Complexomics Study of Two *Helicobacter pylori* Strains of Two Pathological Origins

POTENTIAL TARGETS FOR VACCINE DEVELOPMENT AND NEW INSIGHT IN BACTERIA METABOLISM*[S]

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Helicobacter pylori **infection plays a causal role in the development of gastric mucosa-associated lymphoid tissue (MALT) lymphoma (LG-MALT) and duodenal ulcer (DU). Although many virulence factors have been associated with DU, many questions remain unanswered regarding the evolution of the infection toward this exceptional event, LG-MALT. The present study describes and compares the complexome of two *H. pylori* strains, strain J99 associated with DU and strain B38 associated with LG-MALT, using the two-dimensional blue native/SDS-PAGE method. It was possible to identify 90 different complexes (49 and 41 in the B38 and J99 strains, respectively); 12 of these complexes were common to both strains (seven and five in the membrane and cytoplasm, respectively), reflecting the variability of *H. pylori* strains. The 44 membrane complexes included numerous outer membrane proteins, such as the major adhesins BabA and SabA retrieved from a complex in the B38 strain, and also proteins from the *hor* family rarely studied. BabA and BabB adhesins were found to interact independently with HopM/N in the B38 and J99 strains, respectively. The 46 cytosolic complexes essentially comprised proteins involved in *H. pylori* physiology. Some orphan proteins were retrieved from heterooligomeric complexes, and a function could be proposed for a number of them via the identification of their partners, such as JHP0119, which may be involved in the flagellar function. Overall, this study gave new insights into the membrane and cytoplasm structure, and those which could help in the design of molecules for vaccine and/or antimicrobial agent development are highlighted.** ***Molecular & Cellular Proteomics 9:2796–2826, 2010.***

Helicobacter pylori infection is one of the most common chronic bacterial infections worldwide with up to half of the world's population infected (for a review, see Ref. 1). This infection is involved in the development of various gastroduodenal diseases including two malignant diseases, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT)[1] lymphoma (LG-MALT) (2–5). *H. pylori* infection is associated with ~80% of extranodal marginal zone B-cell lymphomas of MALT type (6). The causal role of this infection in the lymphomatic process was proven by the beneficial effect of *H. pylori* eradication on the regression of lymphoma (7–9). Despite this proof, many questions remain unanswered concerning the mechanism involved in the evolution of *H. pylori* infection toward the development of an LG-MALT. In fact, the development of an LG-MALT is a very exceptional event because fewer than 0.1% of infected patients will develop this cancer. Consequently, few strains are available, and this lymphoma has not been extensively studied as compared with gastric adenocarcinoma or duodenal ulcer (DU) disease. To date, no environmental factor nor genetic host factor has been found, and in contrast to other severe diseases due to *H. pylori* infection, none of the virulence factors known for this bacterium, including the presence of the *cag* pathogenicity island (PAI) or the VacA toxin, could be associated with this pathology except for the *vacAm2* allele (10–12). However, phylogenic analyses, based on DNA array hybridization, revealed that most of the *H. pylori* strains associated with LG-MALT, although lacking the main *H. pylori* virulence factors, cluster separately from strains associated with other pathologies (gastric carcinoma or DU). This, in turn, has led to the assumption that these strains have a specific genetic material content involved in the clinical outcome of LG-MALT (13). Given that the conventional methods used in molecular biology and genetics did not allow the identification of strains with specific virulence genes, it was proposed that other strategies be implemented (11, 12, 14–17). Moreover and despite the availability of 10 different *H. pylori* genome

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[1] The abbreviations used are: MALT, mucosa-associated lymphoid tissue; LG-MALT, gastric MALT lymphoma; BN, blue native; DU, duodenal ulcer; OMP, outer membrane protein; BabA/B, blood group antigen-binding adhesin A/B; Hor, Hop-related; UreA/B, urease α/β subunit; AlpA/B, adherence-associated lipoprotein AlpA/B; SabA, sialic acid-binding adhesin A; PAI, pathogenicity island; FRD, fumarate reductase; IEF, isoelectrofocalization; POR, pyruvate:flavodoxin oxidoreductase; FAS, fatty acid biosynthesis; ACP, acyl carrier protein; ACX, acetone carboxylase; LC-MS/MS, liquid chromatography mass spectrometry.

sequences, there are many "orphan" genes from *H. pylori* for which no function has been attributed, and few data on protein expression are available.

Certain studies have suggested that nearly all biochemical processes are performed by protein complexes (18). The exploration of protein interactions (protein complexes or complexome) is one of the main challenges of functional genomics to get insight into protein function to understand the physiology and pathogenesis of microorganisms. Among the high throughput technologies used to study complexes, blue native/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (BN/SDS-PAGE) is a highly resolvent separation method (19). It was initially described for the separation under native conditions of the membrane protein complexes of mitochondria (20), chloroplasts (21), and more recently bacteria, such as *Paracoccus denitrificans* (22), *Synechocystis* species (23), and *Escherichia coli* (24). It was later applied to the study of whole complexes of eukaryotic cells (25, 26) and of bacteria, *e.g. E. coli* (27) and *H. pylori* reference strain J99 associated with DU (28). This last study led to the description of 13 multiprotein complexes, 11 issued from the cytoplasm and two issued from the membrane that were either partially or totally reported previously in the literature.

In the present study, two-dimensional BN/SDS-PAGE was applied, after technical improvements, to study the whole complexome of two sequenced *H. pylori* strains to determine whether some complexes were specific to one or the other. Because patients with DU are not predisposed to LG-MALT (29), the complexome of the J99 strain associated with DU (30) was compared with that of the B38 strain chosen to be representative of an LG-MALT-specific cluster (13). Protein identification was performed by using liquid chromatography-mass spectrometry (LC-MS/MS). Purification steps, such as gel filtration, liquid isoelectrofocalization (IEF), and ionic column separation, were used to improve the multiprotein complex separation.

EXPERIMENTAL PROCEDURES

Strains Used—*H. pylori* strain B38 was isolated from a 62-year-old French male patient with extranodal marginal zone B-cell lymphoma of MALT type whose lymphoma had regressed after eradication of *H. pylori*. This patient was enrolled in a prospective multicenter study carried out by the Groupe d'Etude Français des Lymphomes Digestifs of the Fédération Française de Cancérologie Digestive (8). The genome of this strain, recently sequenced, is the smallest *H. pylori* genome described to date (13). The B38 strain is lacking in all known pathogenic determinants because it is negative for the entire *cag* PAI; it appears that it does not produce a functional cytotoxin (positive for *vacAs2m2*) and the major adherence factors (absence of *bab*B, *bab*C, *sab*B, and *hom*B genes). This strain is positive for *bab*A2, *ice*A1, and *hop*QII genotypes, and it has a functional *hop*Z gene and non-functional *oip*A and *sab*A genes. The second *H. pylori* strain, J99 (ATCC 700824), was isolated from a patient with DU in the United States (30). The J99 strain is positive for the entire *cag* PAI; it is positive for *vacAs1m1*, *bab*A2, *ice*A1, and *hop*QII genotypes and has functional *hop*Z, *oip*A, and *sab*A genes. Another characteristic of both strains is that they do not carry plasmidic DNA. The genomes of the B38 and J99 strains are available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome). More detailed information about the J99 genome (31) is available at the PyloriGene World Wide Web Server (http://genolist.pasteur.fr/PyloriGene/genome.cgi).

Bacterial Growth Conditions—*H. pylori* B38 and J99 cells were cultured simultaneously and under identical conditions for 48 h on Wilkins-Chalgren agar plates (Oxoid Ltd., Hampshire, UK) supplemented with 10% human blood and the following antibiotics: 1 mg/ml vancomycin (Lilly France S.A., Fergesheim, France), 5 mg/ml cefsulodin (Takeda France S.A., Puteaux, France), 5 mg/ml Fungizone (Bristol-Myers Squibb Co.), and 1 mg/ml trimethoprim (GlaxoSmithKline). The plates were incubated at 37 °C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Bacteria harvested from agar plates were suspended in ice-cold 0.85% NaCl (bioMérieux, Marcy l'Etoile, France). For performance of the two-dimensional BN/SDS-PAGE applied to the *H. pylori* cytosolic and membrane extracts, a total of 3 g of each *H. pylori* strain was frozen at −80 °C. All of the bacteria and sample manipulations (cytoplasmic and membrane preparation) were performed at 4 °C (unless otherwise indicated).

Cytoplasmic Extract Preparations—Bacteria were harvested from a 48-h culture by centrifugation at 6,000 × *g* for 10 min and washed in ultrapure water. Bacteria were suspended in native extraction buffer A (750 mM 6-amino-*n*-caproic acid, 50 mM Tris) supplemented with a 1 mM final concentration of phenylmethanesulfonyl fluoride and passed through a One Shot disruptor (Constant Systems Ltd., Northants, UK) at 2 kilobars (one shot). The lysate was centrifuged at 6,000 × *g* for 20 min, and a 0.2 mg/ml final concentration of DNase I was added to the supernatant for 1 h at 25 °C. Then, the supernatant was centrifuged at 100,000 × *g* for 30 min at 4 °C and filtered with a Miracloth membrane (Calbiochem). The pellet contained membrane (see "Membrane Extract Preparations"). The cytosolic multiprotein complexes contained in the supernatant were desalted. Indeed, for *H. pylori* cytoplasmic extracts, a preliminary dialysis is necessary to obtain highly resolvent gels. Here dialysis was sometimes replaced by a desalting step, which allows the elimination of small molecules and salts, as was described for the purification of the human embryonic kidney cell line HEK293 (25). The final result was the same for dialysis and the desalting step, but the first technique allows sample concentration using a dialysis membrane (cutoff, 14,000 Da) in buffer A with 30% glycerol.

Membrane Extract Preparations—The pellet was resuspended in buffer A with 1 mM phenylmethanesulfonyl fluoride and passed through a One Shot disruptor at 2 kilobars (one shot). The resulting lysate was centrifuged at 6,000 × *g* for 20 min, the pellet was discarded, and the supernatant was centrifuged at 100,000 × *g* for 30 min. The extraction of the protein complexes from the resulting pellet was then carried out by resuspending the membrane in 1 ml of buffer A supplemented with 2% *n*-dodecyl β-D-maltoside detergent (Sigma-Aldrich). This sample was then centrifuged at 100,000 × *g* for 30 min, and the membrane multiprotein complexes contained in the supernatant were separated by two-dimensional BN/SDS-PAGE.

Purification Steps—All of the steps were carried out at 4 °C. Therefore, liquid IEF, exclusion filtration methods, and ionic column separation were used as purification steps before applying the two-dimensional BN/SDS-PAGE.

Liquid IEF purification was used to separate the multiprotein complexes according to their pI in a pH range from 3.5 to 10, 4 to 6, 5 to 7, and 6 to 8. An aliquot of a crude cytosolic sample was analyzed in a Rotofor system (Rotofor Prep IEF Cell, Bio-Rad). The protein mixture was prepared according to the manufacturer's recommendations before filling the Rotofor chamber. The IEF method produced many protein precipitates in the most abundant protein fractions with a pI of

~5–6. Very low protein concentrations were found in the basic fractions, although a concentration step with Vivaspin column (Vivascience, Aubagne, France) was used.

Gel filtration purification was also carried out. An aliquot of crude cytosolic or membrane sample was loaded on a Superdex™ 200 column (Amersham Biosciences). Buffer A was run at a flow rate of 0.3 ml/min using the FPLC ÄKTA (Amersham Biosciences). Multiprotein complexes were recovered in 250-μl fractions. The cytoplasmic sample was separated into four peaks of major interest: 1,000, 450, 220, and 155 kDa. This method allowed the adaptability of the two-dimensional BN/SDS-PAGE acrylamide gradient according to the mass of interest of the complexes.

Complexes were also separated using an ionic column. Crude extract was loaded on a 1-ml HiTrap™ Q XL column (Amersham Biosciences) at a flow rate of 1 ml/min using the FPLC ÄKTA (Amersham Biosciences) and was washed with 5 ml of buffer A before a two-step elution using 5 ml of buffer A supplemented with 250 mM NaCl and 1 M NaCl. The two last fractions were desalted using a dialysis membrane (cutoff, 14,000 Da) (Medicell International Ltd., London, UK).

First Dimension: BN-PAGE—Sample preparation and BN-PAGE were carried out as described previously (20, 28) with the following minor modifications. The gel dimension was 20 cm × 14.5 cm × 1 mm. Separating gels with a linear 4–12, 4–13, 4–13.5, 3–14.6, 4–14.6, 7–14.6, or 7–18% acrylamide gradient gels were used. Anode and cathode buffers contained 50 mM Tris, 75 mM glycine, and only the cathode buffer was supplemented with 0.004% Serva Blue G (Serva, Heidelberg, Germany). Before loading the sample, 2 μl of buffer B (500 mM 6-amino-*n*-caproic acid, 5% Serva Blue G) was added. The gel was run overnight at 4 °C at 1 watt. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and Albumin (66 kDa) (GE Healthcare, Uppsala, Sweden) were used for each BN-PAGE analysis as molecular mass size standards. Different acrylamide gradients were tested for the BN-PAGE to improve the multiprotein complex separation. A certain balance needs to be found to optimize both focalization and separation of complexes with a mass greater than 60 kDa. A molecular mass could be attributed to the membrane complexes based on a molecular mass marker.

Second Dimension: SDS-PAGE—Individual lanes from BN-PAGE were equilibrated for 5 min in an equilibrating buffer containing 1% SDS (w/v), 125 mM Tris, pH 6.8 and then dipped into equilibrating buffer supplemented with 50 mM dithiothreitol (Sigma-Aldrich) for 15 min. Individual lanes were subsequently soaked in equilibrating buffer supplemented with 125 mM iodoacetamide (Sigma-Aldrich) for 15 min. An ultimate washing step lasting 5 min was performed in the equilibrating buffer without supplement. Individual lanes were placed on a glass plate at the usual position for stacking gels. After covering with the second glass plate, the gel was brought into a vertical position. Then the 10, 13, or 15% acrylamide separating gel mixture was poured. After polymerization, the stacking gel mixture was poured.

Gel Staining—Silver staining was performed using a silver staining kit (Sigma-Aldrich) according to the manufacturer's instructions. Coomassie Brilliant Blue G-250 (Bio-Rad) was also used for gel staining. After two ultrapure water washings of 3 min each, the gels were placed overnight in an incubation solution (10% ammonium sulfate, 0.1% Coomassie Brilliant Blue G-250, 3% orthophosphoric acid, 20% ethanol). Gels were washed twice for 1 min in ultrapure water and twice for 1 h in 5% acetic acid.

In-gel Protein Digestion—Silver-stained proteins separated by SDS-PAGE were excised and destained using the PROTSIL2 silver staining kit (Sigma-Aldrich) according to the manufacturer's instructions. Spots were subsequently washed in ultrapure water until completely destained. The solvent mixture was removed and replaced by acetonitrile. After shrinking of the gel pieces, acetonitrile was removed, and the gel pieces were dried in a vacuum centrifuge. They were then rehydrated in 10 ng/μl trypsin (Sigma-Aldrich) and 50 mM ammonium bicarbonate and incubated overnight at 37 °C. Ammonium bicarbonate (50 mM) was added to the gel pieces, which were incubated for 15 min at room temperature under rotary shaking. The supernatant was collected, and an ultrapure water/acetonitrile/acetic acid (47.5:47.5:5) solution was added to the gel pieces for 15 min. This step was repeated twice. Supernatants were pooled and concentrated in a vacuum centrifuge to a final volume of 25 μl. Digested products were finally acidified by the addition of 1.5 μl of acetic acid and stored at −20 °C.

On-line Capillary HPLC Nanospray Ion Trap MS/MS Analysis—Peptide mixtures were analyzed by on-line capillary HPLC (LC Packings, Amsterdam, The Netherlands) coupled to a nanospray LCQ™ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Peptides were separated on a 75-μm-inner diameter × 15-cm C_{18} PepMap™ column (LC Packings). The flow rate was set at 200 nl/min. Peptides were eluted using a 5–50% linear gradient of solvent B for 30 min (solvent A was 0.1% formic acid in 5% acetonitrile, and solvent B was 0.1% formic acid in 80% acetonitrile). The mass spectrometer was operated in positive ion mode at a 2-kV needle voltage and a 38-V capillary voltage. Data acquisition was performed in a data-dependent mode consisting of alternatively in a single run full scan MS over the range *m/z* 300–2,000 and full scan MS/MS in an exclusion dynamic mode. MS/MS data were acquired using a 3-*m/z* unit ion isolation window, a 35% relative collision energy, and a 5-min dynamic exclusion duration.

Data Analysis—Data were analyzed by SEQUEST (ThermoFinnigan) against a subset of the NCBI database consisting of *H. pylori* strain protein sequences. Carbamidomethylation of cysteines (+57 Da) and oxidation of methionines (+16 Da) were considered as differential modifications. Only peptides with an Xcorr greater than 1.5 (single charge), 2 (double charge), and 2.5 (triple charge) were retained. In all cases, ΔC_n had to be greater than 0.1.

Bioinformatics Tools—Protein sequences were compared with the GenBank™ database with the Blast program "protein blast" (algorithms: blastp, psi-blast, phi-blast; http://www.ncbi.nih.gov/BLAST/) at the National Center for Biotechnology Information computer server (32). The search tool for interactions of chemicals (STITCH; http://stitch.embl.de/) was used to explore possible interactions between partners of complexes identified with or without chemical intermediaries (33).

RESULTS

Global Presentation of Results

In total, 329 proteins were identified by LC-MS/MS of which 32 were never mentioned in previous proteomics studies (supplemental Table S1). Among these additional proteins, 27 have a "predicted" function deduced from homologs characterized in other organisms (31), and nine proteins correspond to open reading frames (ORFs) annotated as "predicted ORF/hypothetical protein", demonstrating that these ORFs really do encode proteins, such as JHP0628 (HELPY_0684) or JHP0905 (HELPY_0958) annotated as "predicted coding regions" (31).

The basic condition for the identification of a multiprotein complex is that proteins of the same multiprotein complex co-migrate in the first dimension and are found aligned with a similar shape in the second dimension (20). Multiprotein complexes from the membrane were named "MB" and "MJ" for

the B38 and J99 strains, respectively. Those from the cytoplasm were named "CB" and "CJ" for the B38 and J99 strains, respectively. The pattern of most of the complexes presented in this study undoubtedly fulfilled these criteria (20). Examples are provided with complexes MB2, CB29, MJ8, and CJ9 (Figs. 1*B*, 3*M*, 2*B*, and 4*B*, respectively).

The 90 heterooligomeric complexes comprised 49 protein complexes in the *H. pylori* B38 strain and 41 in the *H. pylori* J99 strain. All of these complexes are shown in Figs. 1–4 and are described in Tables I–IV. Fifty proteins whose functions remain to be elucidated appear in heterooligomeric complexes, and 14 of these 50 proteins correspond to genes annotated as predicted coding regions. The presence of these proteins in heterooligomeric complexes, through the identification of their partners, should give more insight into their functions.

As previously pointed out by Schägger *et al.* (34), the same shape benchmark can sometimes be difficult to apply; consequently, the assignment of spots to a complex becomes difficult. In the present study, spots corresponding to FrdA/B/C (MB4 and MJ5 for the B38 and J99 strains, respectively) were found aligned (Figs. 1, *A* and *B*, and 2, *A–C*, *E*, and *J*), but a spot corresponding to FrdA presented a slightly oval shape, whereas spots corresponding to FrdB/C had a rounded and more diffuse shape due to a more diffuse gel migration in the lower approximate M_r range (28). However, these three proteins correspond to the three subunits of the fumarate reductase (FRD) complex of *H. pylori* (35, 36), and FrdA and FrdB were found previously in a membrane complex of the J99 strain using the two-dimensional BN/SDS-PAGE method (28). Consequently, this complex was included given that its third evident partner, FrdC, was identified on most of the gels (Figs. 1, *A* and *B*, and 2, *A–C*, *E*, and *J*). It was also noted that the M_r of FrdB was higher than that of FrdC, whereas their deduced M_r values were in the same range. This apparent migration of FrdB at 31 kDa was reported previously by Birkholz *et al.* (35). The FRD complex, commonly associated with the membrane of *H. pylori* (37), is the key enzyme of the Krebs cycle involved in fumarate respiration in the case of anaerobic growth. This enzyme is indeed constitutively expressed under microaerobic conditions and is essential for *H. pylori* colonization of the mouse stomach (38).

Different migrations of some proteins were observed during the separation in the second dimension electrophoresis as was the case for TsaA (Fig. 3, *A* and *G*, Fig. 4, *A* and *D*); the outer membrane proteins (OMPs) AlpB (MB17 and MB18, Fig. 1, *D* and *E*) and HorK (MJ24, Fig. *2H*); the "predicted glycinamide ribonucleotide synthetase" PurD (CB11 and CJ9, Fig. 3*E* and Fig. 4*B*), and the peptidyl-prolyl cis-trans isomerase C, PpiC (CB24, Fig. 3*I*), suggesting that multiple isoforms of these proteins do exist. Their occurrence can be explained by probable post-translational modifications changing their physicochemical criteria (pI, M_r, and binding affinity). In fact, *H. pylori* proteins are subjected to a high degree of post-translational modification as is the case for TsaA, Pfr, UreA, UreB, and RecA (28, 39, 40) and also for some OMPs such as HopK (41). Different oligomerization states of some complexes were also observed, *e.g.* the BabA-SabA (MB14 and MB15, Fig. 1*C*) and HopM/N-BabB (MJ7 and MJ18, Fig. 2*E*) complexes. This was also previously reported in *H. pylori* (28).

Compared with the previous study performed on the *H. pylori* J99 strain (28), four of the 11 cytosolic heterooligomeric complexes could be totally or partially retrieved. Indeed, modifications in sample preparation were made to increase the number of multiprotein complexes and to confirm certain protein-protein interactions. In the present study, purification steps were used, different amounts of proteins were loaded onto the gels, and various acrylamide concentrations in the first and second dimensions were used. Concerning membrane complexes, the urease complex (MJ1) was partially retrieved, and the FRD complex was completed by the third subunit FrdC (see previous paragraph). Moreover, a partner of SodB, AroQ, and FabZ, described previously to belong to homooligomeric complexes (28), was found (Table III). These partners, hardly or not visible during previous stainings (28), were observed and identified in this study. For this reason and to avoid the description of homooligomeric complexes with an inadequate number of subunits, homooligomeric complexes were not reported in the present study. *Dotted arrows* in Figs. 1–4 indicate proteins that were not attributed to heterooligomeric complexes.

Membrane Protein Complexes

At the membrane level, 19 and 25 heterooligomeric protein complexes composed of 31 and 41 different proteins were identified for *H. pylori* B38 (Fig. 1 and Table I complexes named MB) and J99 (Fig. 2 and Table II complexes named MJ) strains, respectively. Although gel profiles remained similar, only seven heterooligomeric complexes were common to both strains: UreB-UreA, HefC-HefB, AtpA-AtpD, AtpD-HELPY_1461 (JHP1381), FrdA-FrdB-FrdC, AlpB-AlpA, and HopF-HopG.

New Insight into H. pylori Membrane Illustrated by Four Examples—Examples of complexes are presented below and classified by function. The complexes MB3 and MJ3 comprised the α and β chains (AtpA and AtpD) of the predicted F1 segment of the ATP synthase. In fact, the approximate M_r of 550 kDA observed for this complex would correspond to the entire complex of the ATP synthase. Indeed, the *H. pylori* ATP synthase is predicted as a multisubunit enzyme comprising the F0 complex (consisting of three subunits, AtpB, AtpF, and AtpE, forming a proton channel), the F1 complex (consisting of five subunits, AtpA, AtpD, AtpG, AtpH, and AtpC, constituting the catalytic site for ATP synthesis), and an additional subunit named AtpX (predicted ATP synthase F0 B′ chain) (42). The presence of this complex in the membrane is not

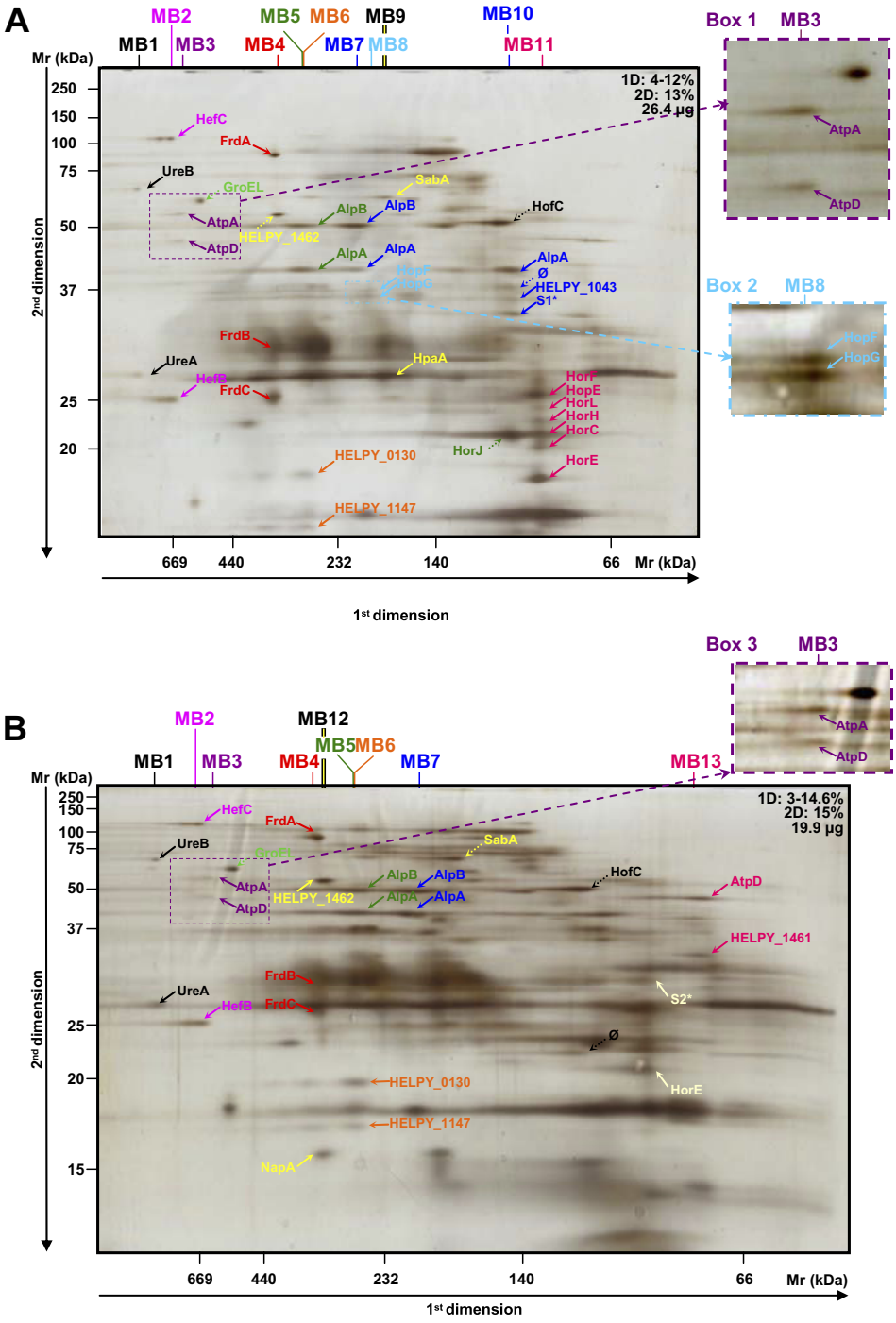

FIG. 1. **Analysis of crude membrane samples of *H. pylori* strain B38.** The first (BN-PAGE) and second dimension gel electrophoreses (SDS-PAGE) were performed with various protein quantities and acrylamide gradients indicated on each gel (*A–E*). *Dotted arrows* indicate proteins that were not attributed to heterooligomeric complexes. Enlargement and second migration of the MB3 complex are shown in *boxes* 1 and 3. Enlargement of the MB8 complex is shown in *box 2*. Protein identifications are presented in Table I. Multiprotein complexes isolated from the membrane of the B38 strain were named MB. * represents spots where different proteins were identified (see Table I). A mixture of proteins was identified in the following spots: spot number S1: HELPY_0856 (one peptide: K ↓ DYKDYLTFFEK ↓ S, coverage = 2.5%, $p = 1.01e^{-7}$) and SdaC (HELPY_0133, one peptide: K ↓ EGLEGIIIQSLK ↓ L, coverage = 2.9%, $p = 5.15e^{-4}$); and spot number S2: PetC (HELPY_1541, one peptide: K ↓ GEHGLNVFINDPQK ↓ L, coverage = 4.9%, $p = 2.03e^{-7}$) and HELPY_0449 (one peptide: K ↓ NLFEIQTHTTK ↓ Q, coverage = 4.3%, $p = 8.46e^{-4}$). ∅, spots for which identification has failed.

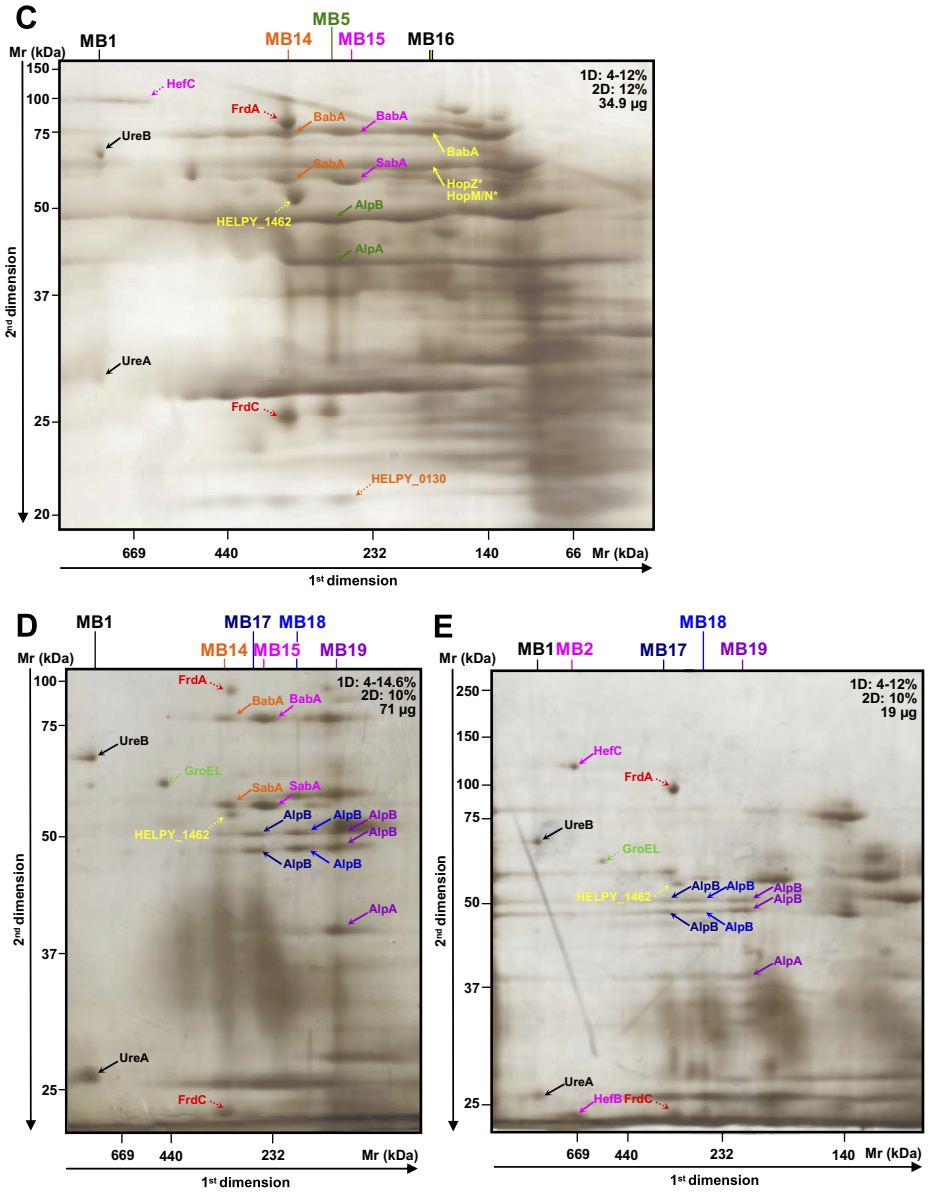

FIG. 1—*continued*

surprising because the β subunit of bacterial ATP synthases exhibits a tight membrane binding property (43), and AtpA was identified previously in the *H. pylori* membrane (44). Functionally, the *H. pylori* ATP synthase would be similar to other bacterial ATPases in that it uses the proton motive force generated by the electron transport chain to synthesize ATP (45, 46). AtpA is very frequently recognized by sera from patients with gastric cancer (47), and AtpD has been shown to be immunogenic (48). This protein, never found in the cytoplasm, was also retrieved with the predicted secreted protein JHP1381 (HELPY_1461) in the membrane fractions from both B38 and J99 strains (MB13 and MJ17). The blastp search for JHP1381 (HELPY_1461) revealed a conserved domain both with the EmrA protein involved in the multidrug resistance efflux pump and the subunit MacA of the macrolide-specific ABC-type efflux transporter from *E. coli*. In this ABC-type efflux transporter (49), MacA is a membrane fusion protein that stimulates the ATPase activity of MacB, a membrane protein that exports macrolide compounds in cooperation with TolC, a multifunctional outer membrane channel (50). Together, all of these observations suggest that JHP1381 is probably involved in efflux resistance and could stimulate the ATPase activity of its MacB counterpart, which is still unidentified in *H. pylori*. Another probable subunit of the ATP synthase, the predicted ATP synthase F1 δ chain (AtpH), was found in an interaction with JHP1494 (MJ25) (approximate M_r of 40 kDa), an uncharacterized protein conserved in numerous Gram-negative bacilli.

MB2 and MJ2 complexes comprised the efflux pump HefB and HefC whose genes are homologs to *E. coli acr*A and *acr*B,

TABLE I

Description of membrane protein complexes identified in H. pylori strain B38 using two-dimensional BN/SDS-PAGE

The complexes presented in the table were all localized on two-dimensional BN/SDS-PAGE gels performed in this study and are represented in Fig. 1. Multiprotein complexes isolated from the membrane of the B38 strain were named MB. The experimental approximate molecular mass is given in kDa. GBAN, GenBank accession number (NCBI Reference Sequence); *n*, the number of peptides; Cov., the protein sequence coverage (percentage) of the peptides.

Complex no.	Molecular mass	Fig. 1 gel letter	Protein[a]	Previously identified	Strain B38[a]				LC-MS/MS information			Strain J99[b]	
					HELPY no.	GBAN	Protein annotation	Molecular mass	*n*	Xcorr	Cov.	JHP no.	GBAN
	kDa							*Da*			%		
MB1	800	A–E	UreB	+	HELPY_0068	YP_003056903	Urease B subunit	61,553	10		25.30	JHP0067	NP_222789
			UreA	+	HELPY_0069	YP_003056904	Urease A subunit	26,465	5		22.30	JHP0068	NP_222790
MB2	670	A, B, E	HefC (AcrB)	+	HELPY_0765	YP_003057504	Membrane fusion protein of the hefABC efflux system	113,657	13		15.50	JHP0554	NP_223272
			HefB (MtrC)	+	HELPY_0766	YP_003057505	Outer membrane protein of the hefABC efflux system	25,898	8		39.74	JHP0553	NP_223271
MB3	550	A, B	AtpA	+	HELPY_1106	YP_003057791	ATP synthase F1, subunit α	54,982	14		32.01	JHP1062	NP_223779
			AtpD	+	HELPY_1104	YP_003057789	ATP synthase F1, subunit β (ATPase subunit β)	51,321	17		47.55	JHP1060	NP_223777
MB4	320	A, B	FrdA	+	HELPY_0195	YP_003057008	Fumarate reductase, flavoprotein subunit	79,968	2	2.43 (2+) 2.73 (2+)	2.80	JHP0178	NP_222899
			FrdB	+	HELPY_0194	YP_003057007	Fumarate reductase, iron-sulfur subunit	27,480	4		22.40	JHP0177	NP_222898
			FrdC	+	HELPY_0196	YP_003057009	Fumarate reductase, transmembrane subunit; putative membrane protein	28,671	5		17.65	JHP0179	NP_222900
MB5	260	A–C	AlpB (HopB)	+	HELPY_0898	YP_003057622	Outer membrane porin and adhesin HopB; putative signal peptide	57,029	7		16.17	JHP0849	NP_223567
			AlpA (HopC)	+	HELPY_0897	YP_003057621	Outer membrane porin and adhesin HopC; putative signal peptide	55,942	6		21.08	JHP0848	NP_223566
MB6	260	A, B	HELPY_0130	+	HELPY_0130	YP_003056952	Conserved hypothetical protein; putative signal peptide	32,642	4		16.80	JHP0119	NP_222840
			HELPY_1147	+	HELPY_1147	YP_003057829	Conserved hypothetical protein; putative signal peptide	20,532	5		33.90	JHP1100	NP_223817
MB7	200	A, B	AlpB (HopB)	+	HELPY_0898	YP_003057622	Outer membrane porin and adhesin HopB; putative signal peptide	57,029	8		20.30	JHP0849	NP_223567
			AlpA (HopC)	+	HELPY_0897	YP_003057621	Outer membrane porin and adhesin HopC; putative signal peptide	55,942	6		18.60	JHP0848	NP_223566
MB8	180	A	HopF (HopX)	+	HELPY_0258	YP_003057062	Outer membrane protein HopF; putative signal peptide	53,024	3		6.17	JHP0237	NP_222958
			HopG (HopY)	+	HELPY_0259	YP_003057063	Outer membrane protein HopG; putative signal peptide	51,724	2	3.56 (2+) 3.62 (2+)	7.01	JHP0238	NP_222959
MB9	200	A	SabA (HopP)	+	HELPY_0642	YP_003057401	Outer membrane protein HopP	72,465	5		11.30	JHP0662	NP_223380
			HpaA	+	HELPY_0563	YP_003057334	Neuraminyllactose-binding hemagglutinin	28,926	4		19.60	JHP0733	NP_223451
MB10[c]	130	A	AlpA (HopC)	+	HELPY_0897	YP_003057621	Outer membrane porin and adhesin HopC; putative signal peptide	55,942	5		16.8	JHP0848	NP_223566
			HELPY_1043	−	HELPY_1043	YP_003057732	Putative metalloprotease; putative membrane protein	46,146	6		16.7	JHP0999	NP_223716
			S1[c]	+	HELPY_0856	YP_003057586	Putative sodium-and chloride-dependent transporter; putative membrane protein	49,292	1	3.46 (1+)	2.5	JHP0449	NP_223167
				+	HELPY_0133	YP_003056954	Amino acid transport protein, HAAAP family; putative membrane protein	46,190	1	3.46 (1+)	2.9	JHP0121	NP_222842
MB11	120	A	HorF	+	HELPY_0698	YP_003057447	Outer membrane protein HorF	30,257	3		10.00	JHP0614	NP_223332
			HopE	+	HELPY_0660	YP_003057417	Outer membrane protein HopE; putative signal peptide	29,784	4		20.10	JHP0645	NP_223363
			HorL	+	HELPY_1515	YP_003058154	Outer membrane protein HorL; putative signal peptide	26,603	4		22.30	JHP1432	NP_224150
			HorH	−	HELPY_0393	YP_003057185	Conserved hypothetical protein; putative signal peptide	27,461	5		30.50	JHP1034	NP_223751
			HorC	+	HELPY_0327	YP_003057128	Outer membrane protein HorC; putative signal peptide	27,590	3		19.18	JHP0307	NP_223027
			HorE	+	HELPY_0456	YP_003057244	Outer membrane protein HorE; putative signal peptide	20,764	4		26.88	JHP0424	NP_223142
MB12	300	B	HELPY_1462	+	HELPY_1462	YP_003058106	Putative outer membrane transporter; putative signal peptide	56,824	9		22.75	JHP1382	NP_224100
			NapA	+	HELPY_0248	YP_003057054	Neutrophil-activating protein NapA (bacterioferritin)	16,677	3		26.39	JHP0228	NP_222949

TABLE I—*continued*

Complex no.	Molecular mass	Fig. 1 gel letter	Protein[a]	Previously identified	Strain B38[a]				LC-MS/MS information			Strain J99[b]	
					HELPY no.	GBAN	Protein annotation	Molecular mass	*n*	Xcorr	Cov.	JHP no.	GBAN
	kDa							*Da*			%		
MB13	80	B	AtpD	+	HELPY_1104	YP_003057789	ATP synthase F1, subunit β (ATPase subunit β)	51,321	19		57.80	JHP1060	NP_223779
			HELPY_1461	+	HELPY_1461	YP_003058105	Conserved hypothetical protein	36,038	11		34.65	JHP1381	NP_224099
MB14	320	C, D	BabA (HopS)	+	HELPY_0880	YP_003057607	Adhesin; putative signal peptide	80,815	7		18.36	JHP0833	NP_223551
			SabA (HopP)	+	HELPY_0642	YP_003057401	Outer membrane protein HopP	72,465	10		24.43	JHP0662	NP_223380
MB15	240	C, D	BabA (HopS)	+	HELPY_0880	YP_003057607	Adhesin; putative signal peptide	80,815	6		12.60	JHP0833	NP_223551
			SabA (HopP)	+	HELPY_0642	YP_003057401	Outer membrane protein HopP	72,465	13		28.24	JHP0662	NP_223380
MB16[d]	180	C	BabA (HopS)	+	HELPY_0880	YP_003057607	Adhesin; putative signal peptide	80,815	5		8.85	JHP0833	NP_223551
			HopZ[d]	−	HELPY_0007	YP_003056850	Outer membrane protein HopZ; putative signal peptide	73,043	6		12.69	JHP0007	NP_222729
			HopM/N[d]	+	HELPY_0231/ HELPY_1317	YP_003057038/ YP_003057989	Outer membrane protein HopM/N; putative signal peptide	75,857	8		15.09	JHP0212/ JHP1261	NP_222933/ NP_223979
MB17	250	D, E	AlpB (HopB)	+	HELPY_0898	YP_003057622	Outer membrane porin and adhesin HopB; putative signal peptide	57,029	4		11.47	JHP0849	NP_223567
			AlpB (HopB)	+	HELPY_0898	YP_003057622	Outer membrane porin and adhesin HopB; putative signal peptide	57,029	3		6.80	JHP0849	NP_223567
MB18	210	D, E	AlpB (HopB)	+	HELPY_0898	YP_003057622	Outer membrane porin and adhesin HopB; putative signal peptide	57,029	2	2.37 (2+) 3.87 (2+)	4.32	JHP0849	NP_223567
			AlpB (HopB)	+	HELPY_0898	YP_003057622	Outer membrane porin and adhesin HopB; putative signal peptide	57,029	3		6.80	JHP0849	NP_223567
MB19	180	D, E	AlpB (HopB)	+	HELPY_0898	YP_003057622	Outer membrane porin and adhesin HopB; putative signal peptide	57,029	4		11.17	JHP0849	NP_223567
			AlpB (HopB)	+	HELPY_0898	YP_003057622	Outer membrane porin and adhesin HopB; putative signal peptide	57,029	5		6.20	JHP0849	NP_223567
			AlpA (HopC)	+	HELPY_0897	YP_003057621	Outer membrane porin and adhesin HopC; putative signal peptide	55,942	5		16.05	JHP0848	NP_223566

[a] Gene product/function and protein molecular mass according to the annotation of strain B38 available in NCBI.

[b] Information available in NCBI; see also the revised annotation of strain J99 in Boneca *et al.* (31) (http://genolist.pasteur.fr/PyloriGene/genome.cgi).

[c] MB10 contains putative additional proteins in the spot number S1: HELPY_0856 (one peptide: K ↓ DYKDYLTFFEK ↓ S, $\Delta M = 0.61503$, score = 3.46, $z = 2$, coverage = 2.5%, $p = 1.01e^{-7}$) and SdaC (HELPY_0133, one peptide: K ↓ EGLEGIIIQSLK ↓ L, $\Delta M = 1.41851$, score = 3.46, $z = 2$, coverage = 2.9%, $p = 5.15e^{-4}$).

[d] MB16 contains a mixture of two proteins (HopZ and HopM/N) in the second spot.

TABLE II

Description of membrane protein complexes identified in H. pylori strain J99 using two-dimensional BN/SDS-PAGE

The complexes presented in the table were all localized on two-dimensional BN/SDS-PAGE gels performed in this study and are represented in Fig. 2. Multiprotein complexes isolated from the membrane of the J99 strain were named MJ. The experimental approximate molecular mass is given in kDa. GBAN, GenBank accession number (NCBI Reference Sequence); *n*, the number of peptides; Cov., the protein sequence coverage (percentage) of the peptides; Pred., predicted.

Complex no.	Molecular mass	Fig. 2 gel letter	Protein[a]	Previously identified	Strain J99[a]				LC-MS/MS information			Strain B38[b]	
					JHP no.	GBAN	Protein annotation	Molecular mass	*n*	Xcorr	Cov.	HELPY no.	GBAN
	kDa							*Da*			%		
MJ1	800	A–C, I, J	UreB	+	JHP0067	NP_222789	Urease B subunit	61,509.5	13		35.10	HELPY_0068	YP_003056903
			UreA	+	JHP0068	NP_222790	Urease A subunit	26,418.4	2	3.45 (2+) 3.30 (2+)	8.80	HELPY_0069	YP_003056904
MJ2	670	A–C, I, J	HefC	+	JHP0554	NP_223272	Cytoplasmic pump proteins of the hefABC efflux system	113,554	4		5.00	HELPY_0765	YP_003057504
			HefB	+	JHP0553	NP_223271	Membrane fusion protein of the hefABC efflux system	25,896.7	6		35.90	HELPY_0766	YP_003057505
MJ3	550	A, B	AtpA	+	JHP1062	NP_223779	Pred. ATP synthase F1 α chain	55,061.1	1[c]	3.03 (2+)	2.20	HELPY_1106	YP_003057791
			AtpD	+	JHP1060	NP_223779	Pred. ATP synthase F1 β chain	51,270.5	2	3.40 (2+) 3.84 (2+)	6.60	HELPY_1104	YP_003057789
MJ4	500	A, C, E, G	GroEL, Hsp60	+	JHP0008	NP_222730	Chaperone and heat shock protein	58,068.6	5		10.62	HELPY_0008	YP_003056851
			Pfr	+	JHP0598	NP_223316	Nonheme iron-containing ferritin	19,170	2	4.11 (3+) 3.20 (2+)	17.40	HELPY_0718	YP_003057465
MJ5	320	A–C, E, J	FrdA	+	JHP0178	NP_222899	Fumarate reductase, flavoprotein subunit	79,934.3	2	3.57 (2+) 3.99 (2+)	4.22	HELPY_0195	YP_003057008
			FrdB	+	JHP0177	NP_222898	Fumarate reductase, iron-sulfur subunit	27,473.6	4		20.80	HELPY_0194	YP_003057007
			FrdC	+	JHP0179	NP_222900	Fumarate reductase, cytochrome *b* subunit	28,722.4	3		10.20	HELPY_0196	YP_003057009
MJ6	180	A	HopF (HopX)	+	JHP0237	NP_222958	Outer membrane protein HopF	52,360	5		12.30	HELPY_0258	YP_003057062
			HopG (HopY)	+	JHP0238	NP_222959	Outer membrane protein HopG	51,837.4	2	3.18 (2+) 4.08 (2+)	6.30	HELPY_0259	YP_003057063
MJ7	140	A, E	HopM/N	+	JHP0212/ JHP1261	NP_222933/ NP_223979	Pred. outer membrane protein HopM/N	75,476.5	2	4.72 (2+) 5.46 (2+)	5.30	HELPY_0231/ HELPY_1317	YP_003057038/ YP_003057989
			BabB	+	JHP1164	NP_223882	Pred. adhesin	76,516.4	3		7.30	/	/
MJ8	200	A, B, H	AlpB (HopB)	+	JHP0849	NP_223567	Outer membrane porin and adhesin	56,583	5		12.90	HELPY_0898	YP_003057622
			AlpA (HopC)	+	JHP0848	NP_223566	Outer membrane porin and adhesin	56,235	10		30.96	HELPY_0897	YP_003057621
MJ9	160	A, B, F	FixN	−	JHP0132	NP_222853	Pred. cytochrome *c* oxidase heme b and copper-binding subunit	55,847.7	1[c]	3.56 (2+)	3.07	HELPY_0147	YP_003056965
			FixP	−	JHP0135	NP_222856	Pred. cytochrome *c* oxidase diheme subunit	32,451.9	3		12.07	HELPY_0151	YP_003056968
			FixO	+	JHP0133	NP_222854	Pred. cytochrome *c* oxidase monoheme subunit	26,421.3	3		13.00	HELPY_0149	YP_003056966
MJ10	800	C	UreB	+	JHP0067	NP_222789	Urease B subunit	61,509.5	10		30.10	HELPY_0068	YP_003056903
			UreA	+	JHP0068	NP_222790	Urease A subunit	26,418.4	6		26.50	HELPY_0069	YP_003056904
			Lpp20	+	JHP1349	NP_224067	Membrane-associated lipoprotein	18,963.8	3		19.40	HELPY_1427	YP_003058075
MJ11	300	C	AlpA (HopC)	+	JHP0848	NP_223566	Outer membrane porin and adhesin	56,235	4		15.40	HELPY_0897	YP_003057621
			HopA	+	JHP0214	NP_222935	Porin protein	52,915.2	4		11.80	HELPY_0233	YP_003057040
			HorK (HopW)	+	JHP1394	NP_224112	Outer membrane protein HorK	42,773.8	3		12.60	HELPY_1474	YP_003058118
MJ12	280	C	HofC	+	JHP0438	NP_223156	Pred. outer membrane protein HofC	59,533	12		17.30	HELPY_0469	YP_003057256
			AlpA (HopC)	+	JHP0848	NP_223566	Outer membrane porin and adhesin	56,235	4		14.20	HELPY_0897	YP_003057621
			HopA	+	JHP0214	NP_222935	Porin protein	52,915.2	9		28.80	HELPY_0233	YP_003057040
MJ13	280	C	HorF	+	JHP0614	NP_223332	Pred. outer membrane protein HorF	30,130.7	4		11.90	HELPY_0698	YP_003057447
			HorJ	+	JHP1362	NP_224080	Outer membrane protein HorJ	28,089.4	4		17.30	HELPY_1441	YP_003058088
			Lpp20	+	JHP1349	NP_224067	Membrane-associated lipoprotein	18,963.8	4		24.00	HELPY_1427	YP_003058075

TABLE II—*continued*

Complex no.	Molecular mass	Fig. 2 gel letter	Protein[a]	Previously identified	Strain J99[a]				LC-MS/MS information			Strain B38[b]	
					JHP no.	GBAN	Protein annotation	Molecular mass	*n*	Xcorr	Cov.	HELPY no.	GBAN
	kDa							*Da*			%		
MJ14	80	D	HopH (OipA)	+	JHP0581	NP_223299	Outer membrane protein HopH	34,144.3	3		16.61	HELPY_0733	YP_003057480
			JHP0368	−	JHP0368	NP_223087	Pred. coding region JHP0368 with no homolog in the databases	27,117.6	2	3.16 (2+) 4.16 (2+)	9.58	HELPY_0393	YP_003057185
			TatB	−	JHP0365	15611433	Pred. Sec-independent protein translocase protein	18,267.4	2	5.18 (2+) 3.19 (2+)	12.50	HELPY_0390	YP_003057182
MJ15	80	D	HopG (HopY)	+	JHP0238	NP_222959	Outer membrane protein HopG	51,837.4	3		7.43	HELPY_0259	YP_003057063
			PpiC	+	JHP0161	NP_222882	Pred. peptidyl-prolyl cis-trans isomerase C	33,885.9	2	4.23 (2+) 4.42 (2+)	9.70	HELPY_0179	YP_003056994
MJ16	80	D	HopE	+	JHP0645	NP_223363	Porin	29,400.8	2	4.47 (2+) 2.24 (2+)	11.10	HELPY_0660	YP_003057417
			HorC	+	JHP0307	15611376	Pred. OMP HorC	27,595.7	1[c]	4.879 (+)	3.70	HELPY_0327	YP_003057128
MJ17	50	D	AtpD	+	JHP1060	NP_223777	Pred. ATP synthase F1 β chain	51,270.5	12		36.46	HELPY_1104	YP_003057789
			JHP1381	+	JHP1381	NP_224099	Pred. secreted protein	36,071.9	3		14.29	HELPY_1461	YP_003058105
MJ18	180	E	HopM/N	+	JHP0212/ JHP1261	NP_222933/ NP_223979	Pred. outer membrane protein HopM/N	75,476.5	9		20.50	HELPY_0231/ HELPY_1317	YP_003057038/ YP_003057989
			BabB	+	JHP1164	NP_223882	Pred. adhesin	76,516.4	4		9.10		
MJ19	130	E	BabA (HopS)	+	JHP0833	NP_223551	Adhesin	80,420.8	6		8.90	HELPY_0880	YP_003057607
			BabB	+	JHP1164	NP_223882	Pred. adhesin	76,516.4	3		6.30		
MJ20	120	E	HopG (HopY)	+	JHP0238	NP_222959	Outer membrane protein HopG	51,837.4	2	2.56 (2+) 4.83 (2+)	5.10	HELPY_0259	YP_003057063
			HopK	−	JHP0857	NP_223575	Pred. outer membrane protein	41,251.5	2	3.58 (2+) 4.36 (2+)	8.60	HELPY_0461	YP_003057249
MJ21	120	E	HorB	+	JHP0117	NP_222838	Pred. outer membrane protein HorB	31,702.4	1[c]	3.15 (2+)	5.90	HELPY_0126	YP_003056949
			HopE	+	JHP0645	NP_223363	Porin	29,400.8	1[c]	2.79 (2+)	5.90	HELPY_0660	YP_003057417
MJ22	320	H	BabA (HopS)	+	JHP0833	NP_223551	Adhesin	80,420.8	4		6.50	HELPY_0880	YP_003057607
			JHP1100	+	JHP1100	NP_223817	Pred. coding region JHP1100 with no homolog in the databases	20,693.6	2	3.12 (2+) 3.00 (2+)	13.51	HELPY_1147	YP_003057829
MJ23	250	H	AlpB (HopB)	+	JHP0849	NP_223567	Outer membrane porin and adhesin	56,583	5		12.90	HELPY_0898	YP_003057622
			HefA	+	JHP0552	NP_223270	Outer membrane protein of the hefABC efflux system	54,458.3	2	2.38 (2+) 2.33 (2+)	5.50	HELPY_0767	YP_003057506
MJ24	80	H	DagA	−	JHP0877	NP_223595	Pred. sodium/D-alanine-glycine symporter	48,466.3	4		13.11	HELPY_0928	YP_003057646
			HorK (HopW)	+	JHP1394	NP_224112	Outer membrane protein HorK	42,773.8	4		13.44	HELPY_1474	YP_003058118
			HorK (HopW)	+	JHP1394	NP_224112	Outer membrane protein HorK	42,773.8	3		9.82	HELPY_1474	YP_003058118
MJ25	40	J	JHP1494	+	JHP1494	NP_224212	Pred. coding region JHP1494	28,325	6		28.85	HELPY_1592	YP_003058226
			AtpH	+	JHP1063	NP_223780	Pred. ATP synthase F1 δ chain	20,154.8	3		20.56	HELPY_1107	YP_003057792

[a] Gene product/function and protein molecular mass according to the revised annotation of strain J99 (31) (http://genolist.pasteur.fr/PyloriGene/genome.cgi).

[b] Information according the annotation of strain B38 available in NCBI.

[c] Only one peptide was identified in these spots: AtpA (R↓HALIVYDDLSK↓H, ΔM = 0.86875, score = 3.03; z = 2, coverage = 2.20%, p = 2.02e^{-7}); FixN (-↓MQENVPLSYDYSISK↓L, ΔM = 0.94, score = 3.56, z = 2, coverage = 3.07%, p = 2.38e^{-7}); HorC (K↓ALFVDEHEFEIGFK↓F, ΔM = −0.57033, score = 4.89, z = 2, coverage = 5.70%, p = 6.38e^{-10}); HorB (R↓GSFHPSNFQVLVNGGIR↓L, ΔM = 1.05764, score = 3.15, z = 2, coverage = 5.90%, p = 7.85e^{-4}); and HopE (K↓YANGALNGFGLNVGYK↓K, ΔM = 1.35787, score = 2.79, z = 2, coverage = 5.90%, p = 3.89e^{-7}).

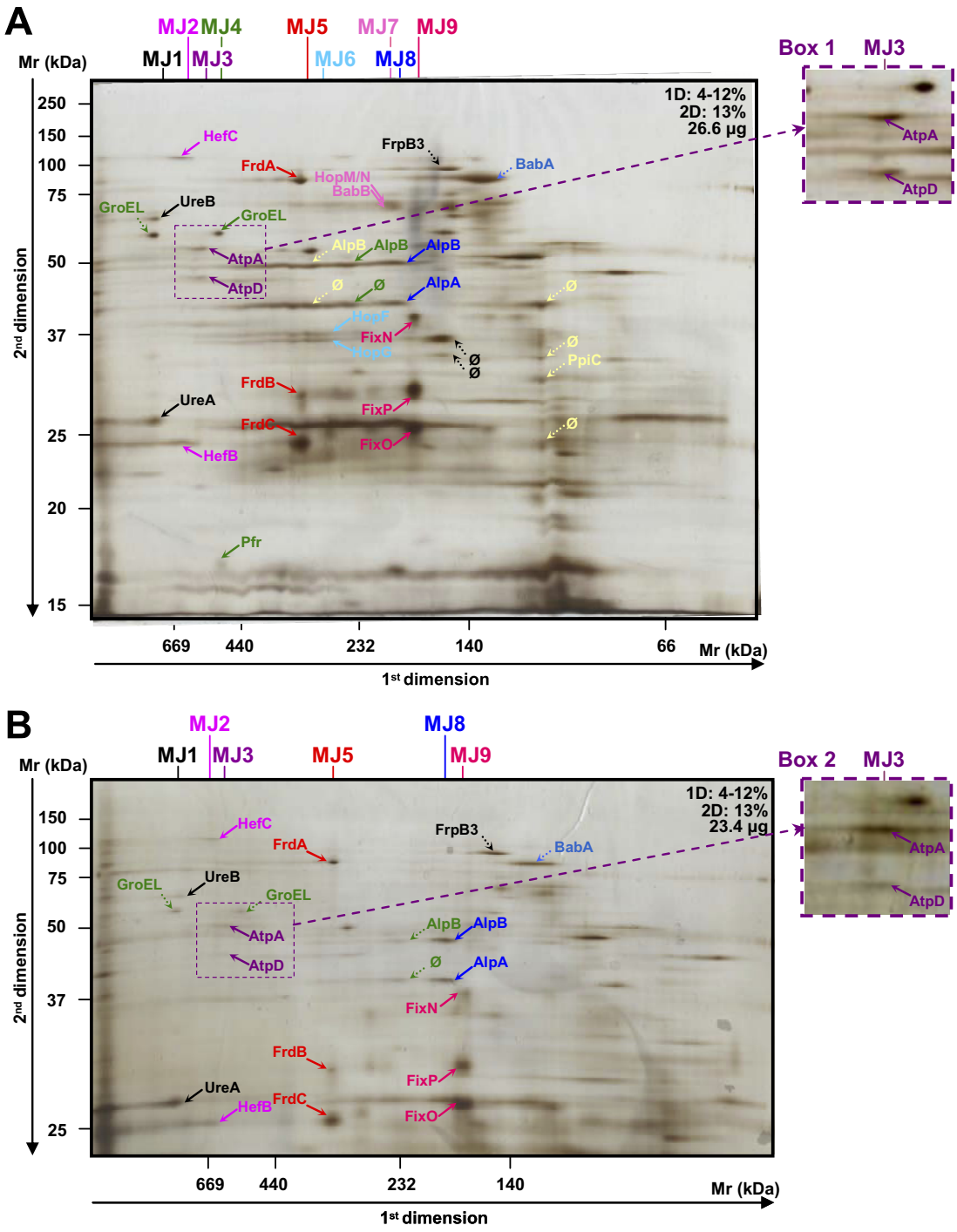

FIG. 2. **Analysis of crude and purified membrane samples of *H. pylori* strain J99.** The first (BN-PAGE) and second dimension gel electrophoreses (SDS-PAGE) were performed with the various protein quantities and acrylamide gradients indicated on each gel (*A–J*). *Dotted arrows* indicate proteins that were not attributed to heterooligomeric complexes. More contrasted pictures of migration of the MJ3 complex are shown in *boxes* 1 and 2. Protein identifications are presented in Table II. Multiprotein complexes from the membrane of strain J99 were named MJ. *A–G* represent the analyses of the crude membrane samples. *H–J* represent the analyses of the fractions eluted when the membrane sample was purified using the ionic column (HiTrap Q column) before applying the two-dimensional BN/SDS-PAGE. *H* corresponds to the directly eluted fraction. *I* and *J* correspond to fractions eluted with 250 mM NaCl from different sample preparations. ∅, spots for which identification has failed.

which encode membrane fusion and resistance-nodulation-division cytoplasmic pump proteins, respectively (51). The resistance-nodulation-division family of efflux systems is widespread among Gram-negative bacteria. They are associated with bacterial resistance to antibiotics. In *E. coli*, AcrA exists as a complex with AcrB on the periplasmic surface of

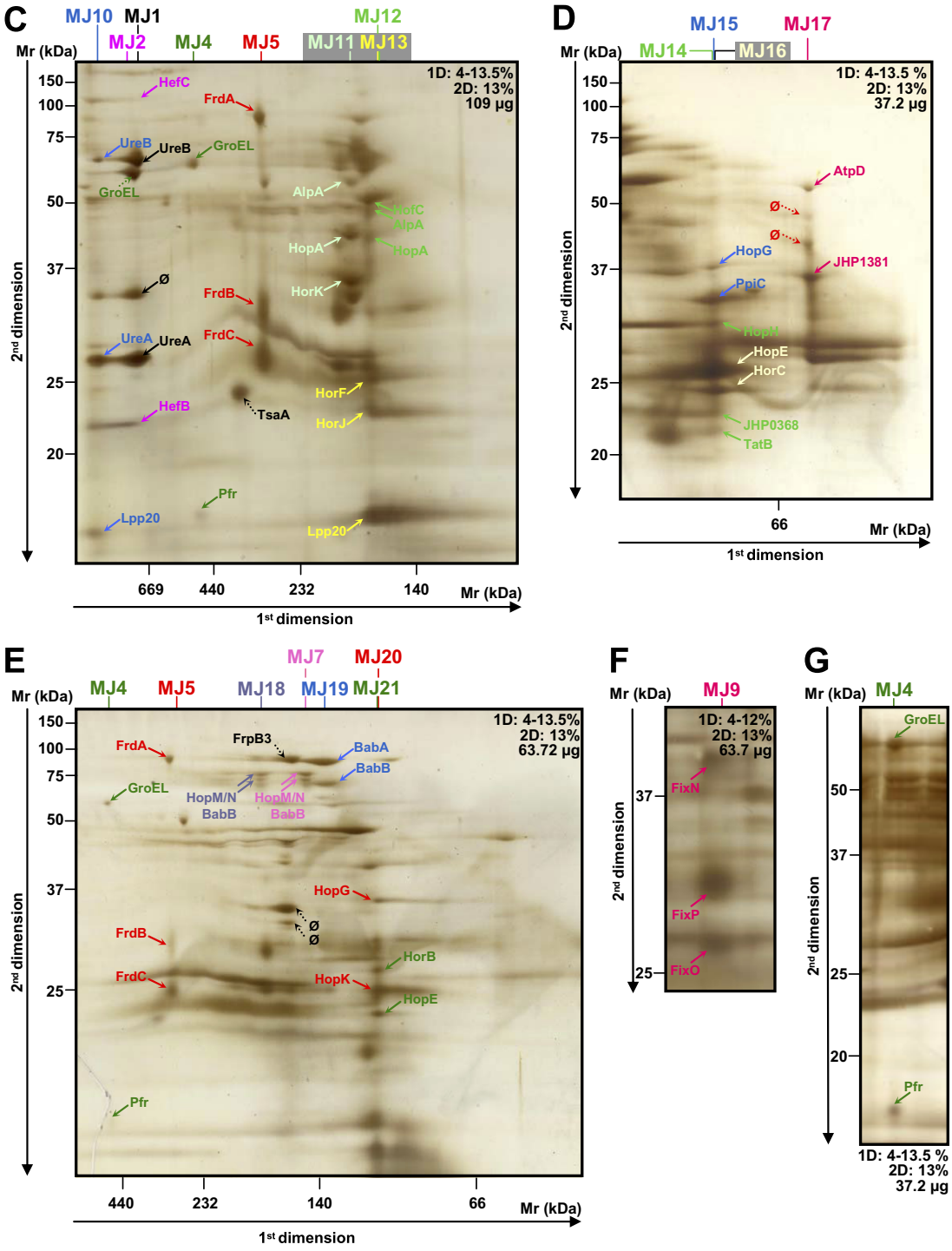

FIG. 2—*continued*

the inner membrane (52). In fact, AcrA, AcrB, and TolC of *E. coli* form a stable intermembrane multidrug efflux complex (53). In *H. pylori*, the genes coding for this proposed efflux system are composed of HefB, HefC, and HefA subunits (corresponding to the AcrA, AcrB, and TolC complex in *E. coli*, respectively) and have been shown to be highly conserved in sequence and organization between multiple *H. pylori* strains and to be expressed both *in vivo* and *in vitro* (54). In *H. pylori*, HefC is involved in energy-dependent multidrug efflux (55), and HefA, a TolC-like protein (56), plays an important role in multidrug resistance (57, 58). MB2 and MJ2 complexes present an approximate M_r of 670 kDa, which would correspond to three homotrimers of each subunit of this proposed efflux system as reported previously in other bacteria (59). Thus, the HefA

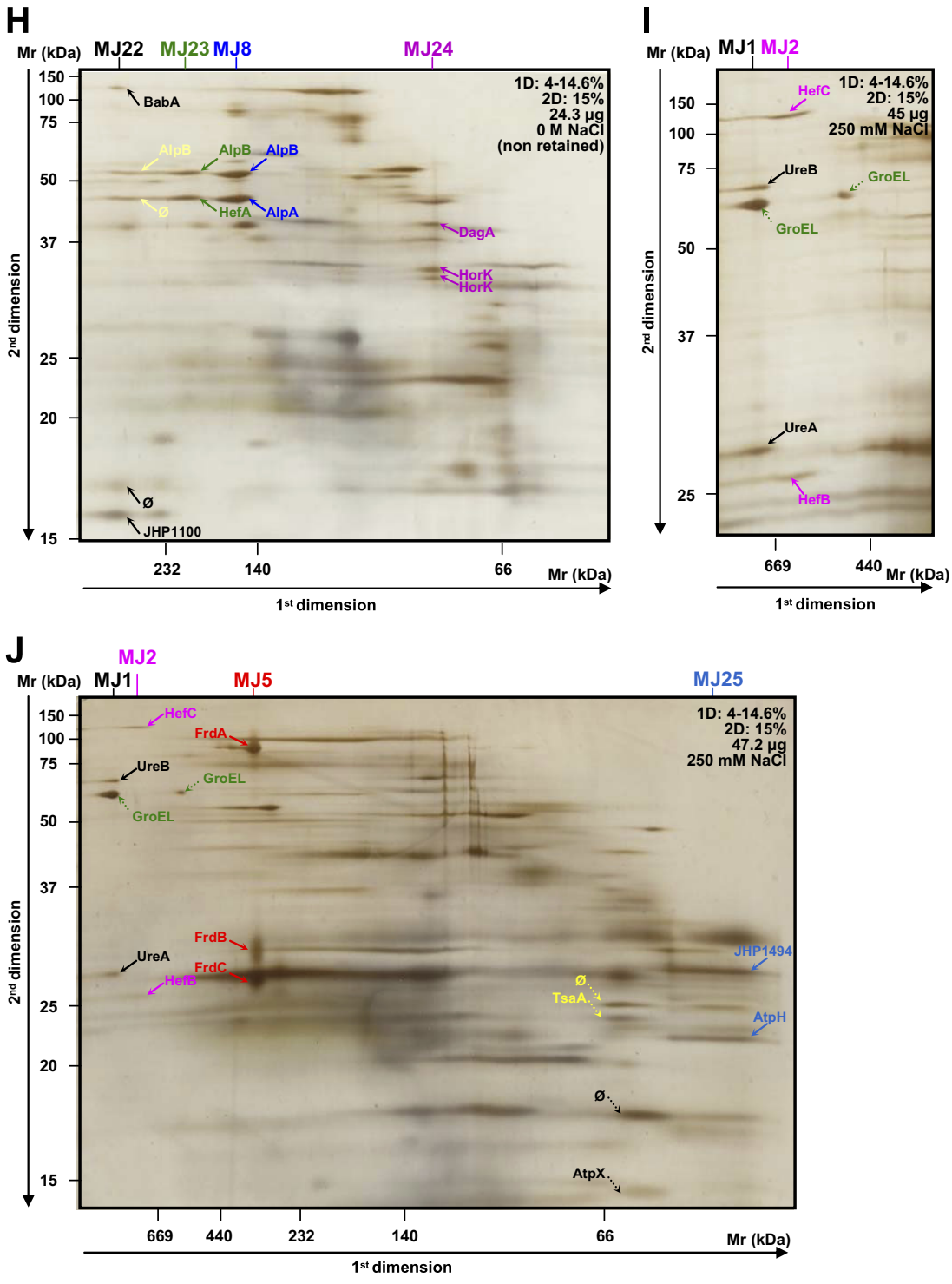

FIG. 2—*continued*

subunit was probably lost during the purification of the complex. It therefore remains to be determined whether HefA corresponds to TolC in this complex.

Another TolC-like protein was identified in the *H. pylori* genome corresponding to JHP1382 (HELPY_1462) (56) and was shown to be active in efflux (57). MB12 is composed of HELPY_1462 and the secreted neutrophil-activating protein NapA (60). Therefore, it is tempting to speculate that the outer membrane efflux protein encoded by JHP1382 (HELPY_1462) participates in the secretion of NapA.

Eight membrane complexes comprising orphan proteins were retrieved (MB6, MB10, MB12, MB13, MJ14, MJ17,

TABLE III

Description of cytosolic protein complexes identified in H. pylori strain B38 using two-dimensional BN/SDS-PAGE

The complexes presented in the table were all localized on two-dimensional BN/SDS-PAGE gels performed in this study and are represented in Fig. 3. Multiprotein complexes isolated from the cytosol of the B38 strain were named CB. The experimental approximate molecular mass is given in kDa. GBAN, GenBank accession number (NCBI Reference Sequence); *n*, the number of peptides; Cov., the protein sequence coverage (percentage) of the peptides.

Complex no.	Molecular mass	Fig. 3 gel letter	Protein[a]	Previously identified	Strain B38[a]				LC-MS/MS information			Strain J99[b]	
					HELPY no.	GBAN	Protein annotation	Molecular mass	*n*	Xcorr	Cov.	JHP no.	GBAN
	kDa							*Da*			%		
CB1	800	A, D, I, J	UreB	+	HELPY_0068	YP_003056903	Urease B subunit	61,553	10		40.76	JHP0067	NP_222789
			UreA	+	HELPY_0069	YP_003056904	Urease A subunit	26,465	18		38.66	JHP0068	NP_222790
CB2	230	A	HELPY_0773	+	HELPY_0773	YP_003057512	Methyl-accepting chemotaxis transmembrane sensory protein (MCP-like protein)	48,157	12		58.78	JHP0546	NP_223264
			FumC	+	HELPY_1301	YP_003057973	Fumarate hydratase class II (fumarase C)	50,773	8		27.00	JHP1245	NP_223963
CB3	200	A, G, H, J	AroQ (AroD)	+	HELPY_0415	YP_003057206	3-Dehydroquinate dehydratase (3-dehydroquinase) (type II DHQase)	18,352	6		49.10	JHP0386	NP_223105
			FlaG	−	HELPY_0614	YP_003057378	Flagellar protein FlaG (*H. pylori* B38)	13,279	2	3.37(2+) 2.29(1+)	19.33	JHP0688	NP_223406
CB4	135	A	MdaB	+	HELPY_0741	YP_003057488	NAD(P)H oxidoreductase (NADPH quinone reductase)	21,524	7		34.02	JHP0573	NP_223291
			Tpx	+	HELPY_1035	YP_003057724	Thiol peroxidase	18,195	2	3.42(2+) 3.37(2+)	15.06	JHP0991	NP_223708
CB5	45	A	NapA	+	HELPY_0248	YP_003057054	Neutrophil-activating protein NapA (bacterioferritin)	16,677	9		70.83	JHP0228	NP_222949
			Rpl7	+	HELPY_1172	YP_003057852	50 S ribosomal protein L7/L12	13,197	3		41.60	JHP1122	NP_223839
CB6	110	B	DnaN	+	HELPY_0852	YP_003057584	DNA polymerase III subunit β	42,002	24		70.60	JHP0452	NP_223170
			TrxR1 (TrxB1)	+	HELPY_0531	YP_003057307	Thioredoxin reductase (TRXR) (TR)	33,378	12		44.10	JHP0764	NP_223482
CB7	200	C	FabI	+	HELPY_0198	YP_003057011	Putative enoyl-(acyl-carrier-protein) reductase	29,868	9		34.18	JHP0181	NP_222902
			FabZ	+	HELPY_1363	YP_003058021	(3*R*)-Hydroxymyristoyl-(acyl-carrier-protein) dehydratase ((3*R*)-hydroxymyristoyl ACP dehydrase)	18,079	4		25.79	JHP1290	NP_224008
CB8	140	D	Edd	+	HELPY_1069	YP_003057758	Phosphogluconate dehydratase (6-phosphogluconate dehydratase)	66,713	4		7.63	JHP1026	NP_207891
			FabI	+	HELPY_0198	YP_003057011	Putative enoyl-(acyl-carrier-protein) reductase	29,868	7		20.73	JHP0181	NP_222902
CB9	130	D	ArgS	−	HELPY_0322	YP_003057123	Arginyl-tRNA synthetase (arginine-tRNA ligase) (ArgRS)E	61,946	14		24.58	JHP0302	NP_223022
			MdaB	+	HELPY_0741	YP_003057488	NAD(P)H oxidoreductase (NADPH quinone reductase)	21,524	3		19.59	JHP0573	NP_223291
CB10	60	E	Tfs	+	HELPY_1558	YP_003058194	Elongation factor Ts (EF-Ts)	39,595	6		18.60	JHP1444	NP_224162
			TsaA	+	HELPY_1565	YP_003058201	Alkyl hydroperoxide reductase	22,105	4		26.26	JHP1471	NP_224189
CB11	50	E	PurD	+	HELPY_1194	YP_003057869	Phosphoribosylamine-glycine ligase (GAR synthetase) (GARS) (glycinamide ribonucleotide synthetase) (phosphoribosylglycinamide synthetase)	47,297	3		8.30	JHP1140	NP_223858
			PurD	+	HELPY_1194	YP_003057869	Phosphoribosylamine-glycine ligase (GAR synthetase) (GARS) (glycinamide ribonucleotide synthetase) (phosphoribosylglycinamide synthetase)	47,297	2	3.98(2+) 3.75(2+)	6.60	JHP1140	NP_223858
CB12	100	F–H	GatB	+	HELPY_0713	YP_003057460	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B (Asp/Glu-ADT subunit B)	52,889	5		13.30	JHP0603	NP_223321
			GatA	+	HELPY_0524	YP_003057302	Glutamyl-tRNA(Gln) amidotransferase subunit A (Glu-ADT subunit A)	49,618	6		20.30	JHP0769	NP_223487
			DnaN	+	HELPY_0852	YP_003057584	DNA polymerase III subunit β	42,002	12		36.60	JHP0452	NP_223170
CB13	800	G, M	UreB	+	HELPY_0068	YP_003056903	Urease B subunit	61,553	18		38.66	JHP0067	NP_222789
			GroEL	+	HELPY_0008	YP_003056851	60-kDa chaperonin (protein Cpn60) (groEL protein)	58,111	28		48.72	JHP0008	NP_222730
			UreA	+	HELPY_0069	YP_003056904	Urease A subunit	26,465	10		40.76	JHP0068	NP_222790
CB14	600	G	GlnA	+	HELPY_0840	YP_003057574	Glutamine synthetase (glutamate-ammonia ligase)	54,280	21		47.40	JHP0461	NP_223179
			TsaA	+	HELPY_1565	YP_003058201	Alkyl hydroperoxide reductase	22,105	7		33.84	JHP1471	NP_224189
CB15	280	G, J	ClpP	+	HELPY_0566	YP_003057337	ATP-dependent Clp protease proteolytic subunit (endopeptidase Clp)	21,427	2	2.29(2+) 5.04(3+)	23.47	JHP0730	NP_223448
			NapA	+	HELPY_0248	YP_003057054	Neutrophil-activating protein NapA (bacterioferritin)	16,677	8		65.28	JHP0228	NP_222949

TABLE III—*continued*

Complex no.	Molecular mass	Fig. 3 gel letter	Protein[a]	Previously identified	Strain B38[a]				LC-MS/MS information			Strain J99[b]	
					HELPY no.	GBAN	Protein annotation	Molecular mass	*n*	Xcorr	Cov.	JHP no.	GBAN
	kDa							*Da*			%		
CB16	210	G, H	SerC	+	HELPY_0631	YP_003057392	Putative aminotransferase, class V	40,955	9		51.54	JHP0673	NP_223391
			Tal	+	HELPY_1468	YP_003058112	Transaldolase	34,967	2	2.74 (2+) 3.28 (2+)	20.3	JHP1388	NP_224106
CB17	130	G, H, J	HELPY_0317	+	HELPY_0317	YP_003057118	Putative NodB-like polysaccharide deacetylase	33,451	2	2.69 (2+) 3.72 (2+)	6.48	JHP0295	NP_223015
			FabZ	+	HELPY_1363	YP_003058021	(3*R*)-Hydroxymyristoyl-(acyl-carrier-protein) dehydratase ((3*R*)-hydroxymyristoyl ACP dehydrase)	18,079	2	2.57 (2+) 2.61 (2+)	10.69	JHP1290	NP_224008
CB18	120	G	TrxR1 (TrxB1)	+	HELPY_0531	YP_003057307	Thioredoxin reductase (TRXR) (TR)	33,378	12		42.44	JHP0764	NP_223482
			FabG	+	HELPY_0816	YP_003057552	3-Oxoacyl-(acyl-carrier-protein) reductase	26,629	2	4.95 (2+) 2.79 (2+)	12.15	JHP0508	NP_223226
CB19	75	G	HELPY_0321	+	HELPY_0321	YP_003057122	Hypothetical protein HELPY_0321	28,512	2	3.46 (2+) 3.79 (2+)	10.32	JHP0301	NP_223021
			HELPY_0581	−	HELPY_0581	YP_003057349	Hypothetical protein HELPY_0581	19,797	3		21.14	JHP0720	NP_223438
CB20	55	G	DnaK	+	HELPY_0109	YP_003056934	Chaperone protein dnaK (heat shock protein 70) (heat shock 70-kDa protein) (HSP70)	66,920	4		11.77	JHP0101	NP_222822
			TsaA	+	HELPY_1565	YP_003058201	Alkyl hydroperoxide reductase	22,105	8		13.10	JHP1471	NP_224189
CB21	45	G	TrxB, TrxB_2	+	HELPY_1138	YP_003057820	Thioredoxin reductase	35,748	21		59.89	JHP1091	NP_223808
			Trx1, TrxA	+	HELPY_0532	YP_003057308	Thioredoxin 1	11,724	2	2.56 (2+) 2.93 (2+)	9.22	JHP0763	NP_223481
CB22	230	H	AspS	+	HELPY_0755	YP_003057498	Aspartate-tRNA ligase (aspartic acid translase) (aspartyl-tRNA synthetase)	65,236	3		5.50	JHP0560	NP_223278
			HELPY_0235	+	HELPY_0235	YP_003057042	Hypothetical protein HELPY_0235	29,400	2	3.78 (2+) 3.73 (2+)	9.10	JHP0216	NP_222937
CB23	100	H	HemE	+	HELPY_0768	YP_003057507	Uroporphyrinogen decarboxylase	38,225	6		21.24	JHP0551	NP_223269
			SodB, SodF	+	HELPY_1036	YP_003057725	Superoxide dismutase	24,416	9		48.36	JHP0992	NP_223709
CB24	380	I	PpiC	+	HELPY_0179	YP_003056994	Peptidyl-prolyl cis-trans isomerase C (PPIase) (rotamase); putative signal peptide	33,847	4		16.05	JHP0161	NP_222882
			PpiC	+	HELPY_0179	YP_003056994	Peptidyl-prolyl cis-trans isomerase C (PPIase) (rotamase); putative signal peptide	33,847	8		26.76	JHP0161	NP_222882
			Tpi	+	HELPY_0197	YP_003057010	Triose-phosphate isomerase (TIM)	26,495	4		17.52	JHP0180	NP_222901
CB25	60	I, K	HELPY_1073	+	HELPY_1073	YP_003057762	Putative zinc-containing alcohol dehydrogenase	38,376	6		25.86	JHP1030	NP_223747
			HELPY_1048[c]	+	HELPY_1048[c]	YP_003057737	Hypothetical protein HELPY_1048	25,451	2	3.71 (2+) 2.70 (2+)	9.42	JHP1004	NP_223721
CB26	120	K	GmhA	+	HELPY_0495	YP_003057281	Phosphoheptose isomerase (sedoheptulose-7-phosphate isomerase)	20,883	3		19.79	JHP0791	NP_223509
			MdaB	+	HELPY_0741	YP_003057488	NAD(P)H oxidoreductase (NADPH quinone reductase)	21,524	2	5.55 (2+) 4.67 (2+	16.49	JHP0573	NP_223291
CB27	380	L	HELPY_0235	+	HELPY_0235	YP_003057042	Hypothetical protein HELPY_0235	29,400	2	4.39 (2+) 3.97 (2+)	9.10	JHP0216	NP_222937
			FabI	+	HELPY_0198	YP_003057011	Putative enoyl-(acyl-carrier-protein) reductase	29,868	2	3.71 (2+) 3.73 (2+)	8.40	JHP0181	NP_222902
			UreA	+	HELPY_0069	YP_003056904	Urease A subunit	26,465	4		17.65	JHP0068	NP_222790
CB28	130	L	Icd	+	HELPY_0025	YP_003056867	Isocitrate dehydrogenase	47,361	5		13.60	JHP0023	NP_222745
			FabZ	+	HELPY_1363	YP_003058021	(3*R*)-Hydroxymyristoyl-(acyl-carrier-protein) dehydratase ((3*R*)-hydroxymyristoyl ACP dehydrase)	18,079	3		20.75	JHP1290	NP_224008
CB29	440	M	PyrC	+	HELPY_0272	YP_003057074	Dihydroorotase (DHOase)	42,096	2	2.42 (2+) 2.81 (2+)	5.04	JHP0251	NP_222972
			PyrB	+	HELPY_0369	YP_003057162	Aspartate carbamoyltransferase (aspartate transcarbamylase) (ATCase)	34,004	4		12.38	JHP0341	NP_223060

TABLE III—*continued*

Complex no.	Molecular mass	Fig. 3 gel letter	Protein[a]	Previously identified	Strain B38[a]					LC-MS/MS information			Strain J99[b]	
					HELPY no.	GBAN	Protein annotation	Molecular mass	*n*	Xcorr	Cov.	JHP no.	GBAN
	kDa							*Da*			%		
CB30	40	N	DnaK	+	HELPY_0109	YP_003056934	Chaperone protein dnaK (heat shock protein 70) (heat shock 70-kDa protein) (HSP70)	66,920	5		10.97	JHP0101	NP_222822
			DmpI	+	HELPY_0908	YP_003057631	Putative tautomerase, DmpI-related protein	7,366	2	2.52 (2+) 3.06 (2+)	38.24	JHP0858	NP_223576

[a] Gene product/function and protein molecular mass according to the annotation of the strain B38 available in NCBI.

[b] Information available in NCBI; see also the revised annotation of strain J99 in Boneca *et al.* (31).

[c] According to this annotation, HELPY_1048 was annotated "hypothetical protein" whereas its corresponding protein in the J99 strain, JHP1004, was annotated "predicted DsbC-like protein" by Boneca *et al.* (31) (http://genolist.pasteur.fr/PyloriGene/genome.cgi).

MJ22, and MJ25). The MB6 complex comprised two proteins whose genes are present in all of the sequenced *H. pylori* strains and were annotated as predicted coding regions with no homolog in the databases, *i.e.* HELPY_0130 (JHP0119) and HELPY_1147 (JHP1100), demonstrating that both of these ORFs encode for proteins present in the membrane. Analysis using the STITCH server (33) also revealed possible interactions between these two proteins and five intermediary proteins: four orphan proteins named JHP1044, HELPY_0788 (JHP0534), HELPY_0795 (JHP0527), JHP0526 and FlbA. FlbA is a membrane protein involved in the coordinated expression of the *H. pylori* flagellar genes, *fla*A and *fla*B, and *flb*A mutants were aflagellate and completely non-motile (61). Using a blastp search, no putative conserved domains were detected for JHP0119, whereas a 56% identity in a 43-residue overlap was revealed with the dynein heavy chain 6 of *Tetrahymena thermophila*, a free-living ciliate protozoa. Dyneins are molecular motor complexes involved in cilium and flagellum movement (62, 63). Taken together, these results suggest that these two orphan proteins, in particular JHP0119, could play a role in the flagellar function of *H. pylori*.

Complexes Involved in H. pylori Adherence—Bacterial adherence is considered to have an important role in the colonization of gastric epithelium by *H. pylori*. Approximately 4% of the *H. pylori* genome encodes at least 32 OMPs (64), but the role of these individual OMPs in *H. pylori* adherence is still poorly understood. The main OMPs associated with *H. pylori* pathogenicity are BabA, SabA, OipA, AlpA, and AlpB, which were all found in different complexes by two-dimensional BN/SDS-PAGE. However, other OMPs, such as HopF (HopX) and the essential OMP for mouse colonization, HopG (HopY) (65), were found together in both strains (MB8 and MJ6). HopG was found also with the predicted OMPs HopK and PpiC in the MJ20 and MJ15 complexes, respectively. HorJ (HopV) interacts with HorF and Lpp20 in the MJ13 complex, and HorK (HopW) is associated with DagA (MJ24) and with both AlpA and HopA (MJ11). All of these complexes contain at least one of four highly conserved OMPs among *H. pylori* strains, *i.e.* HopF, HopG, HorJ, and HorK (HorJ and HopF are porins) (66). The expression of these OMPs/porins does not seem to be regulated by phase variation (67), and they are expressed at the surface of all *H. pylori* strains and appear to be continuously expressed during all stages of *H. pylori* infection (66). In fact, these four OMPs/porins are immunogenic in mice, and the resulting sera recognize specifically the corresponding proteins and no other member of the OMP family, suggesting that the conserved regions do not contain immunodominant epitopes (66) and may constitute an excellent vaccine target because they seem to be constitutively expressed in *H. pylori*.

H. pylori porins are weakly expressed compared with those of other bacteria. In addition to HorJ and HopF, HopE, a non-selective porin allowing the passage of hydrophilic substances by general diffusion (68), was found in complexes

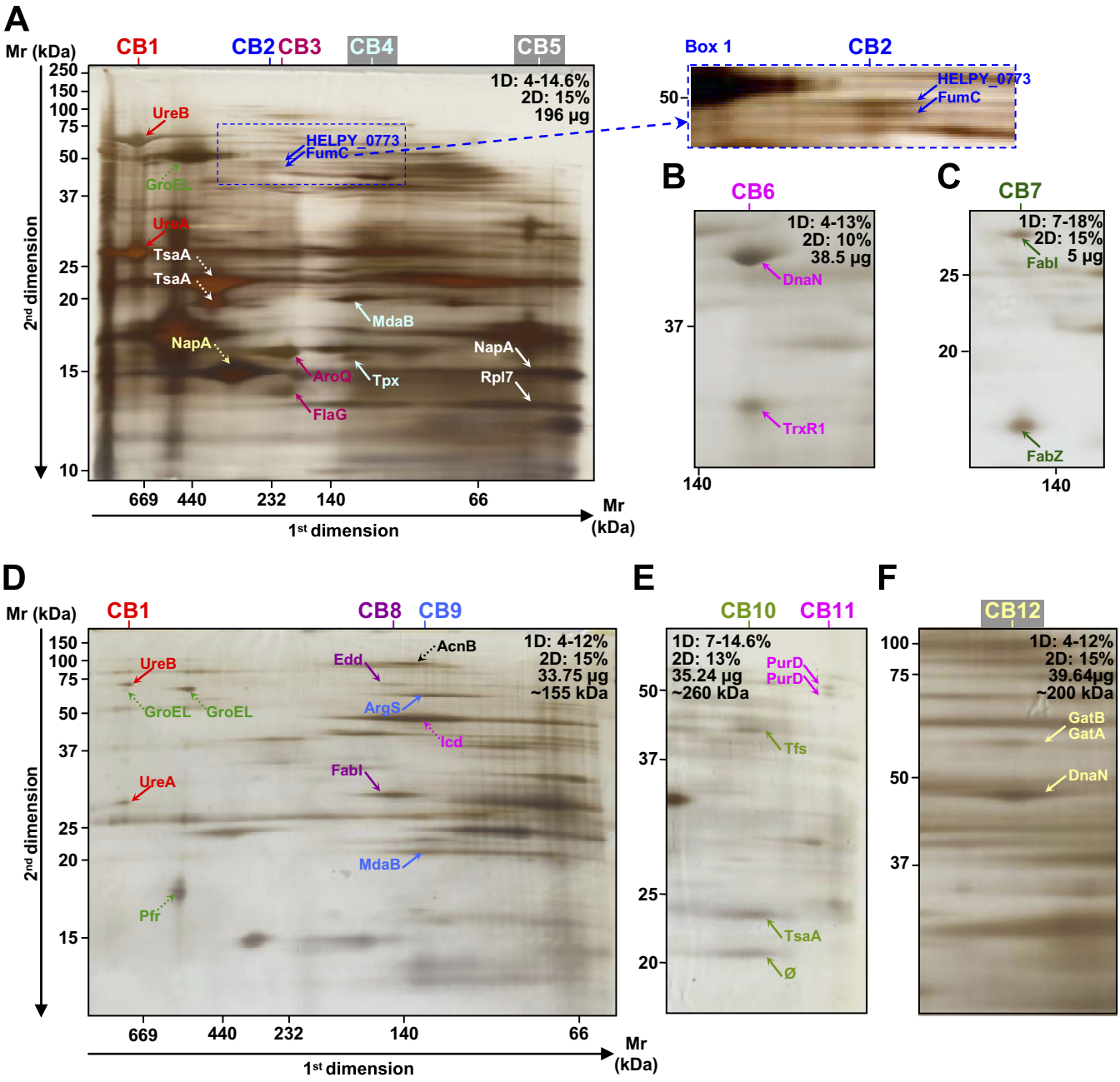

FIG. 3. **Analysis of crude and purified cytoplasmic samples of *H. pylori* strain B38.** The first (BN-PAGE) and second dimension gel electrophoreses (SDS-PAGE) were performed with the various protein quantities and acrylamide gradients indicated on each gel (*A–N*). *Dotted arrows* indicate proteins that were not attributed to heterooligomeric complexes. More contrasted pictures of migration of the CB2 and CB16 complexes are shown in *boxes* 1 and 2, respectively. Protein identifications are presented in Table III. Multiprotein complexes from the cytosol of the B38 strain were named CB. *A–C* represent the analyses of the crude cytoplasmic samples. *D* and *F* represent the analyses of the fractions eluted when the cytoplasmic sample was purified using the gel filtration method (Superdex 200 column) before applying the two-dimensional BN/SDS-PAGE. *G–N* represent the analyses of the fractions eluted by the isoelectrofocalization method (Rotofor system) before applying the two-dimensional BN/SDS-PAGE. ∅, spots for which identification has failed.

MB11, MJ16, and MJ21 comprising proteins rarely studied from the *hor* family: HorB, HorF, HorL, HorH, HorC, and HorE. This can be explained by the fact that HopE forms large channels (68, 186) compared with other porins described in *H. pylori* or in other Gram-negative bacteria. HopE was shown to be antigenic in humans and immunologically conserved with both patients' sera and specific monoclonal antibodies (68). Among the proteins of MB11, HorH, HorE, and HorF were reported previously to be present in the membrane; HorE and HorF are also immunoreactive (69). All of these newly char-

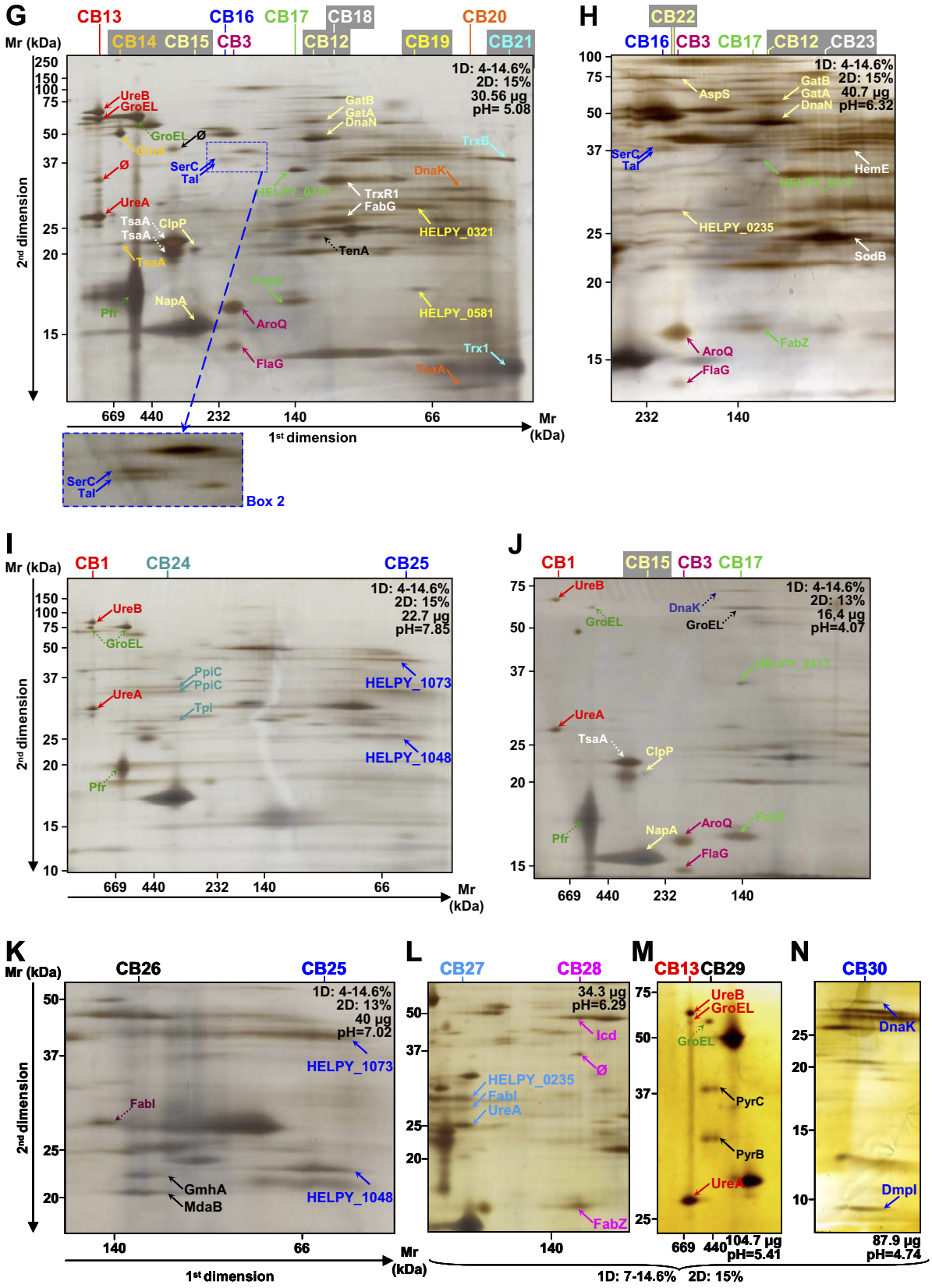

FIG. 3—*continued*

TABLE IV

Description of cytosolic protein complexes identified in H. pylori strain J99 using two-dimensional BN/SDS-PAGE

The complexes presented in the table were all localized on two-dimensional BN/SDS-PAGE gels performed in this study and are represented in Fig. 4. Multiprotein complexes isolated from the cytosol of the J99 strain were named CJ. The experimental approximate molecular mass is given in kDa. GBAN, GenBank accession number (NCBI Reference Sequence); *n*, the number of peptides; Cov., the protein sequence coverage (percentage) of the peptides; Pred., predicted.

Complex no.	Molecular mass	Fig. 4 gel letter	Protein[a]	Previously identified	Strain J99[a]				LC-MS/MS information			Strain B38[b]	
					JHP no.	GBAN	Protein annotation	Molecular mass	*n*	Xcorr	Cov.	HELPY no.	GBAN
	kDa							*Da*			%		
CJ1	800	A, B, D	UreB	+	JHP0067	NP_222789	Urease B subunit	61,509.5	13		35.10	HELPY_0068	YP_003056903
			GroEL	+	JHP0008	NP_222730	Chaperone and heat shock protein	58,068.6	23		45.10	HELPY_0008	YP_003056851
			UreA	+	JHP0068	NP_222790	Urease A subunit	26,418.4	12		41.60	HELPY_0069	YP_003056904
CJ2	110	A	GatB	+	JHP0603	NP_223321	Pred. Glu-tRNA(Gln) amidotransferase subunit B	52,934.9	5		12.90	HELPY_0713	YP_003057460
			GatA	+	JHP0769	NP_223487	Pred. Glu-tRNA(Gln) amidotransferase subunit A	49,584.7	2	2.96 (2+) 3.04 (2+)	8.20	HELPY_0524	YP_003057302
			DnaN	+	JHP0452	NP_223170	Pred. DNA polymerase III β subunit	41,888.1	2	2.73 (2+) 2.78 (2+)	5.60	HELPY_0852	YP_003057584
CJ3	100	A	SodB, SodF	+	JHP0992	NP_223709	Superoxide dismutase	24,384.7	8		41.30	HELPY_1036	YP_003057725
			JHP0631	+	JHP0631	NP_223349	Pred. coding region JHP0631	19,527.2	2	3.56 (2+) 3.82 (2+)	15.70	HELPY_0673	YP_003057426
CJ4	80	A	DnaK	+	JHP0101	NP_222822	Chaperone and heat shock protein 70/DnaK	66,943.4	2	4.30 (2+) 3.98 (2+)	4.70	HELPY_0109	YP_003056934
			GroEL	+	JHP0008	NP_222730	Chaperone and heat shock protein	58,068.6	8		15	HELPY_0008	YP_003056851
CJ5	60	A	Tfs	+	JHP1444	NP_224162	Pred. translation elongation factor Ts	39,700.6	2	2.50 (2+) 2.39 (2+)	6.50	HELPY_1558	YP_003058194
			TsaA	+	JHP1471	NP_224189	Pred. alkyl hydroperoxide reductase	22,112.8	7		42.90	HELPY_1565	YP_003058201
CJ6	50	A	TufA	+	JHP1128	NP_223846	Pred. translation elongation factor Tu	43,568.9	6		18.30	HELPY_1179	YP_003057859
			Pfs	+	JHP0082	NP_222804	Pred. 5′-methylthioadenosine nucleosidase/ *S*-adenosylhomocysteine nucleosidase	24,872	5		23.90	HELPY_0086	YP_003056917
CJ7	150	B	PorA	+	JHP1037	NP_223754	Pyruvate ferredoxin oxidoreductase, α subunit	44,599.6	3		12.00	HELPY_1079	YP_003057768
			RfaD	+	JHP0793	NP_223511	Pred. ADP-L-glycero-D-mannoheptose-6-epimerase	37,196.4	6		21.90	HELPY_0493	YP_003057279
CJ8	110	B	TipA, YihK	+	JHP0432	NP_223150	Pred. GTP-binding protein of the TypA subfamily	66,471.4	8		13.00	HELPY_0464	YP_003057252
			AspA	+	JHP0594	NP_223312	Pred. aspartate ammonia-lyase	51,771	5		12.20	HELPY_0722	YP_003057469
CJ9	40	B	PurD	+	JHP1140	NP_223858	Pred. glycinamide ribonucleotide synthetase	47,348	3		7.10	HELPY_1194	YP_003057869
			PurD	+	JHP1140	NP_223858	Pred. glycinamide ribonucleotide synthetase	47,348	3		10.10	HELPY_1194	YP_003057869
CJ10	150	C	PorG	+	JHP1035	NP_223752	Pyruvate ferredoxin oxidoreductase, γ subunit	20,892	2	3.59 (2+) 2.50 (2+)	10.80	HELPY_1077	YP_003057766
			RfaD	+	JHP0793	NP_223511	Pred. ADP-L-glycero-D-mannoheptose-6-epimerase	37,196.4	5		16.10	HELPY_0493	YP_003057279
CJ11	130	C	FabG	+	JHP0508	NP_223226	Pred. 3-ketoacyl-acyl carrier protein reductase	26,446.1	3		12.60	HELPY_0816	YP_003057552
			SodB, SodF	+	JHP0992	NP_223709	Superoxide dismutase	24,384.7	4		18.30	HELPY_1036	YP_003057725
CJ12	100	C	DnaK	+	JHP0101	NP_222822	Chaperone and heat shock protein 70	66,943.4	10		21.00	HELPY_0109	YP_003056934
			HemE	+	JHP0551	NP_223269	Pred. uroporphyrinogen decarboxylase	38,211.5	3		9.70	HELPY_0768	YP_003057507
CJ13	440	D	JHP0632	+	JHP0632	NP_223350	Pred. *N*-methylhydantoinase	86,415.6	19		26.50	HELPY_0674	YP_003057427
			HyuA[c]	+	JHP0633	NP_223351	Pred. *N*-methylhydantoinase	78,148	3		5.80		
			JHP0631	+	JHP0631	NP_223349	Pred. coding region JHP0631	19,527.2	2	4.37 (2+) 4.54 (2+)	15.70	HELPY_0673	YP_003057426
CJ14	130	D, F	JHP0295	+	JHP0295	NP_223015	Pred. coding region JHP0295	33,489.5	8		43.30	HELPY_0317	YP_003057118
			FabZ	+	JHP1290	NP_224008	Pred. (3*R*)-hydroxymyristoyl-(acyl-carrier-protein) dehydratase	18,067.8	4		25.80	HELPY_1363	YP_003058021
CJ15	120	E	Icd	+	JHP0023	NP_222745	Pred. isocitrate dehydrogenase	47,298.8	6		13.18	HELPY_0025	YP_003056867
			FabG	+	JHP0508	NP_223226	Pred. 3-ketoacyl-acyl carrier protein reductase	26,446.1	6		24.30	HELPY_0816	YP_003057552

TABLE IV—*continued*

Complex no.	Molecular mass	Fig. 4 gel letter	Protein[a]	Previously identified	Strain J99[a]			LC-MS/MS information			Strain B38[b]	
					JHP no.	GBAN	Protein annotation	Molecular mass	*n*	Xcorr	Cov.	HELPY no.	GBAN
	kDa							*Da*			%		
CJ16	120	G	Icd	+	JHP0023	NP_222745	Pred. isocitrate dehydrogenase	47,298.8	15		41.90	HELPY_0025	YP_003056867
			FabI	+	JHP0181	NP_222902	Pred. enoyl-(acyl-carrier-protein) reductase (NADH)	29,874.6	10		33.09	HELPY_0198	YP_003057011

[a] Gene product/function and protein molecular mass according to Boneca *et al.* (31) (http://genolist.pasteur.fr/PyloriGene/genome.cgi).

[b] Information according the annotation of strain B38 available in NCBI.

[c] The gene corresponding to HyuA (JHP0633) is not found in the B38 strain; only three "pseudogenes" correspond to HyuA: HELPY_0677 (Part 1), HELPY_0676 (Part 2), and HELPY_0675 (Part 3).

acterized OMPs and their interacting partners may constitute attractive targets for a vaccine.

The adherence-associated lipoproteins AlpA (HopC) and AlpB (HopB) are encoded by highly homologous genes (64, 67) and were found in the membrane (69). Both lipoproteins are involved in the adherence of *H. pylori* to the gastric epithelium (70) in a different pattern than that observed for the BabA-mediated adherence, suggesting that a different receptor may be involved (71). AlpA and AlpB are required for gastric colonization (72, 73) and are especially recognized by sera from *H. pylori*-infected patients (69, 74). In addition, AlpA/B may induce gastric injury by mediating adherence to gastric epithelial cells and by modulating proinflammatory intracellular signaling cascades (73). Both of these lipoproteins were found in interaction in MB5, MB7, MB19, and MJ8 complexes. Furthermore, AlpA and AlpB are both described as outer membrane porins and adhesins, suggesting that they have multiple activities. In fact, they were retrieved from several complexes (MB10, MB17, MB18, MJ11, MJ12, and MJ23), either alone or together, in association with different OMPs, such as HopA (MJ12); the essential OMP for colonization, HofC (65); HorK; and JHP0999 (HELPY_1043), a putative metalloprotease/putative membrane protein, showing the importance of AlpA and AlpB for the bacteria.

Examples of Membrane Complexes Retrieved from Only One Strain—The proinflammatory OMP OipA (HopH) (75), an adhesin involved in cytoskeleton reorganization (76), was only retrieved from the J99 strain (MJ14), interacting with the predicted Sec-independent protein translocase protein TatB and JHP0368, whose gene was annotated as a predicted coding region with no homolog in the databases. This result is not surprising because the B38 strain has a non-functional *oip*A status.

The major *H. pylori* adhesin is BabA (HopS), which binds to the fucosylated Lewis b blood group antigen (77) and has a closely related paralog, BabB (HopT), whose function has not yet been determined. BabA and BabB are associated in the complex MJ19, which is undoubtedly specific for J99 strain because the B38 strain does not express BabB. BabB was also found to interact with HopM/N (MJ18) in the J99 strain. In the B38 strain (MB16), BabA interacts with HopM/N as well as with the predicted coding region JHP1100 (MJ22), a protein with no homolog in the databases recently reported to be present and immunoreactive in the *H. pylori* membrane (69). Recent studies showed that neither BabA nor BabB could induce an immune response in monkeys (78) and that BabA and BabB were not immunodominant antigens in humans (48, 79). One hypothesis is that the proteins interacting with them in the membrane could mask the BabA epitope and could consequently be exposed and therefore be antigenic; this is probably the case of HopM/N previously shown to be immunoreactive to sera from *H. pylori*-positive patients (44, 80). These BabA/BabB-interacting proteins (JHP1100, HopM/N, and HopZ) represent po-

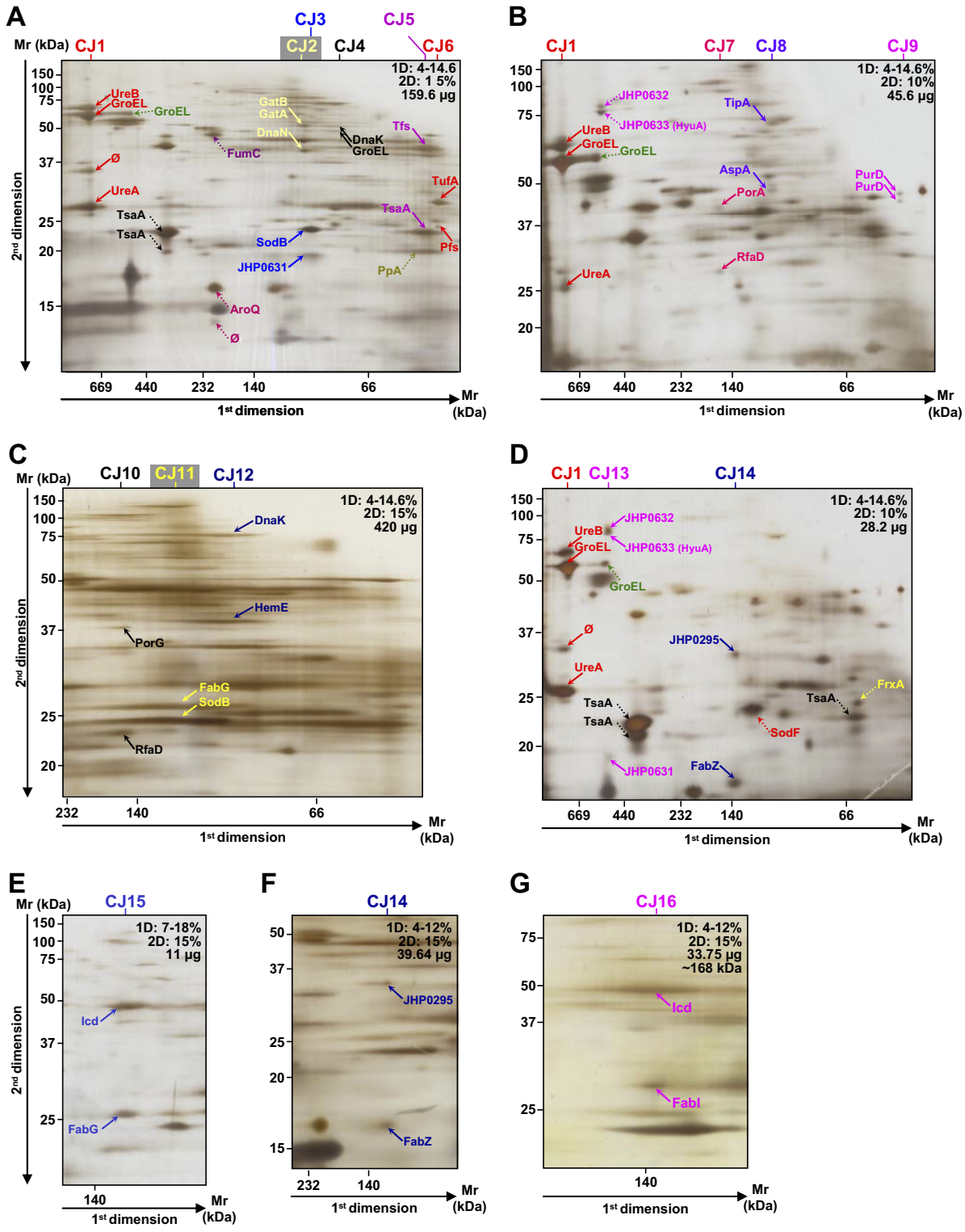

FIG. 4. **Analysis of the crude and purified cytoplasmic samples of *H. pylori* strain J99.** The first (BN-PAGE) and second dimension gel electrophoreses (SDS-PAGE) were performed with the various protein quantities and acrylamide gradients indicated on each gel (*A–G*). *Dotted arrows* indicate proteins that were not attributed to heterooligomeric complexes. Protein identifications are presented in Table IV. Multiprotein complexes from the cytosol of the J99 strain were named CJ. *A–F* represent the analyses of crude cytoplasmic samples. *G* represents the analysis of the fraction eluted at ~168 kDa when the cytoplasmic sample was purified using the gel filtration method (Superdex 200 column) before applying the two-dimensional BN/SDS-PAGE. ∅, spots for which identification has failed.

tential antigen targets for the development of an *H. pylori* vaccine. In the current study, BabA was also associated with SabA in different oligomerization states in the B38 strain (MB14 and MB15); this complex was never retrieved from the J99 strain. SabA (HopP) is the second most well characterized adhesin of *H. pylori*; it binds to sialylated Lewis X antigens and is up-regulated during persistent *H. pylori* infection (81), strengthening the epithelial attachment necessary to achieve successful colonization (82). These BabA-SabA complexes could be potentially implicated in development of malignant diseases because each protein has been associated with gastric cancer (83–86). Indeed, SabA anchors to cellular receptors (81) considered as tumor antigens (87) and gastric dysplasia markers (88). Furthermore, a recent study has shown that BabA-positive strains were associated with an intercellular localization of the bacterium, intestinal metaplasia, and degenerative alterations observed on gastric biopsies (89). Thus, BabA-SabA association could permit strains expressing this complex to reach the intercellular compartment and to persist between host cells even during the development of a malignant disease. The *sab*A gene is among the most divergent genes in the *H. pylori* genome (90), and its "on"/"off" expression is regulated by phase variation (67). Although a non-functional status was found for the *sab*A gene in the B38 strain (11), the corresponding protein is undoubtedly synthesized because it was identified in two complexes. This is not surprising because the SabA expression is frequently switched on or off both *in vitro* (91) and *in vivo* (16).

Although never retrieved from the J99 strain, SabA appears in three complexes in the B38 strain (MB14 and MB15) and also in association with the neuraminyllactose-binding hemagglutinin HpaA (MB9), an antigenic lipoprotein present in the flagella sheath of *H. pylori* and expressed in all strains (69, 92).

Cytosolic Protein Complexes

At the cytosol level, 30 and 16 heterooligomeric protein complexes composed of 47 and 27 different proteins were identified in the *H. pylori* B38 (Fig. 3 and Table III, complexes named CB) and J99 (Fig. 4 and Table IV, complexes named CJ) strains, respectively. Only five complexes were common to both strains: UreB-GroEL-UreA, GatA-GatB-DnaN, DnaK-GroEL, PurD-PurD, and HELPY_0317 (JHP0295)-FabZ.

New Insight into H. pylori Cytoplasm Illustrated by Six Examples—In this study, most of the complexes identified in the cytoplasm contained proteins suspected to be involved in metabolism, which is a prerequisite for virulence. For example, the CB29 complex comprised two proteins predicted to catalyze the second and third steps in the *de novo* pyrimidine biosynthesis pathway, aspartate carbamoyl transferase (PyrB) and dihydroorotase (PyrC), respectively (93). This complex would allow the transformation of carbamoylphosphate into dihydroorotic acid during the *de novo* synthesis of UTP and CTP (94).

The major route for the generation of acetyl coenzyme A in *H. pylori* is via the pyruvate:flavodoxin oxidoreductase (POR), an essential heterotetrameric complex composed of PorA, PorB, PorC (ex-PorG), and PorD (95, 96), which has also been implicated in metronidazole resistance (95, 97). Although PorA, PorB, and PorC subunits were found previously using two-dimensional BN/SDS-PAGE (28), modifications were made during sample preparation that did not allow us to retrieve the POR complex. However, PorA and PorC were found separately in 150-kDa complexes with RfaD (CJ7 and CJ10), a "predicted ADP-L-glycero-D-mannoheptose 6-epimerase". ADP-L-Glycero-D-mannoheptose 6-epimerase is the last enzyme in the pathway for synthesis of ADP-heptose, a precursor of core lipopolysaccharide in Gram-negative bacteria (98). Thus, the interaction of RfaD with PorA and PorC suggests a new activity related to the virulence of *H. pylori* POR.

Several complexes contain proteins predicted to be involved in fatty acid biosynthesis (FAS). The FAS system is divided into two different pathways named FAS I and FAS II based on the architecture of the enzymes involved. In contrast to the large multifunctional enzymes with multiple domains that catalyze various reactions of the FAS I pathway in fungi and mammals, FAS enzymes for bacteria belong to the FAS II pathway where the acyl chain covalently attached to the acyl carrier protein (ACP) is elongated with five enzymes catalyzing consecutively. Thus, the enzymes involved in the FAS II pathway represent a validated yet unexploited and very promising target for antibacterial agent development (99, 100). In *H. pylori*, the elongation phase of fatty acid biosynthesis could imply FabF, FabH, FabG, FabI, and FabZ (94). FabG, FabI, and FabZ were found in 10 different complexes (CB17, CB18, CB27, CB28, CB7, CB8, CJ11, CJ14, CJ15, and CJ16). Each of these three enzymes was found with the predicted isocitrate dehydrogenase Icd (CB28, CJ15, and CJ16), suggesting that the enzymes of the FAS II pathway are closely linked with Icd. Such a link was demonstrated in *Saccharomyces cerevisiae* (101, 102) in which Icd provides NADPH for β-oxidation of polyunsaturated fatty acids. Similarly, Icd could play a role in fatty acid biosynthesis of *H. pylori*. Complexes including orphan proteins associated with enzymes of the FAS II pathway were found: FabG interacts with HELPY_0235 (JHP0892) in CB27, and FabZ is associated with HELPY_0317 (JHP0295) both in B38 and J99 strains (CB17 and CJ14), suggesting a possible role of these two orphan proteins in FAS. FabZ is an important enzyme for the elongation cycles of both saturated and unsaturated fatty acids in the FAS II pathway. With regard to *H. pylori*, FabZ was shown to be immunoreactive (69), and its recent x-ray crystal structure revealed that it maintains its unique features and suggests that it could be inhibited either by occupying the entrance of the tunnel or plugging the tunnel to prevent the substrate from accessing the active site (103). FabZ was also found in complex CB7 with FabI, a predicted

enoyl-ACP reductase (NADH) catalyzing the reduction of the enoyl-ACP resulting from the FabZ reaction. FabI is highly conserved and widely expressed among bacteria with only a single known isoform. This reductase is essential for the bacterial viability of *E. coli* (104) and now appears to be an excellent target for the development of narrow spectrum antimicrobial agents that selectively target pathogens, such as *Mycobacterium tuberculosis* (105, 106) or multidrug-resistant *Staphylococcus aureus* (107). The presence of multiple targets in the FAS II pathway presents the possibility of developing synergistic chemotherapeutic regimes that could intervene simultaneously at multiple points in the biosynthesis of fatty acids (100). Moreover, the identification of certain multiprotein complexes with enzymes involved in FAS II pathway should help in developing new therapeutic strategies by inhibiting the formation of these complexes. Thus, the partners of FabZ and/or FabI could also be targets for antibacterial drugs.

GmhA, a predicted phosphoheptose isomerase involved in the biosynthesis of inner core lipopolysaccharides, was found to be associated with MdaB (CB26), a predicted modulator of drug activity and an important enzyme to fight against oxidative stress (108), suggesting that this complex may be involved in drug resistance.

The essential thioredoxin system of *H. pylori* comprises thioredoxin Trx1 (TrxA; JHP0763) and thioredoxin reductase TrxR1 (TrxB; JHP0764) (109). Trx1 and TrxR1 demonstrate specialized catalytic properties because both form a reductase system for *H. pylori* TsaA/AhpC (110). Trx1 is considered as a stress response element in *H. pylori* as its expression increases dramatically under conditions of oxidative stress (109). Trx1 also acts as an arginase chaperone capable of renaturing the enzyme to a catalytically active state (111). Another predicted thioredoxin reductase, TrxR2 (also named FqrB), was demonstrated to exhibit an NADPH oxidoreductase activity that is part of the pyruvate:ferredoxin oxidoreductase complex (112). Trx1 and TrxR2 were found in complex CB21, suggesting that these enzymes really have multiple functions in the bacteria: TrxR2 could also be implicated in the *H. pylori* thioredoxin system, or conversely, Trx1 could be implicated in the pyruvate:ferredoxin oxidoreductase activity.

Chaperones constitute a functionally related group of proteins increasingly synthesized under heat shock conditions to prevent protein aggregation (113), thus protecting the cell from damage caused by the formation of improperly folded polypeptides (114). Many heat shock proteins play a key role in cellular metabolism under all growth conditions, assisting the folding, assembly, and translocation of cellular proteins (115–117). The presence of DnaK (Hsp70) with TsaA (CB20) is not really surprising because TsaA was shown to switch from a peroxide reductase to a stress-dependent molecular chaperone function (118). The best studied examples of such "molecular chaperones" include the ubiquitous GroEL (Hsp60) and DnaK proteins. In *H. pylori*, the most prominent chaperone is GroEL and its co-chaperone GroES (119), both immunogenic and present in the structure-bound and soluble fractions (48, 120). In the present study, GroEL and DnaK were found in complex CJ4. It was reported recently that the two eukaryotic homologs of these chaperones are able to interact to form a stable complex (121), whereas no similar interaction seems to occur between their prokaryotic counterparts, GroEL and DnaK. However, both of these chaperones are up-regulated by cadmium in *Rhodobacter capsulatus* (122), and they cooperate in their chaperone functions in *E. coli* (123). Moreover, their transcription is negatively regulated by the same repressor in *H. pylori* (124). DnaK was also found with DmpI, a putative tautomerase (CB30), and with HemE, a predicted uroporphyrinogen decarboxylase (CJ12).

Examples of Cytosolic Complexes Retrieved from Only One Strain—The complex including JHP0631, JHP0632, and JHP0633 (HyuA) (28) was again isolated in the J99 strain but not in the B38 strain (CJ13). This was predictable because the protein corresponding to JHP0633 is absent in strain B38. JHP0632 and JHP0633 are annotated as predicted *N*-methylhydantoinase, and JHP0631 is annotated as a predicted coding region. However, JHP0631, JHP0632, and JHP0633 show homologies with the γ (AcxC), α (AcxB) and β (AcxA) subunits, respectively, of the acetone carboxylase (ACX) of numerous bacteria (*Burkholderia* species, *Thauera* species, *Ralstonia* species, *Xanthobacter autotrophicus*, etc.) (125). The approximate M_r of 440 kDa observed for CJ13 is relatively close to that observed for the heterohexameric ACX complex of *X. autotrophicus*, comprising three different polypeptides with M_r values of 86 kDa, 78 kDa, and 19 kDa arranged in an $\alpha 2\ \beta 2\ \gamma 2$ quaternary structure (126). In *H. pylori*, the proposed pathway for the conversion of acetone to acetyl-CoA involves three steps. First, ACX (composed of JHP0632, JHP0633, and JHP0631) is functional and may catalyze the conversion of acetone to acetoacetate. The acetoacetate is subsequently converted into acetoacetyl-CoA by the succinyl-CoA-transferase complex, ScoA/B (JHP0636/JHP0637), and finally transformed by FadA (JHP0638) into two molecules of acetyl coenzyme A that would feed into the TCA cycle to provide energy for the bacteria (125). The fact that two complexes involved in the first two steps of acetone utilization (ACX and ScoA/B complexes) were purified from the J99 strain (28) but never retrieved from the B38 strain is an argument in favor of the specificity of ACX toward the J99 strain. In addition, the transcriptional regulator JHP0403 was shown to strongly activate the transcription of the *acx*ABC and *sco*AB gene cluster (127). ACX is expressed during infection (127) and enhances the ability of *H. pylori* to colonize the mouse stomach (125). Indeed, strain B38, which should be defective for ACX activity, hardly colonizes the mouse.[2] In fact, the *acx*A gene seems to be absent in strain B38 because it would be truncated into

[2] C. Varon, personal communication.

three parts, corresponding to the pseudogenes annotated *helpy_0675*, *helpy_0676*, and *helpy_0677*, localized at the *acx*BC locus (*helpy_0674*/*helpy_0673*). The fact that JHP0631 (AcxC) was retrieved from another complex (CJ3) with SodB, a superoxide dismutase involved in detoxification and oxidative stress resistance, suggests multiple functions for this protein and could explain why its corresponding gene has been conserved in the genome of B38 unlike *acx*A.

Complex CB5, comprising NapA and the predicted ribosomal protein L7/L12 (RpL7/L12), was only retrieved from the B38 strain. Both of these proteins were reported to be among the 20 most abundant proteins in *H. pylori* and to be antigenic (48, 79, 119, 128, 129). NapA was found in the cytoplasm (120) and in the membrane (69) and is also secreted (60). RpL7/L12 was found both in the cytoplasm and membrane fractions (120, 130). A recent study showed that RpL7/L12 is overexpressed in LG-MALT-associated strains when compared with DU-associated strains, suggesting that RpL7/L12 could be used as a biomarker for the differential diagnosis of *H. pylori*-associated clinical outcomes (17). RpL7/L12 was also reported previously to be overexpressed in gastric adenocarcinoma-associated strains (131). Furthermore, NapA was recently proposed as a novel up-regulated biomarker in strains associated with gastric cancer (132) that may play a role in the development of gastric carcinoma (133). Thus, the RpL7/L12-NapA complex would be potentially implicated in the occurrence of gastric cancer. However, it was not possible to determine whether this complex is really specific to the B38 strain associated with LG-MALT because this complex could not be detected in the J99 strain where the RpL7/L12 protein is known to be expressed in smaller quantities (17). Moreover, it was recently demonstrated that NapA is able to prolong the lifespan of monocytes and neutrophils (134), which would contribute to the development of a malignant disease like LG-MALT. This NapA-RplL7/L12 complex is of particular interest because both partners could play a role in the development of LG-MALT. Further studies are necessary to determine the exact role of this complex and of the Rpl7/L12 overexpression in malignant strains.

DISCUSSION

Although *H. pylori* infection is one of the most common bacterial infections worldwide with up to half of the world's population infected (1), questions still remain concerning the evolution of this infection toward gastroduodenal pathologies and especially toward the development of LG-MALT. The fact that it is possible to cure this lymphoma by an antibiotic-based eradication treatment of *H. pylori* suggests an important role of the bacterium in the development of this particular cancer. However, no current known *H. pylori* virulence factors could be associated with the development of this lymphoma (10, 11).

Studies on the *H. pylori* proteome have intensified in recent years, and various strategies have already been applied to examine the *H. pylori* proteome, such as the yeast two-hybrid method (135–138), two-dimensional electrophoresis (119, 120, 129, 139–145), tandem affinity purification (146), and *in silico* analyses (147–150). The extracellular proteome from *H. pylori* was also investigated (60, 151). Several studies examined the proteome of strains associated with different pathologies (47, 48, 79, 80, 128, 131, 143, 152–155), most of them based on immunoproteomics methods. However, only one of these studies included strains associated with LG-MALT (17).

Because the identification of protein complexes is an important step in interpreting protein-protein interaction data, the two-dimensional BN/SDS-PAGE method combined with mass spectrometry has renewed interest because it can be applied to the study of the whole complexome of an organism (24–28). In the current study, this method was used to study the complexome of two *H. pylori* sequenced strains, B38 associated with LG-MALT and J99 associated with DU.

Among the 329 proteins identified, only 145 could be grouped in 90 complexes. Many proteins could not be attributed to complexes for different reasons. In some cases, some visible intense spots could not be identified by LC-MS/MS. Indeed, silver staining is not a quantitative method, and the intensity of a spot does not reflect the quantity of proteins present unlike colloidal blue staining. In addition, some proteins are sometimes poorly ionized during the ionization process before mass spectrometry and therefore cannot be identified. Furthermore, different proteins have sometimes been identified in a single spot, and therefore, the Schägger *et al.* (20, 34) criterion of the "same shape" was not applicable. This is a limit to the method. Moreover, a poor denaturation before the second dimension sometimes occurs and can lead to the identification of a mixture of proteins in the same spot. This descriptive study has nevertheless allowed the identification of genes expressed *in vitro* in *H. pylori* and novel complexes that had not yet been described. The main difficulty encountered during this study was to assign the specificity of the complex to a particular strain because subunits or whole complexes can be lost during the sample preparation or can be hardly visible on the gels, which is the case for complexes whose subunits have a low intensity. It was not possible with such a method to achieve a quantitative complexomics study. However, some complexes specific to each strain could be easily described because some of these proteins do not exist in one strain as is the case with HopM/N-BabB and AcxA/B/C, which are specific to the J99 strain. Complexes retrieved from only one strain, such as BabA-SabA and NapA-RplL7/L12 isolated in the LG-MALT strain, open new fields of research to explore the implication of *H. pylori* in the development of LG-MALT.

Among the 90 heterooligomeric complexes identified in this study (49 in the B38 strain and 41 in the J99 strain), only seven membrane and five cytosolic protein complexes were common to both strains. This result is not surprising because of the huge

genetic variability among *H. pylori* strains. In fact, based on the study of a low number of strains, it appears that 200–400 genes would be variably present in each strain, giving a core of ~1,100–1,300 genes (156–158). However, as mentioned previously, the eventual loss of certain complexes during the preparation steps of the samples must be kept in mind.

The relevance to study complexes from strain B38 is that it is a type II strain (lacking the *cag* PAI), whereas many proteomics studies were performed on type I strains (28, 48, 69, 79, 119, 120). However, the choice to study complexes of a type II strain in LG-MALT is relevant because the presence of the *cag* PAI is not associated with strains isolated from patients with LG-MALT (10, 11). Interestingly, no proteins of the *cag* PAI were identified in the J99 strain, a type I strain, and the absence of *cag* PAI proteins does not seem to modify the complexome, especially in the membrane fraction where some of these proteins are expected to be localized (159). In fact, few studies have reported the presence of *cag* PAI proteins at the membrane level or in culture supernatants (28, 60). Indeed, these proteins are obviously produced in amounts that are below the detection limit of the applied method and are rather detected by immunoblotting (48, 129, 140, 141). Moreover, it was suggested that some proteins of the type IV secretion system contributing to virulence may not be expressed under *in vitro* culture conditions; rather their expression may be dependent on *in vivo* stimuli such as bacterium-host cell contact (160, 161).

In most genomes, ~20–30% of all genes encode membrane proteins (162), and because of their diverse functionality (163–165), they provide one of the most important target groups for drug design (163, 166). However, because of their innate hydrophobic and amphiphilic nature, their low abundance, and their general instability under diverse conditions of purification, membrane proteins are often difficult to purify, produce, and analyze, and as a result, their characterization by proteomics analyses and structural studies has been inadequate (167). Numerous membrane proteins belong to complexes involved in important cellular functions, such as the regulation of energy metabolism, protein trafficking, transport of molecules, and adherence (162, 168). *H. pylori* virulence is due to unique soluble proteins and membrane proteins that allow its survival at acidic pH (169) and successful colonization of the gastric mucosa (65). Most of the reported virulence factors of *H. pylori* are in relation to the membrane because they are 1) secreted, such as urease (170) or the VacA cytotoxin; 2) directly associated with the membrane, such as BabA, HopQ, HopZ, OipA, and SabA OMP; or 3) translocated into infected epithelial cells by the type IV secretion system as is the case for CagA. In addition, many orphan genes of *H. pylori* are believed to be associated with the membrane, *i.e.* putative adhesins, lipoproteins, and other OMPs (30, 31, 64, 67, 171). The identification of proteins that are part of complexes in the *H. pylori* membrane contributes to the elucidation of the membrane function; the challenge is to propose a function for ORFs for which no data are available and, in particular, to identify new virulence factors perhaps hitherto unsuspected. This study suggests a role for some proteins, such as JHP0119 (HELPY_0130), which may play a role in the flagellar function, or isocitrate dehydrogenase, which may have a role in fatty acid biosynthesis.

Some proteomics studies have been carried out to study the membrane of *H. pylori* (69, 120, 139, 142, 172, 173), but few membrane complexes have yet been described (28, 174–177), whereas the yeast two-hybrid method allowed the description of a large set of interactions (135) with a reliability of ~50% (178). Because of their diverse functionality (163–165), membrane proteins provide one of the most important target protein groups for drug design (163, 166). This study allowed the description of 25 and 19 membrane complexes in the B38 and J99 strains, respectively; some of them were found several times. Most of the proteins identified in this study were reported previously to be associated with the membrane (28, 44, 69, 120, 128, 135, 139), validating the membrane sample preparation. Some proteins, such as HopZ and NapA, were reported to be present both in the extracellular compartment (60) and in the membrane (69, 179), indicating the possibility of variable localizations of these proteins. The membrane complexes reported here, comprising numerous proteins involved in *H. pylori* adherence, such as the major adhesins BabA and SabA, the lipoproteins AlpA and AlpB, and numerous porins, are reported to be weakly expressed. In various studies aiming to design a vaccine against *H. pylori*, researchers looked for surface-exposed and/or antigenic proteins (47, 48, 79, 128, 139, 142, 152, 154, 155, 180). Among the antigenic proteins reported in *H. pylori*, most correspond to housekeeping enzymes rather than to antigens associated with the cell envelope (48). Attempts were made to develop a vaccine against *H. pylori* with candidate antigens such as urease (181), catalase (182), and CagA and VacA cytotoxins (183). However, none of these vaccines showed satisfactory protection against the infection. Because the OMP family of *H. pylori* is a very particular family, these OMPs constitute attractive targets for the design of a vaccine. All of the newly characterized OMPs and their interacting partners give new insight into membrane structure. However, a number of the genes encoding these OMPs undergo phase variations in their 5′ region, and therefore, not all strains produce functional proteins (64). To solve this problem, different strategies could be developed. First, conserved regions exist in different *H. pylori* OMPs (64) and could serve as vaccine targets. Indeed, a recombinant protein constructed from a conserved domain of BabA, AlpA, AlpB, and HopZ was shown to be specifically recognized by the patients' sera (184, 185). A second possibility would be to consider a vaccine targeting several OMPs. Indeed, some *H. pylori* OMPs are highly specific to *H. pylori* and would represent potential antigen targets, such as the surface-exposed HorE, HorF, and HopE proteins and their partners, *i.e.* HorC, HorH, and HorL, as well as the BabA-

interacting proteins, *i.e.* SabA, HopM/N, and HopZ. Otherwise, six (HopA, HopE, HopM/N, FrdA, PyrC, and PpiC) of the 14 best candidate antigens to develop a vaccine against *H. pylori* (80) were identified, and their partners could also represent new targets. In addition, the characterization of the functions of individual *H. pylori* OMPs may provide further insight into essential mechanisms for *H. pylori* colonization and persistence in the human gastric mucosa.

With regard to the cytosol, only 46 complexes were identified, whereas 41 were retrieved from the membranes where ~300 proteins are expected to be, showing that two-dimensional BN/SDS-PAGE is better suited to the study of membrane complexes. Most of the proteins identified in the cytosol corresponded to proteins involved in *H. pylori* physiology, *i.e.* glycolysis, tricarboxylic acid cycle, fatty acid biosynthesis, *de novo* purine and pyrimidine biosynthesis, amino acid biosynthesis, catabolic pathway of aromatic compounds, LPS biosynthesis, and translation. Enzymes involved in *H. pylori* metabolism whose structure is very different from their eukaryotic counterparts are very promising targets for the development of new antibacterial molecules. Actually, numerous studies are focusing on such novel targets (99, 100), and it is conceivable to simultaneously target different pathways of bacterial metabolism, a strategy that has remained underexploited in antibacterial molecule development. With regard to *H. pylori*, numerous enzymes are predicted to be involved in metabolism, but few complexes with metabolic enzymes have been reported. This study described such complexes with enzymes involved 1) in the FAS II pathway (FabG, FabI, and FabZ), 2) in the pathway for synthesis of the core lipopolysaccharide (GmhA and ADP-L-glycero-D-mannoheptose 6-epimerase), and 3) in the major pathway for generation of acetyl coenzyme A (the essential pyruvate:flavodoxin oxidoreductase). All of these proteins involved in *H. pylori* physiology and their interacting partners may constitute attractive targets for the design of novel antibacterial agents. These metabolic complexes also involved some proteins whose function is unknown because no counterpart exists in other organisms, such as JH0295 (HELPY_0317) retrieved in association with enzymes involved in the FAS II pathway from both of the strains studied. These proteins deserve full attention and should first be studied in more detail, for example using reverse genetic experiments to determine their implication in the physiology of *H. pylori*.

Conclusions from this study cannot be drawn regarding the pathogenic properties of the strains studied; albeit a hypothesis that two different mechanisms are used by DU- and LG-MALT-associated strains is proposed. In fact, DU strains would be more aggressive via surface expression of certain OMPs via their association in different complexes by mediating adherence to gastric epithelial cells and modulating proinflammatory intracellular signaling cascades. These proteins would therefore be responsible for a strong localized inflammatory response. On the other hand, LG-MALT-associated strains, which seem to be more "insidious" would induce a limited inflammatory response.

CONCLUSION

This study allowed the identification of 329 different proteins of *H. pylori* as well as 49 protein complexes in *H. pylori* strain B38 associated with LG-MALT and 41 protein complexes in strain J99 associated with DU. Twelve of these complexes were common to both strains.

With regard to previously published proteomics comparative studies, this study is the first comparative study of the complexome in *H. pylori* strains. It provides new insight into the membrane and cytoplasm structure that can be used in the design of future molecules for vaccine and/or drug development. Moreover, this is the second study including an *H. pylori* strain associated with LG-MALT (17) and the first comprehensive study of the complexome of an *H. pylori* strain associated with LG-MALT. The resulting availability of the genome of the first *H. pylori* strain associated with LG-MALT should now help pave the way for other studies concerning this very particular cancer.

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