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Comparative proteomics and immunoproteomics of *Helicobacter pylori* related to different gastric pathologies☆

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Abstract

Helicobacter pylori is a Gram-negative bacterium which causes ulcer, atrophic gastritis, adenocarcinoma, or mucosa-associated lymphoid tissue lymphoma. A comparative proteomic and immunoproteomic analysis was chosen to identify the antigenic patterns of three different *H. pylori* strains. These strains were probed against single sera from *H. pylori*-positive patients affected by gastric adenocarcinoma or duodenal ulcer. We found a quite heterogeneous antigenic pattern, both from strain and sera points of view, thus underlying both a strain- and a host-specificity. The different antigenic repertoires introduced the importance of the strain to be used for immunoblotting as a diagnostic test.

Keywords: Immunoproteomic

1. Introduction

Helicobacter pylori is a spiral-shaped, microaerophilic, Gram-negative bacterium which colonizes the apical side of human gastric epithelial cells and mucous layer. Infection by *H. pylori* affects approximately 30% of the population in Western Europe and United States, and about 80% of the population in many developing countries. *H. pylori* is associated with severe pathologies, such as gastric or duodenal ulcer, atrophic gastritis, adenocarcinoma, or mucosa-associated lymphoid tissue lymphoma. Epidemiological studies have demonstrated up to a six-fold increased risk in developing adenocarcinoma for patients infected by *H. pylori*. Accordingly, since 1994 this bacterium was classified by the World Health Organization International Agency for Research on Cancer as a class I carcinogen for humans [1]. Although *H. pylori* is associated with serious diseases, only a subset of infected individuals develop gastroduodenal disease during its lifetime, probably because some stains of *H. pylori* are more pathogenic than other. Thus, the development of disease depends on infecting *H. pylori* strains virulence, host susceptibility and environmental co-factors. DNA typing has established that *H. pylori* is extremely diverse as a species, and it is likely that the varied infection outcomes reflect differences in bacterial genotype as well as genetic, physiological, and immunological factors in human host [2]. Accordingly, there is an urgent need to understand how microbe genetic setup, host genetic setup, host diet and environment impact the outcome of infection. To enhance understanding of *H. pylori* and its interaction with human host, it has been underlined the importance of characterizing proteins and other antigens that various *H. pylori* strains produce, and the human responses to them. Moreover, identification and characterization of predictive biomarkers to different *H. pylori*-related diseases could improve serological tests for detecting and monitoring *H. pylori* infections, and distinguish the outcome of its infection. Characterization of conserved antigens between different strains is beneficial for both

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diagnostic and vaccine purposes. In fact, with the increasing antibiotic resistance of *H. pylori* [3], it appears that the most practical way of controlling and eradicating *H. pylori* will be through the development of effective protein-based vaccines. So far, mainly conserved or very abundant antigens of *H. pylori* including urease A and B subunits, catalase, CagA, VacA, the GroES homologue HspA and NapA have been analyzed for their protective or therapeutic potentials in animal studies. All these antigens have been recognized by serum antibodies, even if none have been shown to be protective in humans [4–9]. Thus, the characterization of further antigens seems important to evaluate valuable combinations of antigens for vaccine purposes. Considering the advantage of an efficacious vaccine, it is important to identify the *H. pylori* proteins that elicit a strong immune response in humans to analyze their capability of conferring protective immunity. In the various preclinical animal models, vaccination has been shown to protect against *H. pylori* challenge infection [10]. Most of vaccines tested so far contain only one or two antigens, although recent studies suggested that combining several protective antigens could substantially increase vaccine efficacy [6,11,12]. Characterization of protein differences between strains may be of particular significance, indicating proteins which could be important as strain markers associated with specific clinical outcomes of *H. pylori* infection. The identification of such proteins might also provide important insights on the molecular mechanism of pathogenesis. According to Correa [13], intestinal gastric cancer may be considered as a multistep process starting from chronic gastritis and progressing through chronic atrophic gastritis, intestinal metaplasia and dysplasia. It is now apparent that this sequence is usually triggered by *H. pylori* infection and affected by a variety of genetic and environmental factors that may act synergistically [14]. Therefore, the comparison of different strains at the protein level (expression from active genes), but not at the DNA level (where both active and inactive genes may be identified), could be very useful to identify characteristic markers for *H. pylori* strains related to different diseases. Particularly, it is very important characterizing strains from patients affected by gastric cancer and identifying markers for this pathology.

Proteomic analysis is a powerful approach for the resolution and identification of proteins in complex biological samples [15–17]. The proteome technology can be used to link certain patterns to specific diseases or modifications of specific marker proteins and to evaluate the proteome map variability; all these data may supplement results of genome and transcriptome typing. Moreover, even when the complete genome of an organism may be determined, only proteomics allow comprehending links between the genetic information and the phenotype-related response. Proteomic analysis has been used to identify disease markers associated with the spectrum of *H. pylori* associated pathologies as well as potential vaccines candidates [18–20]. In fact, this approach combined with Western Blotting using patient sera (immunoproteomics) has been widely used to investigate the humoral immune response against microbial pathogens. Several groups used this approach to detect candidate antigens of *H. pylori* for diagnosis, therapy and vaccine development and to investigate potential association between specific immune responses and manifestations of disease [21–26].

In the present work, we carried out a preliminary and qualitative comparative analysis between strains isolated from duodenal ulcer and gastric carcinoma to identify markers of pathology. Moreover, we also tested single sera from *H. pylori*-positive patients affected by gastric adenocarcinoma or duodenal ulcer with three strains of *H. pylori*, isolated from biopsies of patients with different pathologies (chronic gastritis, duodenal ulcer and diffuse gastric cancer) to highlight possible common/different antigens to be used in development of future specific vaccines.

2. Experimental

2.1. Bacterial strains and culture conditions

Helicobacter pylori strains (Table 1) were isolated from tissues of patients affected by chronic gastritis (strain 328, $cagA^+$ B/D subtype, $vacA^+$ s1/m1 subtype), duodenal ulcer (strain G39, $cagA^+$ BD subtype, $vacA^+$ s1/m1 subtype) and diffuse gastric carcinoma (strain 10K, $cagA^+$ A(I) subtype, $vacA^+$ s1/m1 subtype). The biopsy was streaked onto the surface of Columbia agar containing 5% (v/v) horse blood, 10 mg/L vancomycin, 5 mg/L trimethoprim, 20 U/ml polymyxin B and 5 mg/L cefsulodin. Plates were incubated in a microaerobic environment obtained by using an anaerobic jar with a gas-generating kit for microaerophilic atmosphere (10% CO_2, 6% O_2, 0% H_2, 84% N_2, Oxoid Camp GasPak, Oxoid Unipath, Garbagnate Milanese, Italy). Colonies resembling *H. pylori* were identified by gram's stain as well as oxidase, catalase and urease tests.

2.2. 2D-PAGE (two-dimensional polyacrlamide gel electrophoresis)

Sonicated *H. pylori* bacterial suspensions were solved in 8 M urea, 4% (w/v) CHAPS (3-[(cholamidopropyl)dimethylamino]-1-propanesulfonate), 40 mM Tris, 65 mM dithioerythritol and a trace of bromophenol blue (rehydratation buffer). Fifty micrograms (for analytical runs) or 100 μg (for preparative runs or electrotransfer onto nitrocellulose sheet) of protein samples were brought to a final volume of 600 μl with rehydratation buffer and a trace of bromophenol blue. Proteins were adsorbed onto an Immobiline DryStrip (IPG, Immobilized pH Gradient, 18 cm, pH range 3.0–10 non-linear gradient, Amersham Pharmacia Biotech) for 6–18 h. Isoelectring focusing (IEF) was carried out on a horizontal electrophoresis system Multiphor II (Amersham Pharmacia Biotech, Uppsala, Sweden). The voltage was linearly increased from 300 to 3500 V during the first

Table 1
Helicobacter pylori strains used

Code of strain	Pathology	CagA sub-type	VacA sub-type
328	Chronic gastritis	+, B/D	s1/m1
G39	Duodenal ulcer	+, B/D	s1/m1
10K	Gastric adenocarcinoma	+, A(I)	s1/m1

3 h, and then stabilized at 5000 V for 22 h (total 110 kVh). The IPG strips were then equilibrated in 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS (sodium dodecyl sulfate), 0.05 M Tris–HCl pH 6.8, 2% (w/v) dithioerythritol and later also with 2.5% (w/v) iodoacetamide. Electrophoresis in the second dimension was carried out on a 9–16% polyacrylamide linear gradient gel (18 cm × 20 cm × 1.5 mm) at a constant current of 40 mA, until the dye front reached the bottom of the gel. Analytical and preparative gels were stained with ammoniacal silver nitrate and colloidal Coomassie G250, respectively [27,28]. Digitalized images were obtained by Laser Densitometer (Molecular Dynamics, Sunnyvale, CA, USA) scanning of the gels and then analysed qualitatively and quantitatively by the Melanie II 2D-PAGE software (BioRad, Hercules, CA). Spot intensities were obtained in pixel units and normalized to the total absorbance of the gel.

2.3. Human sera

Human sera (Table 2) were obtained from *H. pylori*-positive patients affected by different diseases, including duodenal ulcer and gastric carcinoma; as a negative control we used a pool of sera from five volunteers without gastric disorders and negative to *H. pylori* infection. Patients were considered positive to *H. pylori* infection when both the CLO and histological tests were positive. All patients were white and recruited from the same geographical area.

2.4. Western blotting

Proteins were electrophoretically transferred from 2D-E gels onto nitrocellulose sheets using a Novablot semidry transblot cell containing 50 mM Tris, 39 mM glycine, 1 mM SDS and 20% (v/v) methanol. Electrotransfer time was 75 min with a current/area of 0.7 mA/cm^2. Contrarily to previous reports, antigens were identified by incubation of the membranes with human sera (dilution of 1:600) for 90 min. Human sera were from patients positive to *Helicobacter pylori* infection. Before and after addition of sera, membranes were blocked for 1 h with 3% (w/v) bovine serum albumin in PBS (phosphate buffered saline) and then washed three times with 0.05% (w/v) Tween-20 in PBS, for 10 min. Immuno-detection was performed with goat anti-human IgG horseradish-peroxidase conjugated Ig (Bio-Rad) at a dilution of 1:3000 followed by enhanced chemiluminescence (ECL kit, Amersham). Before and after the addition of the secondary antibody, the membranes were washed three times with 0.05% (w/v) Tween-20 in PBS, for 10 min.

2.5. Mass spectrometry analysis

Spots from 2-DE were excised from the gel, triturated, alkylated and digested with trypsin as previously reported [29]. Gel particles were extracted with 25 mM NH_4HCO_3/acetonitrile (1:1, v/v) by sonication and peptide mixtures were concentrated. Samples were desalted using μZipTipC18 pipette tips (Millipore) before matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) analysis. Peptide mixtures from 2-DE spots were loaded on the MALDI target together with CHCA (cyano-4-hydroxycinnamic acid) as matrix, using the dried droplet technique. Samples were analysed with a Voyager-DE PRO spectrometer (Applera). Peptide mass spectra for PMF (Peptide Mass Fingerprinting) experiments were acquired in reflectron mode; internal mass calibration was performed with peptides derived from trypsin autoproteolysis. PSD (Post Source Decay) fragment ion spectra were eventually acquired after isolation of the appropriate precursor, as previously reported [29]. In both cases, data were elaborated using the DataExplorer 5.1 software (Applera). ProFound software was used to identify spots from NCBI non-redundant database by PMF experiments [30]. Candidates with ProFound's Est'd *Z* scores >2 were further evaluated by the comparison with M_r and p*I* experimental values obtained from 2-DE. Protein Prospector software was used to confirm spot identification using fragment ions obtained by PSD experiments.

2.6. Edman degradation analysis and database searching

Following preparative 2D-E runs, proteins were electrotransferred onto a PVDF (polyvinylidene difluoride) membrane (Bio-Rad; 0.2 μm pore size) using a semi-dry blotting apparatus (Novablot II; Amersham Pharmacia Biotech, Uppsala, Sweden) [31]. Blots were stained with 0.1% (w/v) Coomassie Brilliant Blue R250 in 50% (v/v) methanol for 1 min, and then destained in an aqueous solution containing 10% (v/v) acetic acid and 40% (v/v) methanol. PVDF membrane spots were excised and transferred to a Hewlett Packard protein sequencer, Model 241 (Palo Alto, CA, USA). Protein sequence tags were used as probes for searches in SWISS-PROT and TrEMBL annotated protein sequence databases, using both BLAST and Blitz softwares.

2.7. Statistical analysis

The statistical analysis was carried out using the Chi-square test with Yates or Mantzel-Haenszel correction and significance was as indicated ($P < 0.05$).

Table 2
Human sera used

Code of human sera	Positivity to *H. pylori* infection	Pathology and histological type	Dilution
E1136	+	Gastric adenocarcinoma	1:600
F824	+	Gastric adenocarcinoma	1:600
F840	+	Diffuse gastric adenocarcinoma	1:600
F123	+	Intestinal gastric adenocarcinoma	1:600
G39	+	Duodenal ulcer	1:600
Hp-negative pool	−	None	1:600

3. Results

Whole cell protein extracts of three different strains of *H. pylori* isolated by endoscopic biopsy from tissues of patients affected by chronic gastritis (strain 328), duodenal ulcer (strain G39), diffuse-type gastric carcinoma (strain 10K) were separated on a 2D-E gel covering the pH 3–10 (IPG non linear gradient) and M_r 200–8 kDa (linear gradient) ranges. Figs. 2A, 3A and 4A show the digitalized images of the gels stained with ammoniacal silver nitrate for strains 328, G39 and 10K, respectively. Using Melanie II 2-D PAGE analysis software, approximately 1650, 1500 and 1650 protein spots were resolved on 328, G39 and 10K 2D maps, respectively.

3.1. Comparative proteomics

To assess variability between *H. pylori* strains, a comparative proteome analysis of G39 and 10K strains was carried out. In both proteome patterns the protein spots were spread over the whole p*I* range 3–10 and the whole M_r range 8–200 kDa. The comparison of the two patterns revealed a genetic variability. Several spots were found at the same position, but positional shifts and differentially present/absent spots were also observed; identified proteins expressed by a single strain are circled and marked on 2D maps (Fig. 1) and are listed in Table 3. In particular, two shifts of horizontal spot series (spots 1 and 2) were observed. Spot 1 (a, b and c), corresponding to aconitate hydratase, had a p*I* 6.42, 6.53, 6.64 and 6.17, 6.23, 6.3 on 2D map of strain G39 and 10K, respectively; spot 2 (a, b and c), corresponding to fructose-bisphosphate aldolase, had a p*I* 6.40, 6.48, 6,56 and 5.99, 6.05, 6.3 on the 2D map of strain G39 and 10K, respectively. Four proteins were present only on the 2D map of G39 (Fig. 1A), namely 6-phosphogluconolactonase (spot 3), *S*-ribosylhomocysteine lyase (spot 4), aliphatic amidase (spot 5) and the hypothetical protein HP0697 (spot 6). On the other hand, five proteins could be considered specific for the strain 10K isolated from gastric carcinoma (Fig. 1B), namely hypothetical protein HP0958 (spot 7), transcription elongation factor greA (spot 8), quinone reactive Ni/Fe hydrogenase large subunit (spot 9), NADPH-flavin oxidoreductase (spot 10) and RecA protein (spot 11). All proteins were identified by a peptide mass fingerprint approach.

3.2. Comparative immunoproteomics

For immunoproteome analysis, proteins from *H. pylori* 328, G39 and 10K strains were electrotransferred onto nitrocellulose membranes and then probed with individual sera from *H. pylori*-positive patients suffering from gastric adenocarcinoma and duodenal ulcer. We used a pool of five sera from *H. pylori*-negative patients, as a negative control. Enhanced chemiluminescence was used to reveal the immunoreactive spots. We chose to use single sera at a 1:600 dilution, which allowed revealing more specific antigens than ones revealed with a 1:200 dilution, commonly used in immunoproteomic studies. Figs. 2–4 show the antigenic patterns obtained when cancer serum F840, duodenal ulcer serum G39 and *H. pylori* -negative pool were hybridized to the whole cell protein lysate of each *H. pylori* strain. The images of blots with the other cancer patients' sera will be available upon request. Only a small proportion of the proteins were antigenic: the average number of spots recognized by sera was 6.6 in strain 328, 10.4 in strain G39 and 15.6 in strain 10K. The immunoreactive spots, circled and marked in Figs. 2–4, are reported in Table 4.

Out of these proteins, 13 spots were identified by gel matching with a previously produced and characterized reference gel [32]. Ten additional antigens were newly identified by a peptide fingerprint MALDI-TOF approach. Spot 20 was not identified

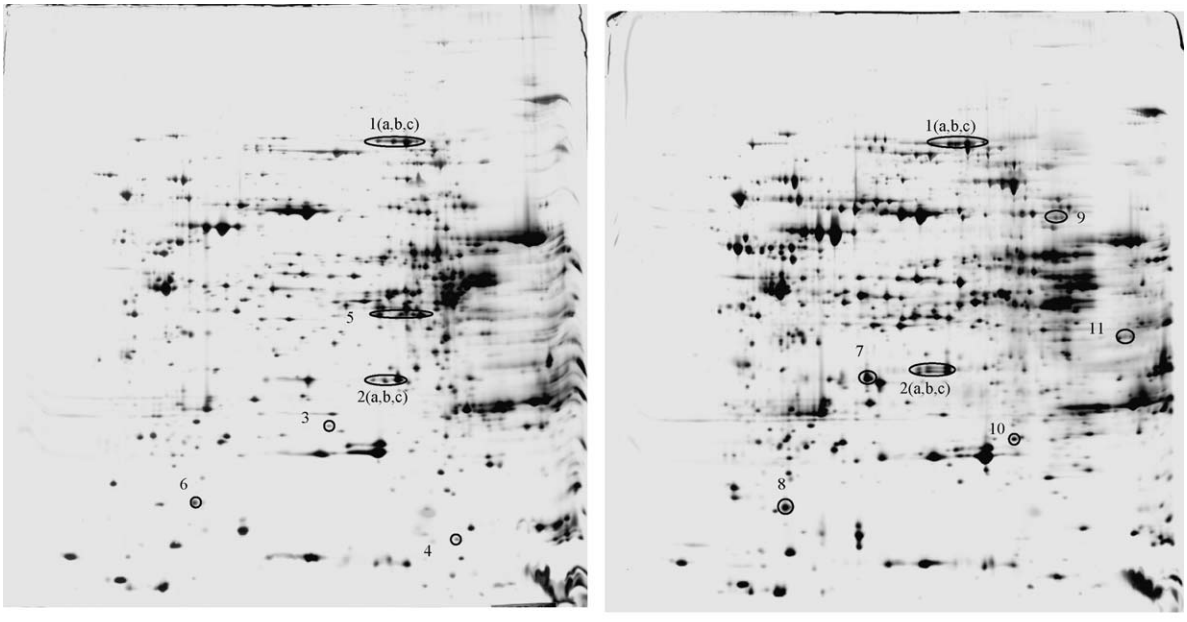

Fig. 1. Comparison between 2D proteomic maps of *H. pylori* strains G39 (A) and 10K (B). Spots of difference are circled.

Table 3
Comparison of protein *H. pylori* patterns of strains G39 and 10K

Spot	Protein	Gene name	Cellular process	Strain G39	Strain 10K
1	Aconitate hydratase 2	*Acnb*	TCA cycle	Shift	Shift
2	Fructose-bisphosphate aldolase	*Fba*	Glycolysis/gluconeogenesis	Shift	Shift
3	6-Phosphogluconolactonase	*Pgl*	Pentose phosphate pathway	+	–
4	*S*-Ribosylhomocysteine lyase	*LuxS*	Quorum sensing	+	–
5	Aliphatic amidase	*AmiE*	Amino acids and amines	+	–
6	Hypothetical protein HP0697	*HP0697*	Unknown function	+	–
7	Protein HP0958	*HP0958*	Motility	–	+
8	Transcription elongation factor greA	*GreA*	Transcription factor	–	+
9	Quinone reactive Ni/Fe hydrogenase large subunit	*HydB*	Electron transport	–	+
10	NADPH-flavin oxidoreductase	*FrxA*	Electron transport	–	+
11	RecA	*RecA*	DNA replication, recombination and repair	–	+

Identified proteins at different position and differentially present or absent.

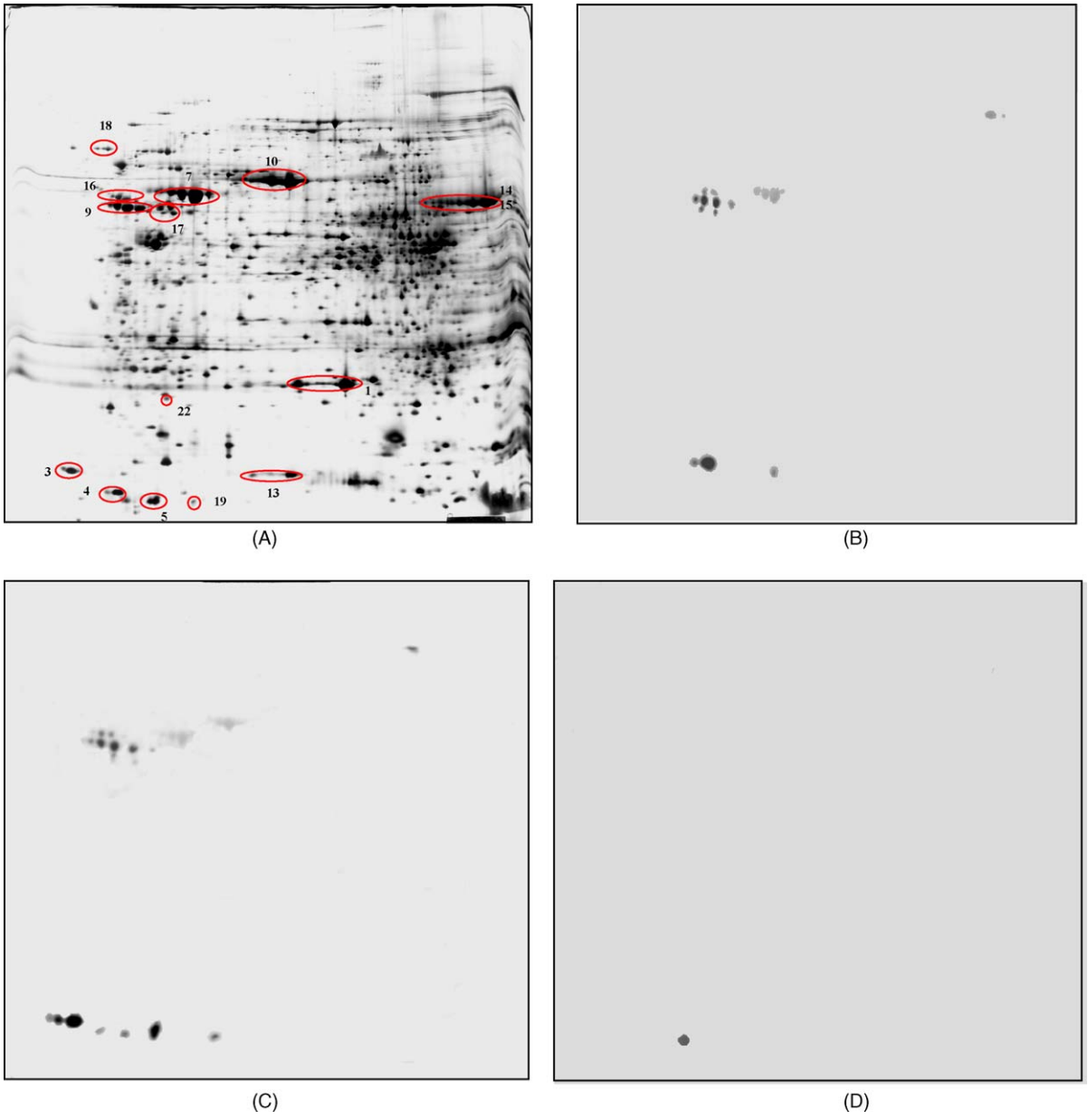

Fig. 2. 2D proteomic map of *H. pylori* strain 328 and detection of *H. pylori* antigens by immunoblotting analysis with human sera. Detected antigens are indicated by numbers as reported in Table 4. (A) Silver stained 2D-PAGE map; (B) immunodetection with serum F840; (C) immunodetection with serum G39; (D) immunodetection with *H. pylori*-negative pool.

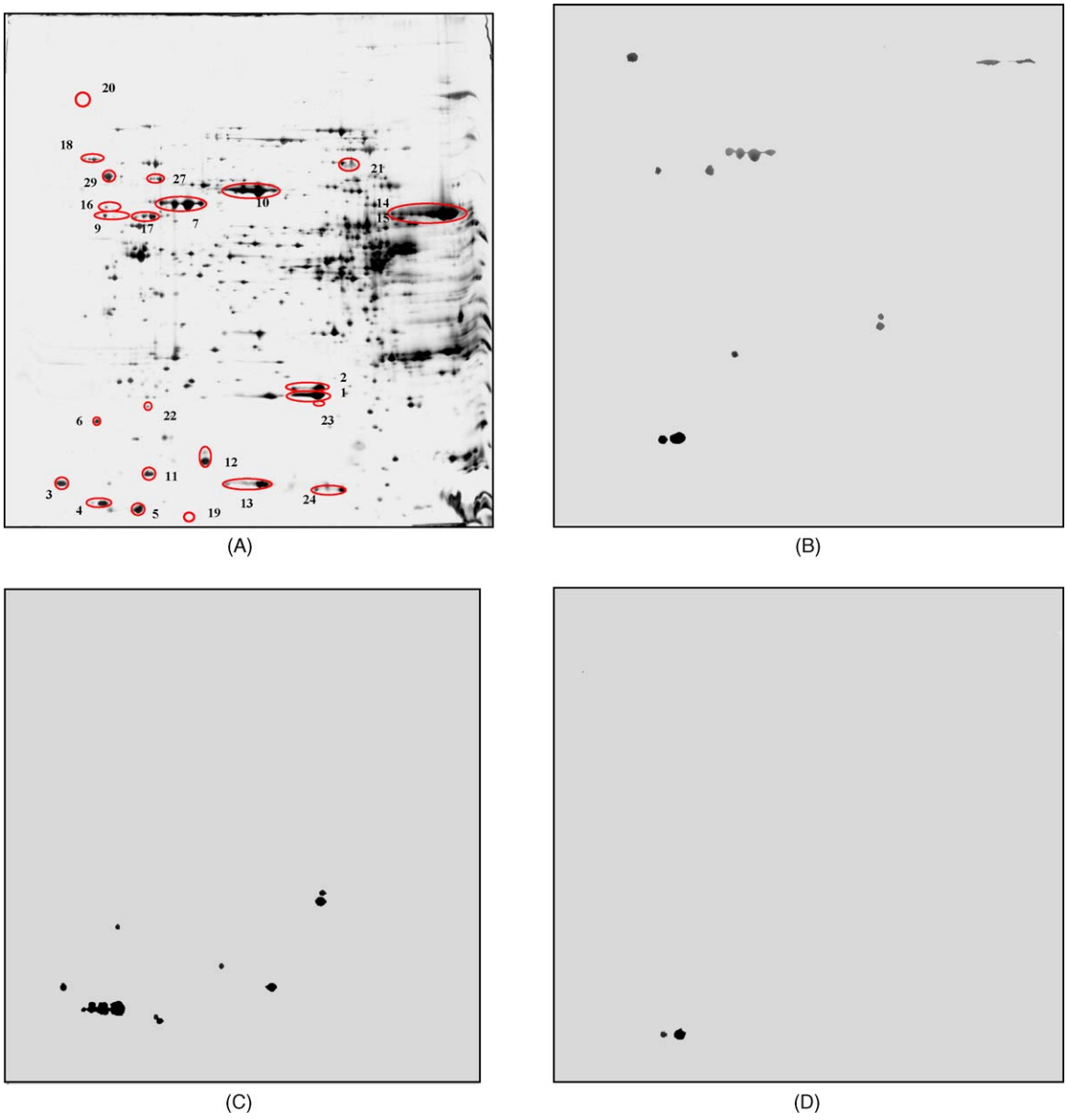

Fig. 3. 2D proteomic map of *H. pylori* strain G39 and detection of *H. pylori* antigens by immunoblotting analysis with human sera. Detected antigens are indicated by numbers as reported in Table 4. (A) Silver stained 2D-PAGE map; (B) immunodetection with serum F840; (C) immunodetection with serum G39; (D) immunodetection with *H. pylori*-negative pool.

since it was not detected in silver or colloidal Coomassie G250 stained-gels, presumably containing less than 1 ng of protein. From the functional point of view, antigenic proteins belonged to different classes. Several proteins were housekeeping enzymes involved in energy metabolism (flavodoxin A, thioredoxin, ATP synthase alpha chain), amino acid biosynthesis (hydantoin utilization protein A), general cellular processes (alkyl hydroperoxidase reductase, chaperone GroEL, neutrophil acvtivating protein A, chaperone GroES, chaperone and heat shock protein 70) and DNA translation (ribosomal protein L7/L12). Several identified proteins have a cytoplasmatic localization, namely alkyl hydroperoxidase reductase (spots 1 and 2), inorganic pyrophosphatase (spot 6), chaperone GroEL (spot 7), non-heme iron-containing ferritin (spot 12), catalase (spots 14 and 15), ATP-dependent Clp protease proteolytic subunit (spot 22) and chaperone GroES (spot 24). In contrast, flavodoxin A, thioredoxin and urease B (spots 3, 5 and 10, respectively) are secreted proteins [33]. Among the identified species, several proteins have been already considered as important pathogenic factors of *H. pylori*, namely urease B, flagellin A and B, catalase, chaperone GroEL, neutrophil acvtivating protein A and non-heme iron-containing ferritin.

On the basis of the distribution of immunoreactive spots within the three strains' proteomes, different antigenic patterns for the three strains analyzed were described, also according to the single sera probed (Table 5). Only a single protein was recognized by each serum in each strain and also by the *H. pylori*-negative pool; this was the case of the two isoforms of

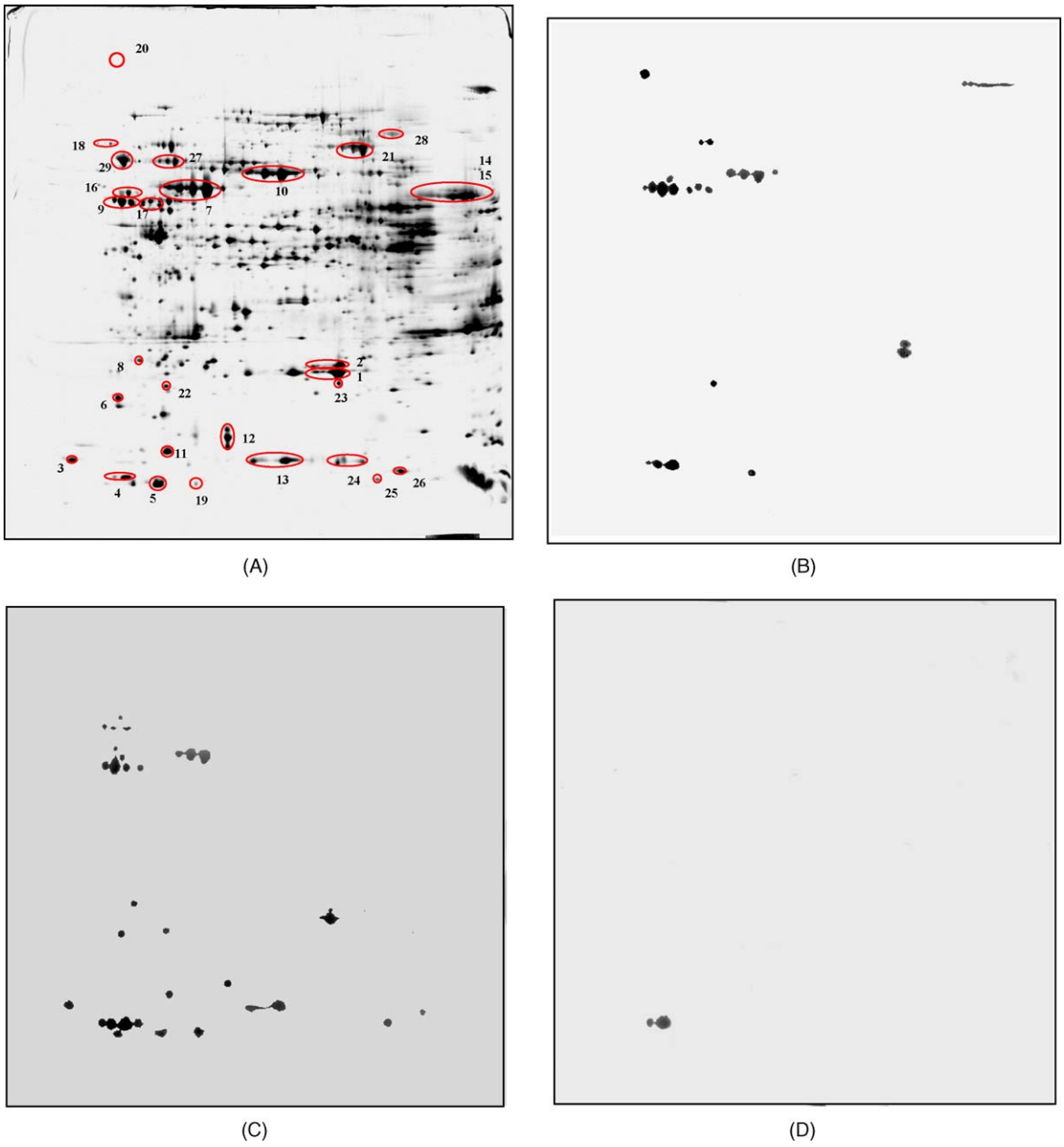

Fig. 4. 2D proteomic map of *H. pylori* strain 10K and detection of *H. pylori* antigens by immunoblotting analysis with human sera. Detected antigens are indicated by numbers as reported in Table 4. (A) Silver stained 2D-PAGE map; (B) immunodetection with serum F840; (C) immunodetection with serum G39; (D) immunodetection with *H. pylori*-negative pool.

the ribosomal protein L7/L12 (spot 4). Previous observations had already shown the reactivity of this protein with negative control sera [24,34].

3.3. Classification of immunoreactive proteins

Immunoreactive protein spots recognized by *H. pylori*-positive sera can be divided into three groups, as summarized in Fig. 5. Group A is represented by proteins recognized by all sera in more than one strain (sub-group A1) or in only one strain (sub-group A2). Sub-group A1 includes two isoforms of alkyl hydroperoxidase reductase (spots 1 and 2), recognized by all sera when probed on strains from diffuse-type gastric carcinoma (10K) and duodenal ulcer (G39), as well as flagellin A and B proteins (spots 9 and 16) recognized by all sera probed on strain 10K and strain isolated by chronic gastritis (328). Sub-group A2 includes ATP synthase alpha chain and the antigen corresponding to spot 19 recognized by all sera in strain 10K, and GroEL in strain 328. On the other hand, group B is represented by proteins recognized only by ulcer serum. Inorganic pyrophosphatase (spot 6) and urease G (spot 8) were immuno-revealed in both strain 10K and strain G39. In contrast, group C included proteins recognized exclusively by gastric carcinoma sera. This group can be divided into three subgroups: proteins immuno-detected by more than one cancer serum in different strains (sub-group C1); proteins recognized by a single cancer serum in more than one

Table 4
Immunoreactive *H. pylori* proteins identified by Edman degradation (ED), gel matching (GM) and mass spectrometry (MS) analysis

Spot	Protein	Gene name	Cellular process/ function	Method of identification	Sequence coverage (%)	McAtee et al. [20]	Kimmel et al. [19]	Jungblut et al. [16]	Haas et al. [17]	Atanassov et al. [18]	Krah et al. [21]
1	Alkyl hydroperoxidase reductase	*TsaA**	Detoxification	GM			+				
2	Alkyl hydroperoxidase reductase	*TsaA**	Detoxification	GM							
3	Flavodoxin A	*FldA*	Electron transport	GM				+			
4	Ribosomal protein L7/L12	*L7/L12*	Protein synthesis and modification	GM		+	+	+	+		
5	Thioredoxin	*TrxA*	Electron transport	GM							
6	Inorganic pyrophosphatase	*Ppa*	Central intermediary metabolism	MS	38						
7	Chaperone and heat shock protein	*GroEL*	Protein folding and stabilization	GM			+	+	+	+	+
8	Urease G	*UreG*	Central intermediary metabolism	MS	24						
9	Flagellin A	*FlaA*	Chemotaxis and motility	GM		+	+			+	
10	Urease B	*UreB*	Central intermediary metabolism	GM		+	+	+			
11	Hypothetical protein HP0697	*HP0697*	Unknown function	GM				+	+		
12	Non-heme iron-containing ferritin	*Pfr*	Transport and binding proteins	MS	46						
13	Neutrophil activating protein A	*NapA*	Detoxification	GM			+				
14	Catalase	*Kata*	Detoxification	GM				+	+		
15	Catalase	*Kata*	Detoxification	GM							
16	Flagellin B	*FlaB*	Chemotaxis and motility	GM		+					
17	ATP synthase alpha chain	*AtpA*	ATP-proton motive force interconversation	MS	34						+
18	Chaperone and heat shock protein 70	*DnaK**	Protein folding and stabilization	GM							
19	5.43/14192		–								
20	4.78/14553		–								
21	Hydantoin utilization protein A	*HyuA*	Amino acid biosynthesis	MS	29				+		+
22	ATP-dependent Clp protease proteolytic subunit	*ClpP*	Degradation of proteins	MS	32	+			+		
23	Alkyl hydroperoxide reductase C22 protein	*AhpC*	Detoxification	MS	63						
24	Chaperone GroES	*GroES*	Protein folding and stabilization	GM			+	+			

25	Hypothetical protein HP0902	*HP0902*	Unknown function	ED									+
26	Thioredoxin	*HP1458*	Electron transport	ED									
27	GTP-binding protein TypA/BipA homolog	*TypA*	Unknown function	MS	38			+					
28	Fumarate reductase flavoprotein subunit	*FrdA*	Anaerobic energy metabolism	GM				+			+		
29	Chaperone and heat shock protein 70	*DnaK**	Protein folding and stabilization	MS	44		+				+		
30	Cytotoxicity associated gene A protein	*CagA*	Inflammatory response modulation	GM		+		+			+		+

Antigenic proteins previously reported by other authors are indicated. Unidentified proteins have been indicated with their pI/M_r coordinates. Asterisk indicates proteins present as different molecular species.

strain (sub-group C2) and proteins recognized by a single cancer serum in only one strain proteins (sub-group C3). Sub-group C1 includes the proteins corresponding to spots 18, 20, 14 and 15. In particular, chaperone-heat shock protein 70 was immuno-revealed by serum F123 probed with strains 10K and G39, by serum F824 probed with strain 328 and serum E1136 probed with strain 10K. Spot 20 of strain 10K was immuno-reactive for sera E1136, F840 and F123. The same protein of strain G39 was revealed by serum F840. Two isoforms of catalase were detected by E1136 probed with all strains and by F123 probed with strain 10K. Sub-group C2 included hydantoin utilization protein A (spot 21) recognized by the serum E1136 when probed with strain 10K and G39. Sub-group C3 included alkyl hydroperoxide reductase C22 protein (spot 23) and fumarate reductase flavoprotein subunit (spot 28), immuno-revealed by serum E1136, as well as GTP-binding protein TypA/BipA homolog (spot 27) by serum F840, when probed with strain 10K.

3.4. Comparison of antigenic patterns for strains probed with the same serum

We observed a strain-specificity by comparing the reactivity to the same sera in three different strains. Table 6 shows the different antigenic patterns observed when the three strains were probed with the same serum. Strain 10K isolated from a patient affected by diffuse-type gastric carcinoma had an antigenic pattern richer than those from gastritis and duodenal ulcer. Thus, the strain's immunogenicity/antigenicity scale could be formulated as gastric carcinoma (10K) > duodenal ulcer (G39) > chronic gastritis (328).

The statistical analysis of immunorecognition of same serum towards different strains proves that cancer serum E1136 recognized a significantly higher number of antigens in strain 10K respecting to the number of antigens of the other two strains (10K versus G39, $P = 0.046$; 10K versus 328, $P = 0.038$; G39 versus 328, $P = 0.009$). Cancer serum F123 recognized a significant number of antigens on strain G39 and strain 10K respecting to strain 328 (G39 versus 328, $P = 0.01$; 10K versus 328, $P = 0.005$), although there was no difference between strain G39 and strain 10K. Ulcer serum G39 recognized a significantly higher number of antigens in strain 10K respecting to strains G39 and 328 (10K versus G39, $P = 0.009$; 10K versus 328, $P = 0.038$), although without any difference between G39 and 328. If we statistically compare the number of immunoreactive proteins of each strain, the strain's immunogenicity/antigenicity scale is confirmed as follows: 10K versus 328, $P = 0.00001$; 10K versus G39, $P = 0.005$; G39 versus 328, $P = 0.03$).

4. Discussion

The ability of *Helicobacter pylori* to provoke pathologies in a sensitive host is determined by several factors, acting, alone or synergistically, in different times of infection. The variety of pathologies related to *H. pylori* is a consequence of host physiological and immunological factors, bacterial genotypes

Table 5
Antigenic patterns of *H. pylori* strains immunorevealed by single human sera

Spot	Protein name	Gene name	*Sera probed on strain 328*						*Sera probed on strain G39*						*Sera probed on strain 10K*					
			E1136	**F824**	**F840**	**F123**	**G39**	**Hp⁻**	**E1136**	**F824**	**F840**	**F123**	**G39**	**Hp⁻**	**E1136**	**F824**	**F840**	**F123**	**G39**	**Hp⁻**
1	Alkyl hydroperoxidase reductase	*AhpC*		+					+	+	+	+	+		+	+	+	+	+	
2	Alkyl hydroperoxidase reductase	*AhpC*							+	+	+	+	+		+	+	+	+	+	
3	Flavodoxin A	*FldA*					+		+			+	+		+			+	+	
4	Ribosomal protein L7/L12	*L7/L12*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	Thioredoxin	*TrxA*					+		+	+		+	+		+				+	
6	Inorganic pyrophosphatase	*Ppa*											+						+	
7	Chaperone and heat shock protein	*GroEL*	+	+	+	+	+		+		+	+			+		+	+	+	
8	Urease	*UreG*																	+	
9	Flagellin A	*FlaA*	+	+	+	+	+		+		+	+			+	+	+	+	+	
10	Urease accessory protein B	*UreB*	+	+			+		+						+			+		
11	Hypothetical protein HP0697	*HP0697*							+			+			+				+	
12	Non-heme iron-containing ferritin	*Pfr*							+			+	+		+				+	
13	Neutrophil activating protein A	*NapA*							+			+	+		+				+	
14	Catalase	*Kata*	+						+						+			+		
15	Catalase	*Kata*	+						+						+			+		
16	Flagellin B	*FlaB*	+	+	+	+	+		+			+			+	+	+	+	+	
17	ATP synthase alpha chain	*AtpA*		+			+		+		+	+			+	+	+	+	+	

18	Chaperone and heat shock protein 70	*DnaK*		+								+			+			+		
19	*pI/Mr* 5.43/14192				+		+		+						+	+	+	+	+	
20	*pI/Mr* 4.78/14553										+				+		+	+		
21	Hydantoin utilization protein A	*HyuA*							+						+					
22	ATP-dependent Clp protease proteolytic subunit	*ClpP*						+			+				+		+		+	
23	Alkyl hydroperoxide reductase C22 protein	*AhpC*													+					
24	Chaperone GroES	*GroES*							+						+					
25	Hypothetical protein HP0902	*HP0902*													+				+	
26	Thioredoxin	*HP1458*													+				+	
27	GTP-binding protein TypA/BipA homolog	*TypA*															+			
28	Fumarate reductase flavoprotein subunit	*FrdA*													+					
29	Chaperone and heat shock protein 70	*DnaK*										+				+		+	+	
30	Cytotoxicity associated gene A protein	*CagA*			+		+				+						+			

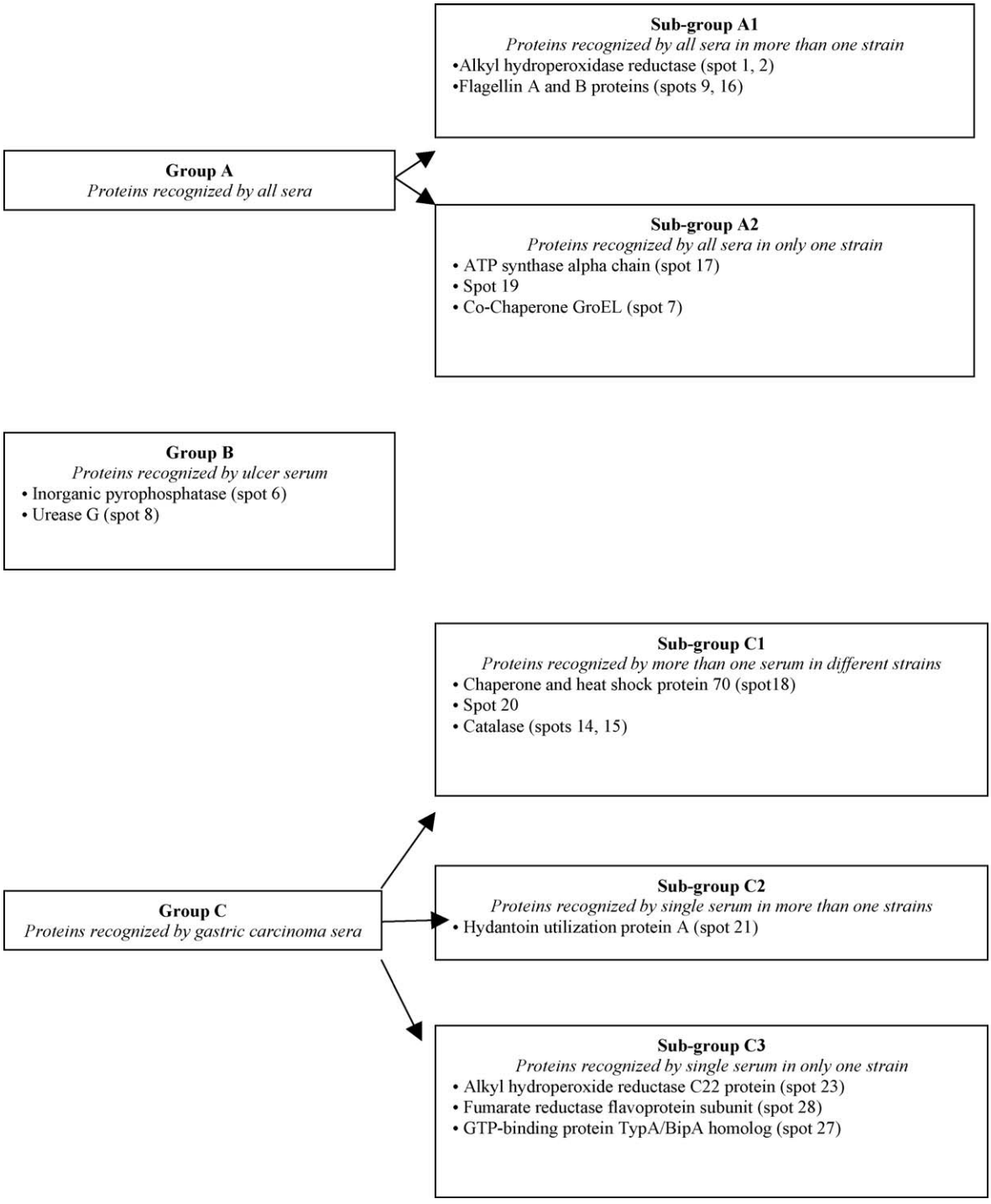

Fig. 5. Classification of immunoreactive *H. pylori* proteins on the basis of serum- and strain-specificity.

variability and host/bacterium geographical localization. In fact, bacterial virulence factors failed to be certainly associated to specific clinical outcome [35], being environmental and host factors probably involved in the fate of *H. pylori* infection as well. On the contrary, the humoral immune response specifically directed against the pathogen is an important host element that can help to discriminate among disease severity. To date, only a single antigenic marker was detected by immunoblotting for gastric B cell lymphoma [36], while larger analysis using pooled human patient sera failed to yield any *H. pylori*-related pathology-specific antigen serological marker [24].

The proteomic approach has been previously used for mapping the protein pattern of *H. pylori* strains with known genome, although only once it was used together with immunoproteomics [21]. More recently, a 2D map of commercial strain NCTC11637 was obtained [37]. Jungblut et al. [21] have published the comparison of proteome maps of *H. pylori* strains 26695, J99 and SS1, standard strains of different origin, but not clinical strains.

Table 6
Comparison of antigenic *H. pylori* patterns of strains probed with the same serum

Spot	Protein name	*Gene name*	Serum E1136			Serum F824			Serum F840			Serum F123			Serum G39		
			Strain 328	Strain G39	Strain 10K	Strain 328	Strain G39	Strain 10K	Strain 328	Strain G39	Strain 10K	Strain 328	Strain G39	Strain 10K	Strain 328	Strain G39	Strain 10K
1	Alkyl hydroperoxidase reductase	*AhpC*		+	+	+	+	+		+	+		+	+		+	+
2	Alkyl hydroperoxidase reductase	*AhpC*		+	+		+	+		+	+		+	+		+	+
3	Flavodoxin A	*FldA*		+	+								+	+	+	+	+
4	Ribosomal protein L7/L12	*L7/L12*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	Thioredoxin	*TrxA*		+	+		+						+		+	+	+
6	Inorganic pyrophosphatase	*Ppa*														+	+
7	Chaperone and heat shock protein	*GroEL*	+	+	+	+			+	+	+	+	+	+	+		+
8	Urease	*UreG*															+
9	Flagellin A	*FlaA*	+	+	+	+		+	+	+	+	+	+	+	+		+
10	Urease accessory protein B	*UreB*	+	+	+	+								+	+		
11	Hypothetical protein HP0697	*HP0697*		+	+								+				+
12	Non-heme iron-containing ferritin	*Pfr*		+	+								+			+	+
13	Neutrophil activating protein A	*NapA*		+	+								+			+	+
14	Catalase	*Kata*	+	+	+									+			
15	Catalase	*Kata*	+	+	+									+			
16	Flagellin B	*FlaB*	+	+	+	+		+	+		+	+	+	+	+		+
17	ATP synthase alpha chain	*AtpA*		+	+	+		+		+	+		+	+	+		+
18	Chaperone and heat shock protein 70	*DnaK*			+	+							+	+			
19	*pI/Mr* 5.43/14192			+	+			+	+		+			+	+		+
20	*pI/Mr* 4.78/14553				+					+	+			+			
21	Hydantoin utilization protein A	*HyuA*		+	+												

Table 6 (*Continued*)

22	ATP-dependent Clp protease proteolytic subunit	*ClpP*				+															+
23	Alkyl hydroperoxide reductase C22 protein	*AhpC*				+															
24	Chaperone GroES	*GroES*		+	+																
25	Hypothetical protein HP0902	*HP0902*			+																+
26	Thioredoxin	*HP1458*			+																+
27	GTP-binding protein TypA/BipA homolog	*TypA*														+					
28	Fumarate reductase flavoprotein subunit	*FrdA*				+															
29	Chaperone and heat shock protein 70	*DnaK*																			+
30	Cytotoxicity associated gene A protein	*CagA*														+					

Instead, Enroth et al. [38] have reported potential differences in proteome patterns of strains isolated from patients affected by different gastric pathologies, although they did not identify any proteins. They did not associate proteins with any specific gastric disease, but suggested the presence of disease specific proteins, which would be diagnostically interesting. Govorun et al. [39] compared the proteomes of four clinical isolates and identified eight proteins characterized by different expression levels or by different p*I* values, but they did not focus on gastric cancer.

In the present study, we carried out a preliminary comparative proteomic analysis aimed to characterize the protein patterns of two different strains, namely G39 and 10K, isolated from patients with duodenal ulcer and gastric carcinoma; we tried identifying proteins related to phenotypes associated with strain variability as potential markers of pathology. We chose these two pathologies since there are statistical evidences that these two types of disease are mutually exclusive. We identified nine proteins that could be potential markers of pathology. Two proteins, aconitase hydratase 2 and fructose-bisphosphate aldolase, were equally present in both strains but showed a shift in the 2D maps, probably as a consequence of amino acid substitutions within genes determining changes in protein p*I* values, as already reported by others [21]. In contrast, the presence or absence of some protein spots on 2D maps may be attributed to repression of expression or complete loss of the corresponding gene from the genome [40], which could be important in specific strains. Regarding the proteins exclusively present in the ulcer strain, many of them play important roles for the bacterium life. In fact, LuxS plays a role in intracellular signalling related to quorum sensing and regulates *flaA* expression with consequences on the infection process [41]. Aliphatic amidase E is an ammonia-producing enzyme conferring *H. pylori* resistance to strong acidity and capacity of damaging tissues during colonization [42]. One protein, HP0697, was hypothetical before the present work, having an unknown function. Notably, one of the protein specific of diffuse gastric adenocarcinoma strain is HP0958, which very recently was found to be an essential motility protein in *H. pylori,* regulating the expression of flagellar genes [43]. GreA is a bacterial elongation factor stimulating an endogenous endonucleolytic transcript cleavage activity of the RNA polymerase [44], but its action is still unstudied in *H. pylori*. Analogously, little is known on *H. pylori* HydB except for a presumable electron transport activity by homology with other bacteria. FrxA is a nitroreductase whose normal role is not known, although it is probably involved in metabolites levels regulation [45]; RecA is involved in DNA repair [46]. Both these proteins had been suspected playing a controversial role in metronidazole resistance of *H. pylori* [47].

Generally, strains of bacterial pathogens have different phenotypes including alterated mode of pathogenicity, and antibiotic and chemical resistance. Comparative proteomics between *H. pylori* strains related to different infection outcomes will allow discovering specific proteins that may become potential targets for diagnostics. In addition, due to the ascertained link between *H. pylori* and gastric carcinoma, this approach could be very useful to find markers of this pathology, by analysing and

characterizing the proteome of strains associated with gastric cancer.

Although it will be necessary to analyse a major number of strains for each disease, this preliminary qualitative study demonstrated the efficacy of this approach; in fact, it allowed identifying proteins to be further explored and eventually used both for laboratory tests which analyse *H. pylori* strain associated with specific disease and for diagnosis of the different pathologies related to this widespread bacterium. Moreover, this approach allowed characterizing proteome patterns that can be used as fingerprints to distinguish isolates and to evaluate if an unsuccessful eradication of *H. pylori* infection could be caused by a persisting infection of the same strains or if the eradication is followed by reinfection with another strain.

In addition to the proteome characterization, we carried out a comparative immunoproteomic analysis aimed to identify the antigenic patterns of different *H. pylori* strains, by probing them against sera from patients affected by diverse gastrointestinal pathologies. We probed human sera on *H. pylori* strains derived from the same geographical area and we probed cancer sera on a cancer strain, too. This is an important novelty of our approach. In fact, there are significant geographical differences between the clinical manifestations of *H. pylori* infections; the link between strain specificity [48] and risk of gastric cancer within specific geographical populations [49,50] has been recently reported. In addition, the use of heterologous strains in diagnostic serological tests was envisaged [51,52].

Our approach was based on the observation that, if serological markers specific for *H. pylori* exist and are indicative of a well defined clinical condition, the comparison between pathological antigenic patterns could be potentially useful to correctly identify such markers, as already proved by others [26]. We chose to adopt individual sera instead of pools; in fact, it has been reported that the use of pooled sera may result in potentially misinterpreted results, since abundance and affinity of antibodies in single sera may strongly vary. As suggested by other authors [22], an extended analysis of antigenic proteins recognized by a large number of individual sera could better lead to the identification of proteins with diagnostic value and potential vaccine candidates. In fact, while several effective serological tests have been produced, none was based on purified antigens, thus requiring a higher sensitivity and specificity. An effective anti-*H. pylori* vaccine is also required, especially because eradication by conventional anti-microbial therapies is often followed by reinfection and urease- or VacA-based vaccines in mouse-*H. felis* models were only partially successful [53,5]. Candidates for vaccine purposes should be proteins from the outer bacterial membrane. In this work, two proteins (flagellin A and flagellin B) in principle meet the criteria for vaccine antigens; in fact, most of antigenic proteins found were described as cytoplasmatic. This phenomenon may be explained on the basis of recent data describing chaperone GroEL and urease expression on cellular membranes determined by a specific secretory system [54] or by autolysis of a subpopulation of *H. pylori* cells that occurs in vitro [55] as well as in vivo [56]. Catalase, another immunogenic cytoplasmic protein, may be located both in the cytosol and periplasmic space [57]. It should be considered that other cytoplasmic *H. pylori* antigens could be secreted by autolysis, a still unknown mechanism to export enzymes on cell surface, or may become adsorbed by outer membrane. Thus, all cytoplasmic antigens detected in this work could also be considered as potential vaccine candidates. Finally, secreted proteins as flavodoxin A, thioredoxin and urease B, being in direct contact with host tissues, may mediate important interaction between *H. pylori* and host.

The identification of pathology-specific antigens would implement new more efficient diagnostic and treatment strategies. Most of antigens found were housekeeping enzymes, which seem to be promising candidates for diagnostic test kits; in fact, they did not cross-react with *H. pylori* negative sera (except ribosomal L7/L12) and were highly conserved among different *H. pylori* strains. In contrast, virulence factors such as CagA and VacA showed sequence variability or may be subjected to a phase variation due to a slipped-strand mispairing, as suggested for several *H. pylori* outer membranes proteins. Moreover, in recent years it has been reported that some of these enzymes could play a role to enhance pathogens' virulence [58].

We identified 30 antigens recognized by *H. pylori* positive sera, 9 out of these (thioredoxin, inorganic pyrophosphatase, urease G, non-heme iron-containing ferritin, proteins corresponding to spots 19 and 20, alkyl hydroperoxide reductase C22 protein, hypothetical protein HP0902 and thioredoxin HP1458) being newly identified and 21 confirmed from other studies. In fact, Kimmel et al. identified 29 proteins which reacted strongly with sera from patients affected by different diseases related to *H. pylori* infection [24], and 8 out of these are in common with antigens detected in this study. They showed a highly variable immune response but revealing no correlation between the presence of particular antibody and the state of disease. On the contrary, other works and the present study demonstrated the presence of antigens related to specific disease. Jungblut et al. [21] identified 32 antigens, 6 out of these recognized only by cancer serum and 1 only by ulcer serum. Another study [22] identified 32 antigenic proteins, 9 out of these found to be immunoreactive also in the present work; they probed sera from patients with active *H. pylori* infection, or with gastric cancer, against proteins of the strain HP26695. Several antigens were differently recognized by sera from gastritis and ulcer patients. Recently, Krah et al. [26] have analysed the reactivity of duodenal ulcer and gastric cancer sera against the HP26695 strain and have demonstrated that four protein species could be useful as markers for gastric carcinoma detection and three out of these were confirmed in this work. Newly reported antigens were Pfr, HP1458, a new thioredoxin protein besides the already known TrxA, involved in the bacterial anti-oxidative response to fight the oxidative action of macrophages and neutrophils, and the hypothetical protein HP0902.

We found a quite heterogeneous antigenic pattern, both from strain and sera points of view, thus underlying both a host- and a strain-specificity, as summarized in antigen classification shown in Fig. 5. Proteins having the features of group A antigens, being pathology-unspecific, could be considered important as vaccine candidates as well as for diagnostic tests to better evaluate a current or previous *H. pylori* infection independently from the

pathology. Group B antigens, Ppa and UreG, being recognized exclusively by ulcer serum in two strains, could be considered as ulcer disease indicators. No recognition of strain 328 may be due to its poorer antigen repertoire. Ppa, an inorganic pyrophosphatase, is a housekeeping gene whose sequence variation allowed Achtman and coworkers defining *H. pylori* clonal grouping from different geographical regions [59]. UreG is an accessory protein required for nickel ion insertion into the urease apoenzyme and is necessary for full enzymatic activity, essential for bacterial colonization [60]. All proteins belonging to group C should represent potential markers of gastric adenocarcinoma. However, serum- and strain-specificity as well as histopathological information on single patient did not allow identification of universal carcinoma markers. Nevertheless, many of group C proteins have already been indicated as tumor markers by other authors, although they limited immunorecognition to a single *H. pylori* strain, the commercial strain 26695. For example, Krah et al. [26] proposed HyuA and GroES as gastric carcinoma markers and a similar conclusion was suggested by Jungblut et al. [21] for TypA, BipA and Kata. In the present work, we enlarged the spectrum of proteins with a potential diagnostic and therapeutic value, by adding AhpC and DnaK. For this latter we found immunoreactivity towards one new molecular species, according to Jungblut's definition [21]. Also for other proteins we revealed differential immunorecognition against specific molecular species (Table 4), thus confirming, on new proteins, an important trend of useful diagnostic markers [26]. DnaK has been defined as a virulence factor in a recent transcriptomic comparative study [61] and one of the early immunogens for *H. pylori*-specific IgM [52]. AhpC is a novel hydroperoxide reductase having a still unknown function. Finally, to date our study has revealed expression and immunorecognition of four conserved hypothetical proteins or proteins with unknown function not described previously at the protein level.

In the present work, we used three strains derived from patients affected by different pathologies. These strains have shown different protein and antigenic repertoires [62], thus introducing the importance of the strain to be used for immunoblotting as a diagnostic test. In fact, the difference of antigenic patterns found between the same sera may be due to the use of different strains as sources of antigens. The present study stresses the importance of using different strains, as a single strain such as the sequenced HP26695 (used in the previous studies) might not encode or express all relevant antigens. Indeed, our immunoblotting data seem to indicate that strain choice can strongly affect immunoproteome results. In particular, HP26695 was isolated from a gastritis patient and might lack cancer-promoting factors and this correlates with the finding that strain 328, again from chronic gastritis, resulted to be the less suitable for such an investigation. On this basis, we propose the use of a defined and representative strain for antigenic preparation contained in specific diagnostic assays. In fact, cancer strains resulted richer in antigens than ulcer strains, which in turn were richer than gastritis ones (Table 5). Analogously, sera from cancer patients were richer of antibodies respecting to sera from ulcer patients (Table 4). In fact, cancer sera provided stronger immunoreactivity even respecting to the case when both homologous strain and serum were adopted (G39 patient). On the other hand, Haas et al. [22] reported that ulcer patients have more anti-*H. pylori* antibodies than gastritis ones, thus completing a congruency between antigen + antibody scale as follows: adenocarcinoma > duodenal ulcer > chronic gastritis. These findings may be explained with the observation that the same scale may be translated to the inflammatory state and tissue damage in all three pathologies [22].

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