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Differential Proteomics of *Helicobacter pylori* Associated with Autoimmune Atrophic Gastritis

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Atrophic autoimmune gastritis (AAG) is a condition of chronic inflammation and atrophy of stomach mucosa, for which development can be partially triggered by the bacterial pathogen *Helicobacter pylori* (HP). HP can cause a variety of gastric diseases, such as duodenal ulcer (DU) or gastric cancer (GC). In this study, a comparative proteomic approach was used by two-dimensional fluorescence difference gel electrophoresis (DIGE) to identify differentially expressed proteins of HP strains isolated from patients with AAG, to identify markers of HP strain associated with AAG. Proteome profiles of HP isolated from GC or DU were used as a reference to compare proteomic levels. Proteomics analyses revealed 27 differentially expressed spots in AAG-associated HP in comparison with GC, whereas only 9 differential spots were found in AAG-associated HP profiles compared with DU. Proteins were identified after matrix-assisted laser desorption ionization (MALDI)-TOF and peptide mass fingerprinting. Some AAG-HP differential proteins were common between DU- and GC-HP (peroxiredoxin, heat shock protein 70 (HSP70), adenosine 5′-triphosphate (ATP) synthase subunit α, flagellin A). Our results presented here may suggest that comparative proteomes of HP isolated from AAG and DU share more common protein expression than GC and provide subsets of putative AAG-specific upregulated or downregulated proteins that could be proposed as putative markers of AAG-associated HP. Other comparative studies by two-dimensional maps integrated with functional genomics of candidate proteins will undoubtedly contribute to better decipher the biology of AAG-associated HP strains.

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INTRODUCTION

Autoimmune gastritis (AG), also known as autoimmune chronic atrophic gastritis or chronic type A gastritis, is an autosomal-dominant disease. AG is characterized by immune-mediated chronic inflammation, mucosal gland atrophy, with increased serum autoantibodies to gastric parietal cells and/or intrinsic factors, hypochlorhydria, vitamin B12 deficiency and, in some cases, neurological symptoms and diffuse metaplasia. In the late stages, patients show a higher risk for developing both neuroendocrine (carcinoid) and glandular (adenocarcinoma) tumors (1–4). In the presence of atrophy, AG is called atrophic AG (AAG).

In Correa's model of gastric carcinogenesis, *Helicobacter pylori* (HP) infection triggers the progressive sequence of gastric lesions from chronic gastritis, gastric atrophy, intestinal metaplasia, dysplasia and finally gastric adenocarcinoma (5).

Metaplastic AAG demonstrates similar histological and clinical findings as those of metaplastic AG related to HP (3). Of note, in the early phases of AG, HP infection may induce autoantibodies to gastric parietal cells, but later HP can spontaneously disappear (3,4,6). Thus, the exact relation between HP and gastric autoimmunity remains controversial as well as the question if HP may be the trigger or a perpetuating hit to AAG (3).

HP is a gram-negative, microaerophilic, spiral-shape bacterium colonizing the human gastric mucosa of more than half of the human world's population. HP preferentially colonizes the antrum of the stomach, for which pH is higher than in the corpus, but during gastritis progression, HP can invade the corpus. In most cases, HP causes asymptomatic gastric infections, but in others, it may progress to symptomatic chronic gastritis, gastric or peptic duodenal ulcer (DU), gastric cancer (GC) or mucosa-associated lymphoid tissue (MALT) lymphoma (7,8). For many years, the molecular cross-talk between HP and human gastric mucosa has been investigated (9–12).

HP strains are extraordinarily numerous, with every individual harboring a

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distinctive bacterial population with clonal variants. Furthermore, HP subclones may be isolated from the same biopsy or biopsies from different stomach locations and HP disease–specific strains may exist (13). Several genetic markers of pathogenicity characteristics for different HP strains have been extensively described; virulence factors associated with gastric carcinogenesis have also been identified, with the HP pathogenesis–related genes mostly residing in the cytotoxin-associated gene (*Cag*) pathogenicity island (9,11,14).

To date, there exists no reliable diagnostics to predict HP-infected patients at risk for developing HP-related pathologies, including AAG, DU and/or GC. Efforts for developing predictive diagnostics have mostly focused on HP disease–related biomarkers explored at gene level (14–18). However, the combination of the most extensively studied factors of pathogenicity *cagA*, *vacA* and *babA* fails in segregating a particular HP-virulent strain associated with a specific pathogenesis.

In this context, after the complete sequencing of four HP strains (19) (strains 26695, J99 and HPAG1: http://cmr.jcvi.org/tigr-scripts/CMR/shared/Genomes.cgi; strain Shi470: NCBI, accession number NC 010698), proteomic technologies hold promise for better disease-specific classification of HP strains. Proteomics allows bacteria to be characterized at the protein level based on the expression from active genes, thus encompassing the limits of DNA level, where both active and inactive genes may be identified and the difference in protein expression cannot been performed. The HP genome contains about 1,600 open-reading frames, 200 of which are known to encode expressed proteins (19,20). The HP proteome has been investigated for many years to characterize the intrinsic HP biology, and two-dimensional maps of several standard strains of different origin (e.g., 26695 from gastritis, J99 from DU, and HP strain Sydney strain 1, CagA negative [*CagA*$^{-}$ *SS1*]) are available (Supplementary Table S1). In parallel, proteomics identified some HP disease markers associated with severe gastric pathologies (e.g., AG, GC and DU; Supplementary Table S2). In particular, proteome components of HP have been investigated to identify functionally active genes, subcellular or secreted/translocated proteins, disease-specific proteins, as well as immunoreactive ones (Supplementary Tables S1, S2).

The more diffuse model for the progression from chronic AG to intestinal metaplasia, dysplasia and finally GC takes into consideration several steps; and it considers the HP infection as acting together with a variety of host genetic and environmental factors (21). Moreover, in patients with DU, more often mild AG occurs, and there is no increased risk of GC compared with risk in the general population (22,23). At present, there is still a lack of proteomics data for markers of patient tendency to develop AAG.

Proteome analysis represents a powerful approach to resolve and identify proteins in complex biological samples. Proteome data supplement data from genome and transcriptome approaches. Only proteomics allows deciphering of the connections between the genetic information and the phenotype-related response(s), even when the complete genome of an organism is determined (24).

In this study, we used a targeted comparative proteomic approach on the basis of two-dimensional fluorescence difference gel electrophoresis (DIGE) to identify differentially expressed proteins of HP isolated from patients with AAG, to be proposed as candidate markers for strains associated with HP-related clinical outcomes of AAG. Proteome profiles of HP isolated from GC or DU were used as a reference to compare AAG proteomic levels. Proteomics analyses revealed 27 differentially expressed proteins of AAG-associated HP in comparison with GC, whereas only nine differential proteins were found for AAG-associated HP profiles compared with DU. Our results may suggest that HP proteomes of HP isolated from AAG and DU share more common proteins than in regards to GC and provide subsets of putative AAG-specific up- or downregulated proteins, which could be proposed as putative markers of AAG-associated HP.

MATERIALS AND METHODS

Patients and Autoimmune Atrophic Gastritis Diagnosis

A total of 18 patients entering the diagnostic criteria of AAG from 2004 to 2010 were evaluated for the presence of past or active HP infection by serum HP-IgG determination (HP-IgG ELISA Biohit, cutoff 30 EIU/mL), histological examination and culture assays within the routine diagnostic workup for HP infection. Positive HP infection was ascertained when at least two parameters showed a positive result at enrollment and/or in previous visits. A total of 10 of 18 (55.5%) patients had a documented HP past or active infection. HP strains were isolated from antrum and/or corpus biopsies in 4 of 6 (66%) patients with active infection (Table 1). Although without atrophy, patient 4 (Table 1) was included in the analyses because of her high levels of antibodies anti-parietal cells (1:160), which increased to 1:1,280 1 month later. Patient 4 is currently undergoing follow-up. This group of patients was compared with both a group of 8 DU-affected patients and another group of 13 GC-affected patients. DU and GC pathologies were used as reference maps for comparative proteomics to individuate both up- or downregulated putative AAG-associated HP proteins, to find a potential marker (panel of markers) for the identification of Hp-related AAG (that is, often asymptomatic but may be associated with pernicious anemia and may be involved in GC development). All patients have been notified of the purpose of the study, and an informed consent has been obtained for all participants. The Internal Review Board of the CRO Institute approved the project as IRB-14-2013.

Bacterial Strains and Culture Conditions

HP strains were isolated from endoscopic biopsy samples from the stomach

Table 1. Characteristics of the patients affected by AAG, from whom HP strains were isolated.

Patient number	Age	Sex	PGI	PGII	PGI/PGII	Gastrin 17	Ab anti-HP	HP histology	Ab anti-PC	Atrophy[a]	Stomach location[b]
1	70	F	22.3	17.4	1.28	103.8	87.9	–	ND	1	Antrum
2	37	F	25	7.26	3.44	40	6.5	–	+	2	Corpus
3	42	F	14.8	10.9	1.36	350.8	100.8	+	+	3	Corpus
4[c]	40	F	107.5	10.3	10.44	2.31	97.8	–	+	0	Antrum and corpus

Ab anti-HP, antibodies against anti-HP; Ab anti-PC, antibodies against parietal cells; anti-HP, antibodies against HP; ND, not determined; PGI, serum pepsinogen I; PGII, serum pepsinogen II.
[a]Atrophy scores according to operative link for gastritis assessment (OLGA) system (adapted considering only a simple sample of antral biopsy).
[b]Stomach location refers to the biopsies from which HP strains were isolated.
[c]Patient at the first visit without atrophy, but with increasing anti-PC Ab (levels increasing from 1:160 to 1:1,280 after 1 month) and still under follow-up.

(corpus and/or antrum). The biopsy specimens were cultured in HP Selective Medium (Bio-Mèrieux, Rome, Italy), incubated at 37°C in a microaerophilic environment (Campygen Oxoid, Basingstoke, Hampshire, UK) for 3–4 d. The cultured bacteria were identified as HP based on gram-negative staining, curved or spiral shape and positivity for catalase, oxidase and urease production. Identification was further confirmed by polymerase chain reaction. Several sweeps of colonies, considered representative of the whole HP population, were subcultured on Columbia sheep blood agar (Kima, Padua, Italy). After bacterial growth, a suspension was obtained and stored at –80°C in microbial storage medium (Microbank; Pro-Lab Diagnostics, Richmond Hill, ON, Canada). Strains were revitalized after a median of 9 months (range 2–98 months) in HP Selective Medium. After expansion in Columbia sheep blood agar, an HP suspension was used for proteome extraction. Bacterial DNA extraction and polymerase chain reaction on the virulence factor genes *CagA*, *CagE* and *VirB11*, mapping into the *Cag* pathogenicity island (*Cag* PAI), *Vac A*, and *Hom A* and *B*, were also performed as previously described (25–27). As already reported, HP was likely isolated from gastric biopsy samples of AAG patients because of an impairment in functional gastric mucosa leading to increasing hypochlorhydria, saprofitic flora overgrowth and progressive disappearance of anti-HP antibodies (28,29). Indeed, most of our patients showed a low incidence of current active infection (6 of 18).

Protein Isolation and Labeling with Cyanine Dyes (CyDyes)

The AAG group of patients was compared with both a group of 8 DU-affected patients and another group of 13 GC-affected patients. DU and GC pathologies were used as reference maps for comparative proteomics to individuate putative up- or downregulated AAG-specific proteins for the identification of AAG.

The HP soluble as well as hydrophobic proteins were methanol/chloroform extracted with DIGE lysis buffer (30 mmol/L Tris, 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS {3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate}). Proteins were recovered at the liquid interphase by three subsequent steps: (a) methanol (MeOH) (4:1, v:v), (b) chloroform (1:1, v:v) and (c) Milli-Q H_2O addition (3:1, v:v), followed by centrifugation (13,000*g* for 5 min at 4°C). After removal of the aqueous upper layer, proteins were MeOH-precipitated (3:1, v:v), and the pellet was washed with ethanol and then resuspended in rehydration buffer for two-dimensional electrophoresis analysis (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 0.5% v/v pharmalytes). Protein concentration was determined by using Bio-Rad Bradford-based protein assay (Bio-Rad, Milan, Italy). Whatever the gastric disease, each individual protein sample extracted from HP isolates of one patient was always kept as a distinct sample. For DIGE labeling, the protein lysates were labeled with CyDyes according to the manufacturer's protocol (CyDye DIGE Fluor minimal dyes; GE Healthcare, Uppsala, Sweden). The sample pairs were mixed with an internal Cy2-labeled standard pool comprising equal amounts of each protein sample, which was used to reduce inter-gel variation. To minimize dye-specific labeling artifacts, Cy3- and Cy5-labeling patterns were swapped among the same group of samples (Supplementary Table S3).

Two-Dimensional DIGE

Proteins were first separated by isoelectrofocusing (IEF) on 11-cm immobilized pH gradient dry strips (IPG) with a nonlinear (NL) pH 3–10 gradient (Bio-Rad). For analytical gels, a pair of Cy3- and Cy5-labeled samples (each 25 μg protein) and 25 μg Cy2-labeled internal standard were pooled and filled up to 200 μL with rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 2% dithiothreitol). Strips were passively rehydrated overnight with the rehydration buffer supplemented with 2% (v/v) IPG buffer, pH 3–10 NL, 50 mmol/L dithiothreitol and 0.1% bromophenol blue, and the protein extracts at room temperature. IEF and second dimension were performed in Protean® IEF and Criterion™ Cells (Bio-Rad), respectively, as previously reported (30). After electrophoresis, analytical gels were washed with Milli-Q H_2O and scanned on a Typhoon Trio™ laser scan-

ner (GE Healthcare) (30). For preparative gels, 360 μg unlabeled protein pooled from amounts of the 4 AAG protein extracts was used, focused for 35 kilovolt hours (kVh) and stained with Coomassie Brilliant Blue CBB G-250 (Bio-Rad). Gel images were acquired on the Typhoon Trio 9400™ laser scanner (GE Healthcare) at 100 μm resolution by using a red excitation wavelength.

Image Analysis

Image analysis was performed by using DeCyder™ software, version 6.5 (GE Healthcare), as previously described (30). Briefly, images were subjected to Difference In-gel Analysis (DIA) to detect, quantify and normalize spots according to the volume ratio of the corresponding spots detected in the Cy2 image of the pooled-sample internal standard, and the Biological Variation Analysis (BVA) module to allow matching of spots from multiple gels, calculate average abundance changes and statistically analyze the differential protein expression. The normalized spot quantities were collectively analyzed as three independent groups: AA, GC and DU, which enabled matching of multiple gel images from different patients to provide statistical data on average abundance for each protein spot among the DIGE gels. Statistical analysis of variance was performed to accurately assess protein expression changes occurring in biological replicates comparing the three groups. Finally, the extended data analysis (EDA) module was used for multivariate analysis of protein expression data, derived from the BVA module, through principal component analysis (PCA) pattern analysis and discriminant analysis. Student *t* test was performed to assess the statistical significance of differentially expressed proteins. On the basis of average spot volume ratio, spots for which relative expression changed at least 1.5-fold between AAG and GC/DU at 95% confidence level (*t* test; $p < 0.05$) were considered to be significant. The spots identified as differentially expressed were subjected to matrix-assisted laser desorption ionization (MALDI)-TOF analysis.

Protein Identification by MALDI-TOF Peptide Mass Fingerprinting

Protein spots of interest were excised from the Coomassie-stained preparative gel using a spot cutter and destained with 25 mmol/L ammonium bicarbonate in 50% acetonitrile. After overnight trypsin digestion, peptides were extracted with 1% (trifluoroacetic acid) TFA, subjected to Zip Tip cleanup (Millipore, Milan, Italy) and eluted with 50% acetonitrile and 0.3% TFA. After mixing the sample with α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (10 g/L CHCA in 50% acetonitrile and 0.3% TFA) (1:1, v:v), peptides were spotted on the MALDI target. The peptide mass fingerprinting measures were performed on a Voyager-DE PRO Biospectrometry Workstation mass spectrometer (AB Sciex, Framingham, MA, USA). After external calibration with Peptide Mix4 (Proteomix) 500–3,500 Da (LaserBio Labs, Sophia-Antipolis Cedex, France), MALDI mass spectra were recorded, collected and processed by using Data Explorer, version 5.1, software (AB Sciex), peak lists were obtained from the raw data under routine laboratory conditions (30) and mass spectra were finally processed using Data Explorer, version 5.1, software (AB Sciex). Database searching was done with the MASCOT search engine, version 2.3 (Matrix Science, London, UK), against the National Center For Biotechnology Information non-redundant protein database (NCBInr) and Swiss-Prot databases, limiting the search to bacterial proteins, allowing for one trypsin missed cleavage and a 0.5-Da mass tolerance error. Protein localizations were attributed according to either the National Center for Biotechnology Information (NCBI) resource (http://www.ncbi.nlm.nih.gov/protein) or, in case of lack of information in the NCBI database, NCBI searches with the publicly available tool PSORTb, version 3.0.2, for bacterial localization prediction against gram-negative bacteria sequences (http://www.psort.org/psortb). For each protein, biological processes and molecular functions were reported according to the Gene Ontology (GO) description in the UniProtKN/TrEMBL database (http://www.uniprot.org/uniprot).

All supplementary materials are available online at www.molmed.org.

RESULTS

Proteoma of HP Strains Isolated from Patients with Autoimmune Atrophic Gastritis

In the HP strains successfully isolated from biopsies of four AAG patients (Table 1), we hereby used DIGE to identify differentially expressed proteins, compared with those isolated from DU and GC biopsies. Figure 1A represents a DIGE proteome map of HP proteins isolated from human biopsies of patient 4 (corpus and antrum samples) to focus on and visualize AAG-associated HP cyanine–labeled proteome maps. Around 1,600 spots were clearly detected and subsequently analyzed by using DeCyder software for differential protein expression. To investigate a possible protein variation depending on the stomach location of HP isolation (corpus versus antrum), protein profiles of HP isolated from corpus were compared with those from antrum. Image analyses by DeCyder of HP differentially expressed proteins revealed that the stomach location was not a parameter significantly influencing the pattern of HP protein expression in our series. Indeed, protein spot maps from antrum and corpus of the same patient analyzed with PCA were close and thus not different; moreover, independently from the patient, within each group (AAG, GC or DU), spot maps from corpus and antro were not placed in distinct subgroups (and thus not significantly different). Therefore, we further continued our analyses by comparing all proteome maps (AAG-HP maps versus DU- or GC-HP maps) independently from the stomach location of HP isolation. All differentially expressed proteins in AAG-associated HP strains are listed in Tables 2 and 3. A total of 15 spots were upregulated in

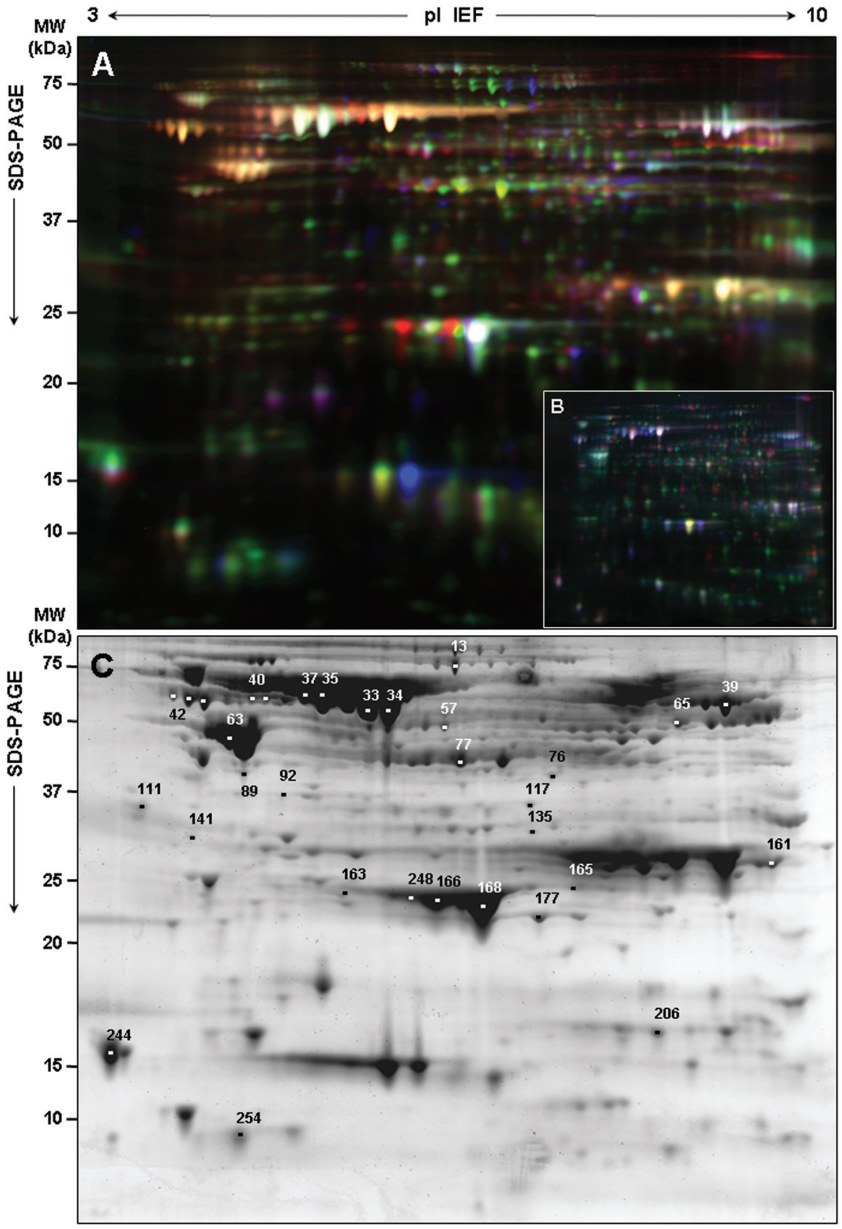

Figure 1. Representative analytical DIGE proteome map of HP from AAG (A) and micropreparative two-dimensional protein map of HP from AAG (B). (A) Proteins were resolved by IEF over the pI 3–10, followed by 8–16% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and overlaid by DeCyder. After extraction, proteins were labeled with Cy3 and Cy5. In particular, this gel (number 19) refers to samples $P102^{cy3}$ (antrum) and $P103^{cy5}$ (corpus) extracted from AAG-associated HP strains. An internal standard comprised of equal amounts of proteins from all samples (AAG, DU and GC) was labeled with Cy2 and included in all gels. (B) Gel number 18 is shown as representative of DIGE maps with proteins extracted from gastric cancer (cy5) comigrated with those extracted from *H. pylori* associated with DU (cy3). (C) Numbered spots indicate the differentially expressed proteins in AAG-associated HP strains in regards to either GC or DU. Identified spots are listed in Tables 2 and 3. Around 300 μg AAG-associated HP unlabeled protein pooled from amounts of samples was resolved by IEF over the pI range 3–10 NL, followed by 8–16% gradient SDS-PAGE and stained with CBB G-250. MW, Bio-Rad two-dimensional molecular weight standards.

AAG compared with GC (Table 2), of which 4 (spots 57, 168, 161 and 166) were also upregulated compared with DU (Table 3). Although a total of 12 spots resulted in downregulation in AAG compared with GC (Table 2), three of them (spots 37, 40 and 42) also downregulated in DU. Two additional spots were downregulated in only DU (spots 117 and 76) (Table 3).

Identification of HP Differentially Expressed Proteins

These 29 spots, indicated with dots in Figure 1B, were excised from micropreparative two-dimensional gels for protein identification by tryptic in-gel digestion and MALDI-TOF analysis. After a Mascot peptide mass fingerprinting database search using the acquired mass values, 20 spots were identified, corresponding to 15 distinct proteins. Details of protein identification, protein score, sequence coverage, theoretical isoelectric point (pI) value and molecular weight as well as GenBank® accession number and average relative change (fold difference) are shown in Tables 2 and 3. Some proteins were found in more than one spot: (a) the probable peroxiredoxin (gi|2507172; alternative names: 26-kDa antigen or thioredoxin reductase) was detected in four spots (166, 168, 244 and 248); (b) the urease β subunit (gi|57014163) was detected in two spots (33 and 34); and (c) the elongation factor Tu (gi|2494256) was detected in two spots (63 and 89).

Autoimmune Atrophic Gastritis Differentially Expressed Protein Involved in Various Biological Processes

Globally, all the identified proteins were involved in the following seven classes of biological processes: (a) protein biosynthesis/DNA translation/tRNA processing (ribosome-recycling or releasing factor, 50S ribosomal protein L30, elongation factor Tu and tRNA pseudouridine synthase A); (b) protein refolding and stress response (60-kDa chaperonin/GroEL and chaperone pro-

Table 2. Differentially expressed proteins of HP isolates related to AAG compared with those of HP isolates related to GC.

Spot number[a]	MW (Da)/pI	GenBank accession no.[b]	Protein annotation	Localization/biological process/ molecular function	Organism	Score/expect/ seq. coverage	Fold difference	*p*	Protein described previously (ref.)
HP proteins upregulated in AAG compared with GC									
13	27557/9.68	gi | 238057731	tRNA pseudouridine synthase A	Cytoplasm/tRNA processing	HP P12	32/1.9e + 002/26	10.19	3.54E-03	n.d.[c]
254	6644/10.96	gi | 226703094	50S ribosomal protein L301	Cytoplasm/ DNA translation/ protein synthesis	*Leptothrix cholodnii* (strain ATCC 51168/ LMG 8142/SP-6)	37/71/30	7.15	4.50E-03	(41)
206		n.i.					5.85	0.049	
168	22335/5.88	gi | 2507172	Probable peroxiredoxin or 26-kDa antigen or thioredoxin reductase	Cytoplasm/peroxidase activity/ oxidoreductase	HP	82/0.0019/52	5.69	3.28E-04	(41–43)
244	22335/5.88	gi | 2507172	Probable peroxiredoxin or 26-kDa antigen or thioredoxin reductase	Cytoplasm/peroxidase activity/ oxidoreductase	HP	75/0.0098/41	2.94	4.56E-03	(41–43)
33	61816/5.64	gi | 57014163	Urease β subunit	Secreted and cytosolic/ pathogenesis/urea catabolic process/hydrolase	HP J99	102/2.1e-005/46	2.5	0.025	(24,42,43,53, 62,75–77)
57	67136/4.99	gi | 226738136	Chaperone protein dnaK or heat shock 70-kDa protein or HSP70	Cytoplasm/protein folding/stress response	HP (strain Shi470)	32/1.8e + 00 2/8	2.44	0.0115	(24,41)
34	61846/5.64	gi | 57014163	Urease β subunit	Secreted and cytosolic/ pathogenesis/urea catabolic process/hydrolase	HP (strain P12)	75/0.0099/37	2.36	5.45E-03	(24,41–43,53, 62,75–77)
39	58706/8.70	gi | 2493545	Catalase	Cytoplasm/hydrogen peroxide catabolic process/peroxidase/ oxidoreductase	HP	106/8.1e-006/30	2.21	1.76E-03	(24,32,55,71, 76,78)
161		n.i.					2.2	8.87E-03	
166	22335/5.88	gi | 2507172	Probable peroxiredoxin or 26-kDa antigen or thioredoxin reductase	Cytoplasm/peroxidase activity/ oxidoreductase	HP	97/6e-005/48	2.15	3.10E-03	(41–43)
177		n.i.					1.87	0.017	
111		gi | 1706274	Bifunctional enzyme cysN/cysC	Cytoplasm/GTP catabolic process/sulfate assimilation	*Mycobacterium tuberculosis*	44/18/14	1.7	7.19E-03	n.d.[c]
77	74074/5.11	gi | 12230111	Flagellar hook–associated protein 2	Secreted/bacterial flagellum/ cell adhesion/flagellum assembly	HP J99	39/41/10	1.6	0.023	(79)
65	20180/5.84	gi | 208432952	Peptidoglycan-associated lipoprotein	Outer membrane/bacterial envelope integrity	HP G27	41/9.1e + 02/17	1.56	0.049	(59)

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Table 2. ***Continued.***

HP proteins downregulated in AAG compared with GC								
35	58321/5.44	gi|226704136	60-kDa chaperonin or GroEL	Cytoplasm/protein refolding/ stress response/ATP binding	HP (strain P12)	68/0.057/23	–6.58	2.84E-03	(24,32,41–43, 53,59,62,75, 77,80)
248	22335/5.88	gi|2507172	Probable peroxiredoxin or 26-kDa antigen or thioredoxin reductase	Cytoplasm/peroxidase activity/ oxidoreductase	HP	44/13/34	–5.31	2.45E-03	(41–43)
37	n.i.						–4.96	9.54E-06	
89	43734/5.17	gi|2494256	Elongation factor Tu	Cytoplasm/GTP catabolic	HP	90/0.00035/33	–2.91	1.22E-05	(41,43,62,75)
141	n.i.			process/protein biosynthesis			–2.6	6.38E-03	
40	55280/5.29	gi|226739893	ATP synthase subunit α or F-ATPase subunit α	Inner membrane/plasma membrane ATP synthesis–hydrolysis coupled proton transport	HP	140/3.2e-009/32	–2.56	5.52E-05	(24,62,75,77)
42	53252/6.04	gi|60392282	Flagellin A	Secreted/bacterial flagellum/ flagellum motility and virulence	HP J99	88/0.00046/30	–2.45	0.025	(24,31,32,43, 60)
165	n.i.						–1.99	0.034	
92	n.i.						–1.99	1.66E-04	
163	20584/5.20	gi|254809526	Ribosome-recycling or releasing factor	Cytoplasm/protein biosynthesis	*Rhodococcus opacus* (strain B4)	32/2.2e + 002/15	–1.93	0.036	(42)
135	n.i.						–1.86	6.50E-04	
63	43734/5.17	gi|2494256	Elongation factor Tu	Cytoplasm/GTP catabolic process/protein biosynthesis	HP	79/0.0037/33	–1.73	1.14E-04	(42,62,77)

[a]spot nr., spot numbers refer to Figure 1.
[b]n.i., not identified.
[c]n.d., not described.

tein dnaK/heat shock protein 70 [HSP70]); (c) catabolic processes (urea: urease β; hydrogen peroxide: catalase), (d) metabolic processes (phosphate: inorganic pyrophosphatase; sulfate: bifunctional enzyme cysN/cysC); (e) energy metabolism (thioredoxin reductase/ 26-kDa antigen, adenosine 5′-triphosphate [ATP] synthase α chain); (f) flagellum assembly/motility and, indirectly, HP virulence (flagellar hook–associated protein 2 and flagellin A); and (g) bacterial envelope integrity (peptidoglycan-associated lipoprotein) (Tables 2, 3). In particular, the differentially expressed proteins in AAG-HP proteomes belonged to all seven classes compared with the GC-HP proteome, whereas they were limited to only four classes (b, d, e and f) if compared with the UD-HP proteome.

Bacterial Localization and Secretion of Selected Proteins

Among the 15 unique identified proteins, the majority has a cytoplasmatic localization (60%), with two proteins being membrane-associated (peptidoglycan-associated lipoprotein and ATP synthase subunit α) and three were secreted (urease β subunit, flagellar hook–associated protein 2 and flagellin A). Several proteins are already considered important HP virulence factors, namely urease β, flagellin A, catalase and chaperone GroEL (Tables 2, 3). With the exception of spots 13 and 111 (tRNA pseudouridine synthase A and bifunctional enzyme cysN/cysC), all the identified proteins were previously described in works about HP proteomes (Tables 2, 3). However, among them, only nine were reported to be related to specific gastric disease(s) (Table 4).

Proteome of HP Isolated from Patients with Autoimmune Atrophic Gastritis and from Patients with DU Showed a Greater Similarity than Those Obtained from Patients with Gastric Cancer

A PCA analysis indicated that AAG-associated HP can be discriminated from DU and GC on the basis of the proteome characterization. The most important

Table 3. Differentially expressed proteins of HP isolates related to AAG compared with those of HP isolates related to DU.

Spot number[a]	MW (Da)/pI	GenBank accession no.[b]	Protein annotation	Localization/biological process/ molecular function	Organism	Score/expect/ Seq. coverage	Fold difference	*p*	Protein described previously (ref.)
Proteins upregulated by AAG compared with DU									
57	67136/4.99	gi|226738136	Chaperone protein dnaK or HSP70	Cytoplasm/protein folding/ stress response	HP (strain Shi470)	32/1.8e + 002/8	4.23	7.50E-04	(24,41)
168	22335/5.88	gi|2507172	Probable peroxiredoxin or 26-kDa antigen or thioredoxin reductase	Cytoplasm/peroxidase activity/ oxidoreductase	HP	82/0.0019/52	2.15	0.0277	(41–43)
161		n.i.					2.09	0.0277	
166	22335/5.88	P21762	Probable peroxiredoxin or 26-kDa antigen or thioredoxin reductase	Cytoplasm/peroxidase activity/ oxidoreductase	HP	97/6e-005/48	1.69	0.0467	(41–43)
Proteins downregulated by AAG in comparison with DU									
37		n.i.					−7.36	1.09E-04	
117		n.i.					−2.3	0.039	
76	19317/5.01	gi|2500043	Inorganic pyrophosphatase	Cytoplasm/phosphate-containing compound metabolic process/ magnesium ion binding	HP	30/2.9E + 002/12	−2.12	0.037	(13,24,62,77)
40	55280/5.29	gi|226739893	ATP synthase subunit α or F-ATPase subunit α	Membrane/ATP hydrolysis coupled proton transport/plasma membrane ATP synthesis coupled proton transport	HP	140/3.2e-009/32	−2.03	7.36E-03	(13,24,62,75)
42	53252/6.04	gi|60392282	Flagellin A	Secreted/bacterial flagellum/ flagellum motility and virulence	HP (strain J99)	88/0.00046/30	−2.45	0.0897	(13,24,31,43)

[a]Spot numbers refer to Figure 1.
[b]n.i., Not identified.

principal component, PC1, explains the variation and discriminates against the biological samples according to group (AAG, DU and GC) (Figure 2). PC2 is correlated with intragroup variability. In the score plot visualization mode (Figure 2A), each full black circle inside the ellipse is a significantly expressed protein, which contributes to discriminate spot maps (Figure 2B). In the loading plot, we see that there is more variability in GC-associated HP spot maps then in both DU- and AAG-associated ones. In particular, AAG-related HP proteome maps are positioned between DU and GC but are closer to DU-related maps (Figure 2B).

Peroxiredoxin, HSP70, F-ATPase and Flagellin A Are the HP proteins More Specifically Associated with Autoimmune Atrophic Gastritis

Among the all–AAG-HP differential proteins, we focused on three upregulated spots (168, 166: peroxiredoxin; 57: HSP70) and two downregulated spots (40: F-ATPase; 42: flagellin A), which we found of particular interest because of their common variation in level in AAG versus both GC and DU. We analyzed their contents in each AAG patient as log standard volume, as calculated by the DeCyder software, and we evaluated the presence of a possible association between protein up-/downregulation and the HP strain characterization at the levels of *CagA*, *CagE*, *VirB11*, *VacA* and *Hom* genes and the available clinical data of autoimmunity and atrophy (Figure 3A, Table 1). In our analyzed HP isolates associated with AAG, the genotyping results on the three virulence genes within the *Cag* PAI (*CagA*, *CagE* and *VirB11*), on VacA polymorphisms and on Hom selection are shown in Figure 3B. Patient 4 represents successful HP isolation from both antrum and corpus. Furthermore, for this patient, we illustrate in Figure 3A that there is no significant difference in protein log standard abundance between corpus and antrum, even if there is an apparent difference in spot 42.

Table 4. List of differential proteins found in HP associated with AAG and previously reported as HP proteins associated with gastric disease(s).

Our findings	Protein	Gastric disease
Upregulated in AAG^{HP} versus only GC^{HP}	Urease β subunit	CG, GC and DU (24); GC or CG>DU (62)
	Catalase	Early GC (32); CG, GC and DU (24); CG, GC and DU (55)
	Peptidoglycan-associated lipoprotein	CG and DU (59)
Upregulated in AAG^{HP} versus both GC^{HP} and UD^{HP}	Chaperone protein dnaK or HSP70	GC and DU (24)
	Peroxiredoxin	[a]
Downregulated in AAG^{HP} versus only GC^{HP}	60-kDa chaperonin or GroEL	Early GC (32); CG, GC and DU (24); GC>DU>CG (62); CG and DU (59)
	Elongation factor Tu	CG (32); GC>CG>DU (62)
Downregulated in AAG^{HP} versus only UD^{HP}	Inorganic pyrophosphatase	GC and DU (24); CG, GC and DU (62); CG and GC (13)
Downregulated in AAG^{HP} versus both GC^{HP} and UD^{HP}	ATP synthase subunit α or F-ATPase subunit α	CG, GC and DU (24); CG, GC and DU (62)
	Flagellin A	Early GC (32); CG, GC and DU (24)

AAG^{HP}, HP associated with autoimmune atrophic gastritis; DU^{HP}, HP associated with DU; GC^{HP}, HP associated with gastric cancer.
[a]Unfound association till the present.

DISCUSSION

With the exception of two works (31,32), which were based on a DIGE approach, our study is the first to analyze the HP protein isolated from patients with different gastric diseases by two-dimensional DIGE approaches. Moreover, to our knowledge, at present, there is a lack of comparative proteomics information among maps of clinical HP strains isolated from patients affected by AAG and those of clinical HP strains isolated from patients affected by DU or GC. Identification and characterization of the HP-related proteins isolated from AAG patients is important, since it is known that AAG related to HP infection may be a risk factor for further disease development into GC (33,34).

In this study, we focused on the comparative proteome of HP associated with AAG versus those of HP associated with DU or GC by using the DIGE approach (35). Protein profiles of HP isolated from AAG patients (corpus or antrum) were compared with reference maps of HP associated with corpus or antrum protein maps of DU or GC patients (Supplementary Table S3). The stomach location did not significantly influence the pattern of HP protein expression in our series. Moreover, protein profiles analyzed by PCA (Figure 2) succeeded in discriminating AAG-related HP from those associated with both DU and GC. Interestingly, even patient 4 without atrophy was included in the PCA-evidenced AAG group.

A total of 29 distinct spots were differentially regulated, of which 20 were identified by MALDI-TOF MS and database searches as 15 distinct proteins. It is interesting to note that all 15 proteins were already identified in HP proteome, with the exception of two (the tRNA pseudouridine synthase A, spot 13, and the bifunctional enzyme cysN/cysC, spot 111), but only 9 were previously described in the HP proteome associated with a gastric disease (urease β subunit, catalase, peptidoglycan-associated protein, HSP70, GroEL, elongation factor Tu, inorganic pyrophosphatase, ATP synthase subunit α and flagellin A).

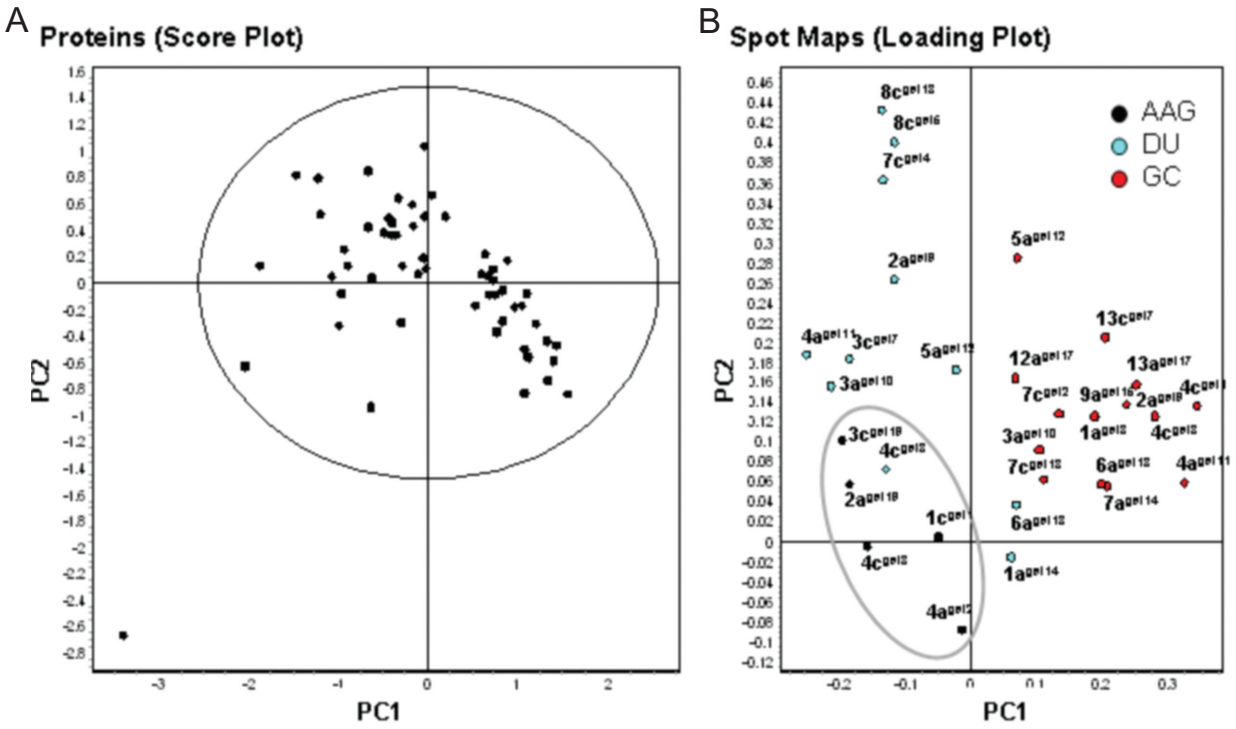

Figure 2. Principal component analysis of HP proteins isolated from AAG, DU and GC patients. (A) Score plot showing an overview of the proteins. Each circle represents a protein. The ellipse represents a 95% significance level. Proteins outliers can either be very strongly differentially expressed proteins or mismatched spots. (B) Loading plot showing an overview of the spot maps from the three groups AAG, DU and GC. Each circle represents a spot map. The ellipse groups the five spot maps from AAG, which can be separated from DU and GC ones and only one DU sample (4Cgel8). AAG-associated HP spot maps are displayed in black; those from GC and DU are displayed in red and blue, respectively. For each spot map, abbreviations indicate the identifier of the patients reported in Table 3 and include the diagnosis (AAG, GC, DU), the stomach location of HP isolation (a, antrum; c, corpus), and a superscript gel number in the Deyder work flow.

A total of 15 spots were upregulated in AAG compared with GC, of which four spots (57, 161, 166 and 168) were also upregulated compared with DU; these four spots were thus considered as proteins of the hallmarks characterizing HP strains associated with the AAG disease. The four spots were involved in the biological processes of "protein folding/stress response" and "oxidoreductase," and they corresponded to a probable peroxiredoxin (or 26-kDa antigen or thioredoxin reductase; spots 166, 168) and a chaperone protein dnaK (or heat shock 70-kDa protein or HSP70, spot 57), with spot 161 not being identified.

Among these two specifically upregulated proteins of AAG, the peroxiredoxin is HP-specific (36). In *Escherichia coli*, in addition to its protein disulfide isomerase activity, the peroxiredoxin may interact with unfolded/denatured proteins similarly to molecular chaperones, and it can also promote the functional folding of citrate synthase after urea denaturation (37). Overall, the members of the peroxiredoxin family (PRX) are considered thiol-specific antioxidant proteins, which confer a protective role in cells. It has been demonstrated that HP-associated gastric inflammation cause epithelial cell damage by induction of oxidative and nitrosative stress, which moreover plays an important role in gastric carcinogenesis (38). Recently, Bettington and Brown (1) clearly evidenced how the inflammation of autoimmune gastritis displayed more eosinophil and lymphocyte infiltrations of the basal epithelium than other forms of chronic gastritis. To ensure its pathogenesis and persistence, HP has evolved a wide range of mechanisms of reactive oxidative species detoxification, including peroxiredoxin production (39,40). In this context, it is tempting to suggest that during AAG HP may produce more antioxidant molecules than in DU and GC. In our work, interestingly, this protein was identified in four distinct differential spots (166, 168, 244 and 248), three of them (spots 166, 168 and 244) upregulated with regard to GC and, moreover, spots 166 and 168 were also upregulated with regard to DU. The occurrence of peroxiredoxin in HP proteomes was previously described (41–43), but to our knowledge, this is the first time that its upregulation was associated with an AAG disease state.

In parallel, together with peroxiredoxin protein, we found the chaperone protein dnaK hallmark upregulated pro-

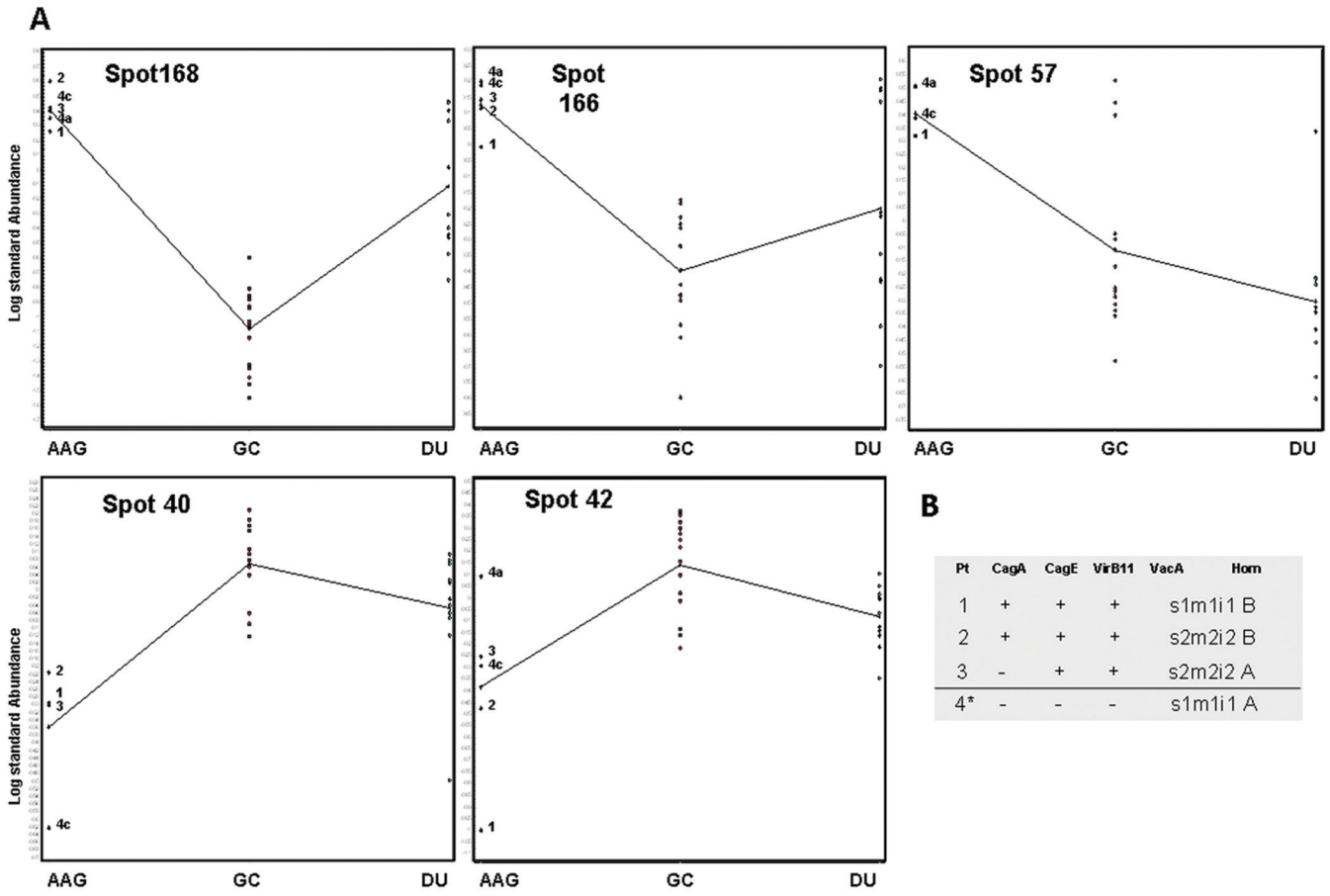

Figure 3. Protein contents of selected HP spots and HP gene characterization in the analyzed atrophic autoimmune gastritis-affected patients. (A) Protein content, expressed as log standard volume, as calculated by the DeCyder software, is represented for spots 168, 166 (peroxiredoxin), 57 (HSP70), 40 (F-ATPase subunit) and 42 (flagellin A) in the four patients of Table 3, with "4a" and "4c" above being for antrum and corpus, respectively. (B) The presence (+) or absence (–) of the *CagA*, *CagE* and *VirB11* genes, together with the polymorphisms for *VacA* and *Hom* genes are shown for each patient. *Patient at the first visit without atrophy, but with increasing anti-PC antibody (levels increasing form 1:160 to 1:1,280 after 1 month) and still under follow-up.

tein in AAG. The chaperone dnaK family is ubiquitous in bacteria and eukaryotes, and it is usually associated with the co-chaperones GrpE and HSP40 (dnaJ). The chaperone protein dnaK is a major surface-exposed HP antigen with significant homology with other bacterial dnaKs (41,44,45) and, similarly to the peroxiredoxin, it was implicated in protein folding and stress response (41,46). Moreover, at the surface of HP, the chaperone protein dnaK may act as a stress-induced surface adhesin capable of mediating the recognition of sulfatide glycolipid receptors on gastric epithelial cells (47). Data have suggested that high levels of this protein may allow a more abundant colonization of HP into the host cells and thus favor the development of a gastric lesion (48). Interesting, chaperone protein dnaK, even from different HP strains, has been previously found immunoreactive toward more than one gastric cancer serum (24), suggesting its potentiality as a marker for GC. To date, this is the first report of HP-related chaperone dnaK expression in the AAG state.

The other seven proteins upregulated in AAG-associated HP proteomes compared with the only GC-associated ones were identified as follows: a tRNA pseudouridine synthase A, a functional enzyme cysN/cysC, a 50S ribosomal protein L30, a urease β subunit, a catalase, a flagellar hook–associated protein 2 and a peptidoglycan-associated lipoprotein.

The tRNA pseudouridine synthase A is thought to play a role in the initiation of translation (49). Recently, it was discovered that this protein can be induced by stress, thus proposing a regulatory role in survival, and that, by mediating a nonsense-to-sense codon conversion, it may represent new means of generating coding or protein diversity (50). This protein (spot 13) has not been identified previously in HP proteome, like the spot 111 and spot 254, which may be involved in the synthesis of activated sulfate upon oxidative stress and the structure of the 50S ribosomal subunit, respectively (51,52).

Two spots (33 and 34) were identified as urease β subunit. The HP urease is known to be critical for bacterial survival in the highly acidic environment such as in human gastric mucosa by the hydrolysis of urea to yield ammonia and carbon dioxide, thereby buffering HP periplasma and cytoplasm. Because of its effective role for HP survival, urease is one of the most abundant proteins synthesized by HP (41). This protein has been already described in HP proteomes obtained from different gastric diseases (24,53). In particular, Pyndiah *et al.* (53) showed a higher intensity of urease B (UreB) in HP isolates from patients with chronic gastritis or GC compared with isolates from patients with DU. Of note, Kobayashi *et al.* (54) showed that HP urease can stimulate *in vitro* innate B-cells to secrete a number of autoantibodies and suggest a new paradigm of innate-dependent B-cell initiation of autoimmunity related to the urease protein. Accordantly, we speculate that higher content of UreB founded in AAG-associated HP protein profiles may thus be associated with a more favorable status for an autoimmune development pathognomic of AGG condition.

Similarly to peroxiredoxin, HP catalase is known to allow HP to resist against the highly oxidative stress coming from the H_2O_2 generation at the infection sites in gastric epithelial cells (40). HP catalase acts synergistically together with other decomposing proteins to detoxify the cell from aggressive oxygen metabolites, thus preventing the misfolding or unfolding of proteins under long-term stress conditions. For these reasons, HP catalase is included among the HP virulence factors, and it has been reported as an important enzyme in different HP-related disorders such as gastritis, GC and DU (24,32,55). Probably the overexpressed catalase found in AAG compared with GC in our series is associated with either a more accentuated oxidative stress occurring during AAG state with respect to the GC ones, or, alternatively, an environmental selection of bacteria with a higher capability to counteract oxidative stress by enzyme overexpression. Accordantly, Ni *et al.* (56) reported a higher expression of human 8-oxoguanine DNA *N*-glycosylase 1 (hOOG1), which prevents the oxidative DNA damage in AAG compared with the GC condition.

Flagellar hook–associated protein 2 is a protein required for the morphogenesis and elongation of the flagellar filament, and it is considered as essential to colonize and establish infection in gastric mucosa because of its essential role in HP motility (57). While the peptidoglycan-associated lipoprotein has been described as essential for bacterial survival and pathogenesis, its exact role in virulence has not been clearly defined (58). Its presence in HP proteome has been reported in both CG and DU (59), but at present, there is a lack of data about its differential expression in AAG-related HP. It may be tempting to hypothesize that the HP envelope may change its protein contents/composition depending on the particular host physiology/disease status.

A total of 12 spots were downregulated in AAG compared with GC, of which 3 spots (37, 40 and 42) were also downregulated as compared with DU. They were identified as follows: ATP synthase subunit α (or F-ATPase subunit α; spot 40) and flagellin A (3 spots put together into spot 42), with spot 37 not being identified. These downregulated proteins may come from alterations in metabolic processes more specific of AAG than both DU and GC.

Flagellin A is the subunit protein that, together with flagellin B, polymerizes to form the bacterial filaments. The protein was reported in HP proteome by several works (31,43,60), as well as in the proteomes of some gastric disease–associated HP strains (32). The important role of flagellin A in both bacterial motility and virulence is well documented (61). Since flagellin A was downregulated in the only AAG-associated HP, we may speculate that a decrease in the virulence of AAG-HP strains may partly be related to a decrease in motility.

The ATP synthase subunit α (or F-ATPase subunit α) is a regulatory subunit of a protein producing ATP from adenosine 5′-diphosphate (ADP) in the presence of a proton gradient across the membrane. Its occurrence in HP proteomes is also documented in some bacterial strains associated with CG, GC and DU (24,62). In the pH range of 3.5–5.0, HP is known to maintain the proton motive force (PMF) across its periplasmic membrane, ensuring a continued supply of energy through ATP synthesis (63). In the presence of a high local acid concentration, the protective mechanism fails to keep up with hydrogen ion influx, ATP synthesis declines and the bacterium dies, or at least loses virulence (64,65). The lower content of F-ATPase found in AAG-related HP strains may be associated with a decrease in their overall survival rates.

These data all together seem to evidence that AAG-associated HP strains may be less motile and less able to maintain their proton motive force than both DU-HP and GC-HP. However, the negative effect for HP survival may be compensated for by a higher capacity to neutralize the high local hydrogen concentration found in HP isolated from patients with AAG compared with concentrations isolated from patients with DU or GC.

The remaining spots resulted in downregulation in HP proteome associated with AAG compared with only the HP proteome of GC and corresponded to a 60-kDa chaperonin or GroEL, an elongation factor Tu, a ribosome-recycling or -releasing factor, a probable peroxiredoxin or 26-kDa antigen or thioredoxin reductase, and four spots not identified.

The 60-kDa chaperonin or GroEL (or HSPB) is known as a highly abundant heat shock protein (66), which is also involved in inflammatory responses and autoimmunity (66) through the stimulation of inflammatory and gastric cells with the production of interleukin (IL)-8 cytokine (67).

Inflammation and IL-8 production were found to be higher in GC than in

normal gastric tissue (68) and directly correlate with angiogenesis (69). Thus, the down-expression of GroEL found in AAG might have a protective role in GC development by reducing inflammation and IL-8 cytokine production. In agreement with our data showing downregulation of GroEL in AAG-associated HP proteomes, Park *et al.* (62) reported the lowest contents of GroEL in patients with chronic gastritis, followed by DU and GC, with the highest levels being in HP from GC patients.

The elongation factor thermo unstable (EF-Tu) plays a central role during the selection of the correct amino acids throughout the elongation phase of translation (70). An additional property in interaction with the extracellular matrix of infected host cells was also proposed by Backert *et al.* (71). High levels of EF-Tu expression in HP isolates from GC patients have been reported (32,62). In our work, the lowest content of EF-Tu in the HP proteome associated with AAG was accompanied by a low content of another protein involved in protein biosynthesis: the ribosome-recycling or -releasing factor, responsible for the release of ribosomes from mRNA at the termination of translation (72). This is the first time that this protein was found in the proteome of HP associated with gastric diseases. Both downregulation of EF-Tu and releasing factor proteins suggest an overall lower level of protein synthesis of HP associated with AAG with respect to HP isolated from GC.

Finally, one protein was specifically downregulated in the HP proteome isolated from an AAG patient compared with the DU-isolated patient: the inorganic pyrophosphatase (spot 76). This enzyme converts one molecule of inorganic pyrophosphate into two phosphate ions by a highly exergonic reaction. This protein was identified in HP proteome isolated from chronic gastritis, DU and GC (13,24,62), with a higher expression in GC with respect to the proteome of HP from patients with gastritis (73).

A pivotal role in HP-induced pathogenesis is played by the virulence factors included in the *Cag* PAI: CagA, the cytotoxin-associated protein translocated in gastric epithelial cells; CagE, the protein involved in IL-8 expression; the type IV secretion system, VirB11 (9); the vacuolating cytotoxin, VacA; and the outer-membrane protein, Hom (74). In our patients, the contents of the proteins of interest (spots 168, 166, 57, 40 and 42) did not seem to correlate overall with the HP virulence or the atrophy grade. However, patient 4 (negative for all the *Cag* PAI virulence genes and without atrophy) appeared to behave differently from others for the downregulated spot 40; this finding was hypothesized to be in some way related to the absence of atrophy.

CONCLUSION

We have successfully performed DIGE differential proteomics analysis of HP strains isolated from AAG patients and identified some proteins that had not been characterized in AAG-HP before. The presence of some common versus differential proteins in AAG-HP versus DU- and/or GC-HP shows a certain level of different physiology of HP depending on the gastric disease. A higher antioxidant activity was found in AAG-associated HP strains, which were hypothesized to be less motile/virulent and able to neutralize the high local hydrogen concentration, as well as to accomplish protein biosynthesis and related processes, in comparison with DU- or GC-associated HP. Some of the identified proteins may provide some new information on understanding the mechanism of the differential HP behavior in human stomach disease(s) and indicate potential protein markers for the specific detection of AAG-related HP. In particular, it may be interesting to screen some of these found AAG-associated HP antigens (e.g., peroxiredoxin, chaperone protein dnaK) from HP-affected patients through cheap and fast technical approaches based on noninvasive samples (e.g., feces) (study in course in our laboratory). Finally, further studies should be conducted to confirm a specific functional role of some identified proteins of interest in HP strains associated with AAG disease.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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