The proteins were detected by silver staining optimized for these gels (Jungblut and Seifert, 1990). For identification of proteins, 200–300 μg of protein were applied and in the second dimension 1.5 mm thick gels were used. The proteins were stained by Coomassie brilliant blue R250 (Eckerskorn *et al*., 1988) or G250 (Doherty *et al*., 1998), or negative staining (Fernandez-Patron *et al*., 1995).

Peptide mass fingerprinting

The first 20 proteins were identified by in-gel tryptic digestion using a peptide collecting device to concentrate and wash the peptide mixture (Otto *et al*., 1996). The original procedure was improved during this investigation (S. Lamer and P. R. Jungblut, in preparation). Working with volatile buffer, decreased trypsin concentrations and in volumes below 20 μl allowed the identification of weakly stained Coomassie Blue G-250 protein spots starting with only one excised spot. The peptide solution was mixed with an equal volume of a saturated α-cyano-4-hydroxy cinnamic acid solution in 50% acetonitrile, 0.3% TFA and 2 μl was applied to the sample template of a MALDI-MS (Voyager Elite, Perseptive). Data were obtained using the following parameters: 20 kV accelerating voltage, 70% grid voltage, 0.050% guide wire voltage, 100 ns delay and a low mass gate of 500.

Peptide mass fingerprints were searched using the program MS-FIT (http://prospector.ucsf.edu/ucsfhtml/msfit.htm) reducing the proteins of the NCBI database to the Helicobacter proteins and to a molecular mass range estimated from 2-DE ± 20%, allowing a mass accuracy of 0.1 Da for the peptide mass. In the absence of matches the molecular mass window was extended. 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.

Dependency of pH

H. pylori was cultivated on serum plates as described above.

The pH of the medium was adjusted to 5, 6, 7 and 8. For each pH value, three independent cultivations were performed and from each one the proteins were separated by a small gel 2-DE method (Jungblut and Seifert, 1990). The spot intensities were determined by scanning and spot detection (Topspot, Algorithmus). To confirm four of the detected variants large 2-DE gels of pH 5 and pH 8 samples were analysed.

Immunoblotting

For immunostaining, the proteins were transferred from the 2-DE gels onto PVDF membranes (ImmobilonP, Millipore) by semidry blotting (Jungblut *et al.*, 1990) using a blotting buffer containing 100 mM borate, 20% methanol and pH 9.0. The blotting time was 2 h with a current of 1mA/cm^2. The gels were divided in two equal-sized parts (13 $\times$ 19 cm) to avoid too high temperatures during blotting. Antigens were detected by incubation of the membranes with human sera in a dilution of 1:200, a secondary antibody (anti-human polyvalent immunoglobulins, G, A, M, peroxidase conjugated, Sigma A-8400) at a dilution of 1:10000. Before the addition of serum, the membrane was blocked with 5% skim milk, 0.05% Tween-20 in PBS for at least 1 h at room temperature. All washing steps were performed with PBS, 0.05%Tween 20. After blocking the membrane was washed three times for 5 min, the sera were incubated with the membrane for 1 h at room temperature. Before and after the addition of the secondary antibody the membranes were washed four times for 15 min in PBS, 0.05% Tween 20. The washed membrane was incubated with 30 ml per membrane of a 1:1 mixture of Enhanced Luminol Reagent and Oxidizing Reagent for 1 min [Renaissance Western Blot Chemiluminescence Reagent for ECL Immunostaining (NEN)]. The detection reagent was drained off and the membrane wrapped in a foil. The foil was overlaid with Kodak BioMax MR1 film for an exposure time of 5 min. For localization the proteins were stained on the membranes by Coomassie brilliant blue R-250.

Database construction

Gels were digitized after scanning with a UMAX Mirage IIse scanner using the TOPSPOT software (Algorithmus). Before spot detection the gels were divided into six sectors, which were automatically spot detected and afterwards interactively corrected. The resulting map files were introduced together with gif files and identification data collected within an access database into the 2-DE database (Mollenkopf *et al.*, 1999).

Acknowledgements

We would like to thank A. B. Roznowski and B. Wiedenmann from the Department of Hepatology and Gastroenterology, Humboldt-University Berlin for providing the three sera for immunoblotting. A. Walduck is acknowledged for her help in the preparation of the manuscript.

References

Aebersold, R., and Leavitt, J. (1990) Sequence analysis of proteins separated by polyacrylamide gel electrophoresis: towards an integrated protein database. *Electrophoresis* **11**: 517–527.

Alm, R.A., Ling, L.S., Moir, D.T., King, B.L., Brown, E.D., Doig, P.C., *et al.* (1999) Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen Helicobacter pylori. *Nature* **397**: 176–180.

Atherton, J.C., Peek, R.M.J., Tham, K.T., Cover, T.L., and Blaser, M.J. (1997) Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori. *Gastroenterology* **112**: 92–99.

Aucher, P., Petit, M.L., Mannant, P.R., Pezennec, L., Babin, P., and Fauchere, J.L. (1998) Use of immunoblot assay to define serum antibody patterns associated with Helicobacter pylori infection and with H. pylori-related ulcers. *J Clin Microbiol* **36**: 931–936.

de Bernard, M., Papini, E., de Filippis, V., Gottardi, E., Telford, J., Manetti, R. *et al.* (1995) Low pH activates the vacuolating toxin of Helicobacter pylori, which becomes acid and pepsin resistant. *J Biol Chem* **270**: 23937–23940.

Bijlsma, J.J., Gerrits, M.M., Imamdi, R., Vandenbroucke-Grauls, C.M., and Kusters, J.G. (1998) Urease-positive, acid-sensitive mutants of Helicobacter pylori: urease-independent acid resistance involved in growth at low pH. *FEMS Microbiol Lett* **167**: 309–313.

Bijlsma, J.J., Vandenbroucke-Grauls, C.M., Phadnis, S.H., and Kusters, J.G. (1999) Identification of virulence genes of Helicobacter pylori by random insertion mutagenesis. *Infect Immun* **67**: 2433–2440.

Bizzozero, G. (1893) Über die schlauchförmigen Drüsen des Magendarmkanals und die Beziehung ihres Epithels zu dem Oberflächenepithel der Schleimhaut. *Arch Mikr Anat* **42**: 82–152.

Chen, M., Lee, A., and Hazell, S. (1992) Immunisation against gastric helicobacter infection in a mouse/Helicobacter felis model. *Lancet* **339**: 1120–1121.

Clyne, M., Labigne, A., and Drumm, B. (1995) Helicobacter pylori requires an acidic environment to survive in the presence of urea. *Infect Immun* **63**: 1669–1673.

Corthesy-Theulaz, I.E., Bergonzelli, G.E., Henry, H., Bachmann, D., Schorderet, D.F., Blum, A.L., *et al.* (1997) Cloning and characterization of Helicobacter pylori succinyl CoA: acetoacetate CoA-transferase, a novel prokaryotic member of the CoA-transferase family. *J Biol Chem* **272**: 25659–25667.

Cover, T.L., and Blaser, M.J. (1992) Purification and characterization of the vacuolating toxin from Helicobacter pylori. *J Biol Chem* **267**: 10570–10575.

Crabtree, J.E. (1998) Eradication of chronic Helicobacter pylori infection by therapeutic vaccination. *Gut* **43**: 7–8.

Czinn, S.J., Cai, A., and Nedrud, J.G. (1993) Protection of germ-free mice from infection by Helicobacter felis after active oral or passive IgA immunization. *Vaccine* **11**: 637–642.

Doherty, N.S., Littman, B.H., Reilly, K., Swindell, A.C., Buss, J.M., and Anderson, N.L. (1998) Analysis of changes in acute-phase plasma proteins in an acute inflammatory response and in rheumatoid arthritis using two-dimensional gel electrophoresis. *Electrophoresis* **19**: 355–363.

Doig, P., Austin, J.W., and Trust, T.J. (1993) The Helicobacter pylori 19.6-kilodalton protein is an iron-containing protein resembling ferritin. *J Bacteriol* **175**: 557–560.

Dunkley, M.L., Harris, S.J., McCoy, R.J., Musicka, M.J., Eyers, F.M., Beagley, L.G., *et al.* (1999) Protection against Helicobacter pylori infection by intestinal immunisation with a 50/52-kDa subunit protein. *FEMS Immunol Med Microbiol* **24**: 221–225.

Dunn, B.E., Perez-Perez, G.I., and Blaser, M.J. (1989) Two-dimensional gel electrophoresis and immunoblotting of Campylobacter pylori proteins. *Infect Immun* **57**: 1825–1833.

Eckerskorn, C., Jungblut, P., Mewes, W., Klose, J., and Lottspeich, F. (1988) Identification of mouse brain proteins after two-dimensional electrophoresis and electroblotting by microsequence analysis and amino acid composition analysis. *Electrophoresis* **9**: 830–838.

Evans, D.J.J., Evans, D.G., Kirkpatrick, S.S., and Graham, D.Y. (1991) Characterization of the Helicobacter pylori urease and purification of its subunits. *Microb Pathog* **10**: 15–26.

Evans, D.G., Karjalainen, T.K., Evans, D.J.J., Graham, D.Y., and Lee, C.H. (1993) Cloning, nucleotide sequence, and expression of a gene encoding an adhesin subunit protein of Helicobacter pylori. *J Bacteriol* **175**: 674–683.

Evans, D.J.J., Evans, D.G., Takemura, T., Nakano, H., Lampert, H.C., Graham, D.Y., *et al.* (1995) Characterization of a Helicobacter pylori neutrophil-activating protein. *Infect Immun* **63**: 2213–2220.

Faulde, M., Schroder, J.P., and Sobe, D. (1992) Serodiagnosis of Helicobacter pylori infections by detection of immunoglobulin G antibodies using an immunoblot technique and enzyme immunoassay. *Eur J Clin Microbiol Infect Dis* **11**: 589–594.

Faulde, M., Cremer, J., and Zoller, L. (1993) Humoral immune response against Helicobacter pylori as determined by immunoblot. *Electrophoresis* **14**: 945–951.

Fernandez-Patron, C., Calero, M., Collazo, P.R., Garcia, J.R., Madrazo, J., Musacchio, A., *et al.* (1995) Protein reverse staining: high-efficiency microanalysis of unmodified proteins detected on electrophoresis gels. *Anal Biochem* **224**: 203–211.

Forman, D., Newell, D.G., Fullerton, F., Yarnell, J.W., Stacey, A.R., Wald, N., *et al.* (1991) Association between infection with Helicobacter pylori and risk of gastric cancer: evidence from a prospective investigation. *Br Med J* **302**: 1302–1305.

Frazier, B.A., Pfeifer, J.D., Russell, D.G., Falk, P., Olsen, A.N., Hammar, M., *et al.* (1993) Paracrystalline inclusions of a novel ferritin containing nonheme iron, produced by the human gastric pathogen Helicobacter pylori: evidence for a third class of ferritins. *J Bacteriol* **175**: 966–972.

Gerhard, M., Lehn, N., Neumayer, N., Boren, T., Rad, R., Schepp, W., *et al.* (1999) Clinical relevance of the Helicobacter pylori gene for blood-group antigen-binding adhesin. *Proc Natl Acad Sci USA* **96**: 12778–12783.

Gilbert, J.V., Ramakrishna, J., Sunderman, F.W.J., Wright, A., and Plaut, A.G. (1995) Protein Hpn: cloning and characterization of a histidine-rich metal-binding polypeptide in Helicobacter pylori and Helicobacter mustelae. *Infect Immun* **63**: 2682–2688.

Goddard, A., and Logan, R. (1995) One-week low-dose triple therapy: new standards for Helicobacter pylori treatment. *Eur J Gastroenterol Hepatol* **7**: 1–3.

Gomez-Duarte, O.G., Lucas, B., Yan, Z.X., Panthel, K., Haas, R., and Meyer, T.F. (1998) Protection of mice against gastric colonization by Helicobacter pylori by single oral dose immunization with attenuated Salmonella typhimurium producing urease subunits A and B. *Vaccine* **16**: 460–471.

Gomez-Duarte, O.G., Bumann, D., and Meyer, T.F. (1999) The attenuated Salmonella vaccine approach for the control of Helicobacter pylori-related diseases. *Vaccine* **17**: 1667–1673.

Humphery-Smith, I., Cordwell, S.J., and Blackstock, W.P. (1997) Proteome research: complementarity and limitations with respect to the RNA and DNA worlds. *Electrophoresis* **18**: 1217–1242.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (1994) Schistosomes, liver flukes and Helicobacter pylori. *IARC Monogr Eval Carcinog Risks Hum* **61**: 1–241.

Ilver, D., Arnqvist, A., Ogren, J., Frick, I.M., Kersulyte, D., Incecik, E.T., *et al.* (1998) Helicobacter pylori adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science* **279**: 373–377.

Jiang, Q., Hiratsuka, K., and Taylor, D.E. (1996) Variability of gene order in different Helicobacter pylori strains contributes to genome diversity. *Mol Microbiol* **20**: 833–842.

Jungblut, P.R., and Seifert, R. (1990) Analysis by high-resolution two-dimensional electrophoresis of differentiation-dependent alterations in cytosolic protein pattern of HL-60 leukemic cells. *J Biochem Biophys Methods* **21**: 47–58.

Jungblut, P., Eckerskorn, C., Lottspeich, F., and Klose, J. (1990) Blotting efficiency investigated by using two-dimensional electrophoresis, hydrophobic membranes and proteins from different sources. *Electrophoresis* **11**: 581–588.

Jungblut, P., Otto, A., Zeindl-Eberhart, E., Pleissner, K.P., Knecht, M., Regitz-Zagrosek, V., *et al.* (1994) Protein composition of the human heart: the construction of a myocardial two-dimensional electrophoresis database. *Electrophoresis* **15**: 685–707.

Jungblut, P.R., Schaible, U.E., Mollenkopf, H.J., Zimny-Arndt, U., Raupach, B., Mattow, J., *et al.* (1999a) Comparative proteome analysis of Mycobacterium tuberculosis and Mycobacterium bovis BCG strains: towards functional genomics of microbial pathogens. *Mol Microbiol* **33**: 1103–1117.

Jungblut, P.R., Grabher, G., and Stöffler, G. (1999b) Comprehensive detection of immunorelevant Borrelia garinii antigens by two-dimensional electrophoresis. *Electrophoresis* **20**: 3611–3622.

Kimmel, B., Bosserhoff, A., Frank, R., Gross, R., Goebel, W., and Beier, D. (2000) Identification of Immunodominant Antigens from *Helicobacter pylori* and Evaluation of Their Reactivities with Sera from Patients with Different Gastroduodenal Pathologies. *Infect Immun* **68**: 915–920.

Klaamas, K., Held, M., Wadstrom, T., Lipping, A., and Kurtenkov, O. (1996) IgG immune response to Helicobacter pylori antigens in patients with gastric cancer as defined by ELISA and immunoblotting. *Int J Cancer* **67**: 1–5.

Kleanthous, H., Lee, C.K., and Monath, T.P. (1998) Vaccine development against infection with Helicobacter pylori. *Br Med Bull* **54**: 229–241.

Klose, J., and Kobalz, U. (1995) Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. *Electrophoresis* **16**: 1034–1059.

Labenz, J., and Borsch, G. (1995) Toward an optimal treatment of Helicobacter pylori-positive peptic ulcers. *Am J Gastroenterol* **90**: 692–694.

Lage, A.P., Godfroid, E., Fauconnier, A., Burette, A., Butzler, J.P., Bollen, A., *et al.* (1995) Diagnosis of Helicobacter pylori infection by PCR: comparison with other invasive techniques and detection of cagA gene in gastric biopsy specimens. *J Clin Microbiol* **33**: 2752–2756.

Lamarque, D., Gilbert, T., Roudot-Thoraval, F., Deforges, L., Chaumette, M.T., and Delchier, J.C. (1999) Seroprevalence of eight Helicobacter pylori antigens among 182 patients with peptic ulcer, MALT gastric lymphoma or non-ulcer dyspepsia. Higher rate of seroreactivity against CagA and 35-kDa antigens in patients with peptic ulcer originating from Europe and Africa. *Eur J Gastroenterol Hepatol* **11**: 721–726.

Lee, A., O'Rourke, J., De Ungria, M.C., Robertson, B., Daskalopoulos, G., and Dixon, M.F. (1997) A standardized

mouse model of Helicobacter pylori infection: introducing the Sydney strain. *Gastroenterology* **112**: 1386–1397.

Marchetti, M., Arico, B., Burroni, D., Figura, N., Rappuoli, R., and Ghiara, P. (1995) Development of a mouse model of Helicobacter pylori infection that mimics human disease. *Science* **267**: 1655–1658.

Marchetti, M., Rossi, M., Giannelli, V., Giuliani, M.M., Pizza, M., Censini, S., *et al.* (1998) Protection against Helicobacter pylori infection in mice by intragastric vaccination with H. pylori antigens is achieved using a non-toxic mutant of E. coli heat-labile enterotoxin (LT) as adjuvant. *Vaccine* **16**: 33–37.

Marshall, B.J., and Warren, J.R. (1984) Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **1**: 1311–1315.

Mattsson, A., Quiding-Jarbrink, M., Lonroth, H., Hamlet, A., Ahlstedt, I., and Svennerholm, A. (1998) Antibody-secreting cells in the stomachs of symptomatic and asymptomatic Helicobacter pylori-infected subjects. *Infect Immun* **66**: 2705–2712.

McAtee, C.P., Lim, M.Y., Fung, K., Velligan, M., Fry, K., Chow, T.P., *et al.* (1998a) Characterization of a Helicobacter pylori vaccine candidate by proteome techniques. *J Chromatogr B Biomed Appl* **714**: 325–333.

McAtee, C.P., Fry, K.E., and Berg, D.E. (1998b) Identification of potential diagnostic and vaccine candidates of Helicobacter pylori by 'proteome' technologies. *Helicobacter* **3**: 163–169.

McAtee, C.P., Lim, M.Y., Fung, K., Velligan, M., Fry, K., Chow, T., *et al.* (1998c) Identification of potential diagnostic and vaccine candidates of Helicobacter pylori by two-dimensional gel electrophoresis, sequence analysis, and serum profiling. *Clin Diagn Lab Immunol* **5**: 537–542.

McGee, D.J., and Mobley, H.L. (1999) Mechanisms of Helicobacter pylori infection: bacterial factors. *Curr Top Microbiol Immunol* **241**: 155–180.

Megraud, F. (1997) How should Helicobacter pylori infection be diagnosed? *Gastroenterology* **113**: S93–S98.

Michetti, P., Corthesy-Theulaz, I., Davin, C., Haas, R., Vaney, A.C., Heitz, M., *et al.* (1994) Immunization of BALB/c mice against Helicobacter felis infection with Helicobacter pylori urease. *Gastroenterology* **107**: 1002–1011.

Moayyedi, P., Sahay, P., Tompkins, D.S., and Axon, A.T. (1995) Efficacy and optimum dose of omeprazole in a new 1-week triple therapy regimen to eradicate Helicobacter pylori. *Eur J Gastroenterol Hepatol* **7**: 835–840.

Mollenkopf, H.J., Jungblut, P.R., Raupach, B., Mattow, J., Lamer, S., Zimny-Arndt, U., *et al.* (1999) A dynamic two-dimensional polyacrylamide gel electrophoresis database: the mycobacterial proteome via Internet. *Electrophoresis* **20**: 2172–2180.

Müller, E.-C., Thiede, B., Zimny-Arndt, U., Scheler, C., Prehm, J., Müller-Werdan, U., Wittmann-Liebold, B., Otto, A., and Jungblut, P. (1996) High-performance human myocardial two-dimensional electrophoresis database – edition 1996. *Electrophoresis* **17**: 1700–1712.

Nilsson, I., Ljungh, A., Aleljung, P., and Wadstrom, T. (1997) Immunoblot assay for serodiagnosis of Helicobacter pylori infections. *J Clin Microbiol* **35**: 427–432.

Nomura, A., Stemmermann, G.N., Chyou, P.H., Kato, I., Perez-Perez, G.I., and Blaser, M.J. (1991) Helicobacter pylori infection and gastric carcinoma among Japanese Americans in Hawaii. *N Engl J Med* **325**: 1132–1136.

Odenbreit, S., Wieland, B., and Haas, R. (1996) Cloning and genetic characterization of Helicobacter pylori catalase and construction of a catalase-deficient mutant strain. *J Bacteriol* **178**: 6960–6967.

Odenbreit, S., Till, M., Hofreuter, D., Faller, G., and Haas, R. (1999) Genetic and functional characterization of the alpAB gene locus essential for the adhesion of Helicobacter pylori to human gastric tissue. *Mol Microbiol* **31**: 1537–1548.

Otto, A., Thiede, B., Müller, E.-C., Scheler, C., Wittmann-Liebold, B., and Jungblut, P. (1996) Identification of human myocardial proteins separated by two-dimensional electrophoresis using an effective sample preparation for mass spectrometry. *Electrophoresis* **17**: 1643–1650.

Parsonnet, J., Friedman, G.D., Vandersteen, D.P., Chang, Y., Vogelman, J.H., Orentreich, N., *et al.* (1991) Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med* **325**: 1127–1131.

Radcliff, F.J., Hazell, S.L., Kolesnikow, T., Doidge, C., and Lee, A. (1997) Catalase, a novel antigen for Helicobacter pylori vaccination. *Infect Immun* **65**: 4668–4674.

Riley, M. (1993) Functions of the gene products of Escherichia coli. *Microbiol Rev* **57**: 862–952.

Segal, E.D., Shon, J., and Tompkins, L.S. (1992) Characterization of Helicobacter pylori urease mutants. *Infect Immun* **60**: 1883–1889.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* **68**: 850–858.

Solnick, J.V., Josenhans, C., Suerbaum, S., Tompkins, L.S., and Labigne, A. (1995) Construction and characterization of an isogenic urease-negative mutant of Helicobacter mustelae. *Infect Immun* **63**: 3718–3721.

Telford, J.L., Ghiara, P., Dell'Orco, M., Comanducci, M., Burroni, D., Bugnoli, M., *et al.* (1994) Gene structure of the Helicobacter pylori cytotoxin and evidence of its key role in gastric disease. *J Exp Med* **179**: 1653–1658.

Tomb, J.F., White, O., Kerlavage, A.R., Clayton, R.A., Sutton, G.G., Fleischmann, R.D., *et al.* (1997) The complete genome sequence of the gastric pathogen Helicobacter pylori. *Nature* **388**: 539–547.

Warren, J.R. (1983) Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* **1**: 1273–1275.

Weel, J.F., van der Hulst, R.W., Gerrits, Y., Roorda, P., Feller, M., Dankert, J., *et al.* (1996) The interrelationship between cytotoxin-associated gene A, vacuolating cytotoxin, and Helicobacter pylori-related diseases. *J Infect Dis* **173**: 1171–1175.

Xiang, Z., Censini, S., Bayeli, P.F., Telford, J.L., Figura, N., Rappuoli, R., *et al.* (1995) Analysis of expression of CagA and VacA virulence factors in 43 strains of Helicobacter pylori reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. *Infect Immun* **63**: 94–98.

Zevering, Y., Jacob, L., and Meyer, T.F. (1999) Naturally acquired human immune responses against Helicobacter pylori and implications for vaccine development. *Gut* **45**: 465–474.