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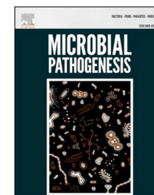

The function of the HtrA protease in maintaining homeostasis of the human pathogen *Helicobacter pylori*

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A B S T R A C T

Helicobacter pylori is a Gram-negative bacterium known to cause persistent infections in humans. HtrA protease is one of the most important secreted virulence factors of this pathogen and is responsible for damaging intercellular junctions between the gastric epithelial cells. Although this protein is regarded as essential in *H. pylori*, very little is known about its function in the bacterial cell physiology. In this work, we attempted to expand the knowledge in this aspect. We found that deletion of the *htrA* gene caused significant alterations in the membrane proteome, including changes in the content of many outer membrane proteins. This translated into modifications in the cell surface properties, affecting the function of the outer membrane as a barrier in the mutant cells. Interestingly, maintaining the homeostasis of cellular envelope was dependent mainly on the chaperone activity; the role of proteolytic activity was far less important in this respect, considering the proteomic data and the surface properties of the bacteria. *In vitro*, HtrA degraded several *H. pylori* proteins, including proteins involved in virulence: oncogenic effector CagA, whose degradation products accumulated in *H. pylori* Δ*htrA* cells, and the iron-regulated OM protein FrpB4.

In conclusion, the results presented in this work underline the very important role of the HtrA protein in the maintenance of the cellular envelope proteostasis in *H. pylori*. Disrupted homeostasis in the absence of HtrA function can significantly impair the physiology of the whole *H. pylori* cell, which may affect its virulent properties.

1. Introduction

The Gram-negative bacterium *Helicobacter pylori* is a persistent pathogen that infects over half of the world's human population. Although most of the time *H. pylori* infections are asymptomatic, they induce a chronic inflammation of the gastric mucosa. As a result, stomach or duodenal ulcers may develop in 5–10 % of cases. Furthermore, *H. pylori* infection leads to development of gastric cancer: adenocarcinomas in approximately 1 % and in less than 0.1 % cases to gastric MALT lymphoma [1]. For this reason, the World Health Organization (WHO) recognized *H. pylori* as the leading cause of gastric cancer and classified it as a type I carcinogen [2].

H. pylori colonizes exclusively the human gastric mucosa and is equipped with various adaptations for long-term existence in the demanding acidic environment. First, it possesses systems for neutralizing the external microenvironment by producing ammonia, mainly via urea metabolism by urease. The spiral shape of the bacterial cell and the presence of flagella enable the bacteria to evade the harsh gastric environment and localize near the surface of gastric epithelial cells. There, specific adhesins allow them to attach to host cell receptors. The

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long-term persistence of *H. pylori* in the host organism is associated with production of numerous virulence factors that can contribute to evasion of the host defense, affect host cell signaling pathways, damage of the intercellular junctions, and influence other processes involved in host-pathogen interactions. The most important secreted virulence factors directly related to alterations of gastric epithelium include cytotoxin-associated gene A (CagA), vacuolating cytotoxin A (VacA), the Neutrophil-activating Protein (Hp-NAP), γ-glutamyl transpeptidase (Ggt), and High temperature requirement protease A (HtrA) (reviewed in Ref. [3,4]).

HtrA is a member of the evolutionary conserved HtrA serine protease family. In Gram-negative bacteria, HtrA homologs localize to the cellular envelope and in some cases are also secreted to the extracellular environment (reviewed in Ref. [5]). These proteins are involved in maintaining cell homeostasis by acting as components of the extracytoplasmic protein quality control system (EPQCS). HtrA proteins are believed to function through two distinct activities, proteolytic and chaperone. They degrade abnormal proteins in the cellular envelope and prevent formation of the protein aggregates. Moreover, they seem to be involved in the periplasmic transit of the outer membrane proteins (OMPs) before their incorporation into the outer membrane (OM) bilayer (reviewed in Ref. [6]). However, the latter function is a subject of debate [7,8].

In pathogenic bacteria, HtrA homologs play important roles related also to virulence. For example, *Borrelia burgdorferi* HtrA mediates maturation of the BB0323 protein which is essential for spirochete infectivity [9]. Secreted HtrAs are direct virulence factors that facilitate bacterial dissemination in the host organism by degrading the extracellular components, including extracellular matrix proteins and/or damaging the cell-to-cell junctions (reviewed in Ref. [5,10]). In case of *H. pylori,* HtrA participates in paracellular migration of bacteria across the epithelial barrier. In particular, it degrades components of the adherence junctions (E-cadherin) [11], tight junctions (occludin, claudin-8) [12], and desmosomes (desmoglein-2) [13]. This process is very important because the CagA is injected into the host cell at the basolateral side, but not at the apical epithelial surface [12]. For this reason, HtrA is regarded as a potential therapeutic target to combat *H. pylori* infections.

While the involvement of HtrA in bacterial infections has been intensively investigated, less is known about functions of HtrA within the *H. pylori* cell. It was demonstrated that bacteria lacking the *htrA* gene (Δ*htrA*) are more sensitive to elevated temperatures, non-physiological pH values, osmotic shock, and antibiotic puromycin, compared to the parental wild type strain [14,15]. Interestingly, the chaperone activity of HtrA is sufficient for *H. pylori* survival under various stress conditions. Bacteria expressing the protease-deficient HtrAS221A variant, which retains chaperone activity, can grow at elevated temperatures (41 °C), in the presence of ionic osmotic stressors, puromycin, metronidazole, or under basic pH conditions, unlike *H. pylori* Δ*htrA*. The proteolytic activity seems to be indispensable only at low pH [14]. However, the importance of HtrA in bacterial cell physiology seems to extend significantly beyond its role in EPQCS, because in the vast majority of the tested *H. pylori* strains it was not possible to delete the *htrA* gene or replace it with the *htrAS221A* variant [16]. The only strain which tolerated *htrA* mutations was *H. pylori* N6 [17] which became a model *H. pylori* strain to study the role of HtrA. What makes the N6 strain so unique has not been explained. Nevertheless, even in this strain, deletion of the *htrA* gene, or its replacement with *htrAS221A*, results in suppressor mutations grouping in the *secA* gene. SecA protein is a key component of the SEC translocon, responsible for protein export from the cytoplasm. It is most likely that mutations in the *secA* gene result in reduced efficiency of protein translocation across the inner membrane and thus reduce concentration of unfolded proteins in the periplasm (and consequently reduce a risk of increased folding stress in the absence of HtrA). The reasons for the essential role of HtrA in *H. pylori* cells remain unclear. Of note, HtrA substrates in bacterial cells have not yet been identified, making it difficult to determine which pathway is impaired by the loss of HtrA function.

There are no data in the literature describing natural HtrA substrates derived from *H. pylori*. In order to address the existing knowledge gap regarding the role of HtrA in the physiology of the *H. pylori* cell, the studies presented in this work were undertaken. The *in vitro* assay revealed that HtrA degrades several *H. pylori* proteins, including some proteins related to virulence. Furthermore, it was observed that the absence of HtrA resulted in alterations to the composition of the OM, consequently leading to its impaired functionality as a protective barrier.

2. Materials and methods

2.1. Plasmids and strains

The strains and plasmids used in this study are listed in Table 1.

2.2. Bacterial growth conditions

2.2.1. Growth in solid medium

H. pylori was cultivated on GC agar (Oxoid, Germany) supplemented with 10 % donor horse serum (Biowest, France), proteose peptone (Oxoid, Germany), 1 % vitamin mix, antibiotic mix: vancomycin (5 μg/ml), polymyxin B (2500 mU/ml), trimethoprim (5 μg/ml) and amphotericin B (4 μg/ml) [18]. Bacteria were incubated for 2 days at 37 °C in anaerobic jars containing microaerophilic gas mixture: 6 % O_2, 7.1 % CO_2, 3.6 % H_2, 83.3 % N_2).

2.2.2. Growth in liquid medium

Bacteria from the solid culture were collected and resuspended in 10–12 ml liquid medium consisting of brain–heart infusion (BHI), 10 % fetal bovine serum (FBS, BioWest), 1 % vitamin mix, antibiotic mix: vancomycin (2.5 μg/ml), polymyxin B (1250 mU/ml), trimethoprim (2.5 μg/ml) and amphotericin B (2 μg/ml). The bacteria were cultured 6–8h at 37 °C with shaking 140 rpm in anaerobic jars containing microaerophilic gas mixture: 6 % O_2, 7.1 % CO_2, 3.6 % H_2, 83.3 % N_2 to OD_{595nm} = 0.5–0.6. Then, new cultures were prepared with a starting OD_{595nm} of 0.05 (volume 12 ml) and carried out overnight, at 37 °C.

2.3. Plasmid construction

The plasmid FrpB4_UZ was constructed using overlap extension PCR cloning according to Ref. [19]. In short, using PCR reaction we added the regions overlapping with plasmid sequence (primers P1/P2)

Table 1
Bacterial strains and plasmids.

Strain/plasmid	Genotype	References/ source
H. pylori N6	Wild type strain	[60]
H. pylori N6 Δ*htrA*	*secAR837K* Δ*htrA*, KanR	[17]
H. pylori N6 Δ*htrA/ htrA wt*	*secAR837K* Δ*htrA/htrAN6*, CmR	[17]
H. pylori N6 Δ*htrA/ htrA S221A (SA)*	*secAR837K* Δ*htrA/htrAN6 S221A*, CmR	[17]
DH5α	*supE44 1lacU169 (φ80 lacZ1M1) hsdR17 endA1 gyrA96 thi-1 relA1*	[61]
E. coli BL21DE3	*F – ompT hsdSB(rB – mB –) gal dcm*	Novagen
E. coli BL21(DE3) pLysS	*F –, ompT, hsdSB(rB, mB–), dcm, gal, λ(DE3), pLysS*, CatR	Promega
pUZN10	pET26b, wt *htrA* from the *H. pylori* N6 strain with C-terminal His6-tag, KanR	[17]
pUZN11	pET26b, *htrAS221A* from the *H. pylori* N6 strain with C- terminal His6-tag, KanR	[17]
FrpB4_UZ	pET26b, *frpb4* (*hp_1512*) from the *H. pylori* N6 strain with C -terminal His6-tag, KanR	This work
Tip-alpha	pQE31, *tip-alpha* (*hp_0596*) from *H. pylori*	[62]

(S_Table 1) to the *frpB4 (HP_1512)* gene (overlapped insert obtained). Genomic DNA from N6 strain was used as a template. Next, using Phusion DNA polymerase we performed PCR reaction with an overlapped insert and circular vector pET26b. The template plasmid was then degraded by the *Dpn*I enzyme (overnight incubation at 37 °C) and the reaction mixture was used to transform *Escherichia coli* DH5α competent cells. The correctness of the obtained constructs was verified by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany).

2.4. H. pylori cell fractionation

H. pylori strains (N6Δ*htrA*, N6Δ*htrA/htrA wt*, N6Δ*htrA/htrA SA*) were cultured in liquid medium to the stationary phase of growth (OD_{595nm} = 0.8–1.0). For the membrane-enriched fractions (MEFs), 50 ml aliquots of the cultures were centrifuged at 4,000×*g* for 10 min at 4 °C and the cells were washed twice with 10 ml of the phosphate saline buffer (PBS). The pellets were then resuspended in 10 ml of PBS and sonicated for 4.5 min (10 pulses of 10 s each at 10 s intervals, amplitude of 15 %). The lysates were centrifuged at 2,000×*g* for 10 min at 4 °C. The pellets were discarded and the supernatants were subjected to ultracentrifugation at 45,000×*g* for 1 h at 4 °C. The pellets were resuspended in 1 ml of PBS containing 10 % glycerol and stored at −80 °C. To obtain total lysates, 10 ml aliquots of liquid cultures were centrifuged at 4,000×*g* at 4 °C and washed twice with 10 ml of PBS. The pellets were then resuspended in 2 ml of PBS with 10 % glycerol and sonicated for 4.5 min by application of 10 pulses of 10 s each at 10 s intervals and an amplitude of 15 %. The lysates were stored at −80 °C. All experiments were performed in triplicate for each strain. Protein amounts were measured using Bradford (RotiQuant, Carl Roth, Germany).

2.5. 2D-DIGE

We performed proteomic analysis of the whole cell lysates of the *H. pylori* N6 Δ*htrA* and *H. pylori* N6 Δ*htrA/htrA S221A* strains and fractions of insoluble proteins (termed "membrane enriched fractions"; MEFs), which contain mainly membrane proteins and protein aggregates. As a control, we used the *H. pylori* N6 Δ*htrA/htrA wt* complementation strain, in which the wild type *htrA* gene was restored to the original locus on the chromosome. This approach allowed us to compare the effect of the lack of HtrA (Δ*htrA*) or its proteolytic activity (*htrAS221A*) on the protein composition of the cell, and to minimize problems in interpreting the results due to the effects of an additional mutation in the *secA* gene (present in all strains used in the experiments) [17]. The whole cell and MEF protein fractions were prepared using the TCA/acetone precipitation method. Bacterial cells and MEFs (obtained as described above) were resuspended in the lysis buffer (1.5 % SDS, 50 mM DTT, pH 8.0) and incubated for 3 min at 97 °C. The cells were then sonicated for 45 s (5 s pulses followed by 5 s intervals, 15 % amplitude). To the bacterial lysates and MEF suspensions cold acetone (100 %, −20 °C) and TCA (100 %) were added in a 1:8:1 ratio and gently vortexed. Protein precipitation was carried out at −20 °C for 1 h. The samples were subsequently centrifuged at 21743 RCF at 4 °C for 15 min. The supernatants were discarded, and the pellets were washed with 1 ml of cold acetone. The samples were centrifuged again, and the pellets were further washed with 3 ml of cold acetone. Excess acetone was removed and the samples were left to air-dry to remove any residual acetone. Prior to 2D DIGE analysis, the samples were resuspended in rehydration buffer (ReadyPrep 2-D Rehydratation/Sample Buffer 1, Biorad) and disrupted using an ultrasonic bath (20 °C, 10–30 min).

2D-DIGE experiments were performed as described in Ref. [20]. Differentially abundant spots were those that: (i) had an average ratio (fold change) ≥1.5 or ≤ -1.5; and (ii) had $P < 0.05$ on Student's *t*-test (FDR applied). The differentially abundant spots were then analyzed using the Extended Data Analysis module for principal component analysis (PCA) and hierarchical clustering. The total and MEF proteomes of mutant strains (*H. pylori htrAS221A* and *H. pylori* Δ*htrA*) were compared with those of the complemented strain, that was used as control.

To identify the proteins of the differentially abundant spots, two 2D-DIGE picking gels (160 μg of membrane/total proteins) were prepared and separated. After Coomassie blue staining, spots of interest were excised, destained overnight with 25 mM ammonium bicarbonate in 50 % acetonitrile, dehydrated with 100 % acetonitrile, dried. Identification of proteins was performed according to the standard in-gel digestion procedure using trypsin [21]. Spectra were recorded on a TripleTOF 5600+ spectrometer (Sciex Framingham, MA, United States) equipped with a Ekspert MicroLC 200 Plus System (Eksigent, Redwood City, CA, United States) on the column ChromXP C18CL column (3 μm, 120 Å, 150 × 0.3 mm). The gradient used during separation was 11–42.5 % B (solvent A 0 % aqueous solution, 0.1 % formic acid; solvent B 100 % acetonitrile, 0.1 % formic acid) in 60 min. Instrument parameters were used as described previously [22]. Protein identification was performed in PeaksStudio software (Bioinformatics Solution Inc.) against the *Helicobacter pylori* database.

2.6. SWATH-MS

The sequential window acquisition of all theoretical mass spectra (SWATH-MS) method was used to quantify proteins. Experiments were performed in a looped product ion mode with the spectrometer set to high sensitivity focus. For the quantitative analysis, a spectral library was created with the group file data processing in PeakView v. 2.2 (SCIEX), with settings described in detail by Lewandowska [23]. A joint search for library generation included all measurements conducted in DDA mode. For database search, ProteinPilot 4.5 software (Sciex) was used, along with the *Helicobacter pylori* database. All files from SWATH experiments were searched against the prepared library. All data were processed in Perseus software [24]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [25] partner repository with the dataset identifier PXD062402 and PXD062510. Cytoscape StringApp was used for the interactome network visualization [26].

2.7. SDS-PAGE, immunoblotting and antibodies

Proteins were separated by SDS-PAGE using 10, 12, or 15 % gels with or without 0.5 % 2,2,2- Trichloroethanol (TCE) (Merck). For the whole cell lysates and MEF analysis, the equal amounts of protein (usually 5 μg) were used as demonstrated in S_Fig.1. Gels with TCE were visualized by the TCE-UV method [27]. The separated proteins were stained by Coomassie Brilliant Blue (Merck) or blotted onto PVDF membrane or nitrocellulose and blocked in 3 % BSA or Rotiblock (Carl Roth, Germany) for 30–60 min. Membranes were incubated with primary antibodies (Table 2) for 2h at room temperature or overnight at 4 °C, washed and incubated with rabbit or mouse HRP-coupled secondary antibodies (Table 2) for 1.5 h at room temperature or overnight at 4 °C. The antigens were visualized with ECL prime solution (Amersham™, MedChemExpress) using an BioRad imaging system (ChemiDoc XRS, BioRad) or Radiance Plus (#AC2103, Azure Biosystems, USA) using Azure c400 imaging system (Azure Biosystems, USA).

2.8. Cleavage assays

The *H. pylori* HtrA protein variants were expressed and purified exactly as described previously [15,28].

For the whole *H. pylori* cell lysate cleavage assay, we used a strain lacking the *htrA* gene to preserve potential substrates of this protease. Since HtrA is a protein that protects cells against the effects of exposure to low pH and elevated temperature [14,15], incubations were performed both in conditions considered physiological and in the above-mentioned stress conditions. In particular, 50 μg of the whole N6 Δ*htrA* lysate were incubated with 5 μg of purified wt HtrA in buffers

Table 2
List of antibodies used in this study.

Antigen	Type	Dilution	Source
anti-CagA-N-term	rabbit polyclonal	1:2500	[63]
anti-CagA-C-term	rabbit polyclonal	1:25 000	[63]
anti- $HtrA_{Hp}$	rabbit polyclonal	1:10 000	[17]
anti-HisTag	mouse monoclonal	1:2500	#66005, ProteinTech
anti-Tip-alpha	rabbit polyclonal	1:500	[62]
anti- HP_1350	rabbit polyclonal	1:10 000	A gift from the laboratory of Prof. S. Wessler
anti- HP_1012	rabbit polyclonal	1:10 000	A gift from the laboratory of Prof. S. Wessler
anti- HP_0657	rabbit polyclonal	1:10 000	A gift from the laboratory of Prof. S. Wessler
Secondary antibody anti-rabbit	Goat polyclonal	1:10 000	#31462, Thermo Fisher Scientific
Secondary antibody anti-mouse	Goat polyclonal	1:10 000	#31446, Thermo Fisher Scientific

containing 200 mM NaCl, 50 mM HEPES pH 6.2 or 200 mM NaCl, 50 mM sodium phosphate pH 5.0 (total 50 μl reaction mixture) for 2 h at 37 or 50 °C. The reactions were stopped by adding 50 μl of 2x Laemmli buffer and heating (10 min, 95 °C).

For CagA cleavage assay, 50 μg of the whole N6 Δ*htrA* lysate were incubated with 1 μg of HtrA (wt or S221A) in buffers containing 100 mM NaCl, 25 mM HEPES pH 6.2 or 100 mM NaCl, 25 mM acetate pH 5.0 (total 100 μl reaction mixture) for 16 h at 37 °C. The reactions were stopped by adding 25 μl of 4x Laemmli buffer and heating (10 min, 95 °C). The aliquots of 25 μl of each sample were resolved by 12 % SDS-PAGE and subjected to western blotting using anti- CagA-N-term or anti-CagA-C-term antibodies.

For FrpB4 cleavage assay, 50 ml liquid culture of *E. coli* BL21(DE3) pLysS carrying the plasmid FrpB4_UZ was grown at 37 °C in Luria-Bertani (LB) broth supplemented with 50 μg/ml kanamycin to OD_{600} 0.6. The expression of *frpb4* was induced for 3 h with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were centrifuged (10 min, 5,000×*g*) and the pellet was suspended in 25 mM HEPES 7.5, 100 mM NaCl, 20 % glycerol. Cells were lysed by sonication and stored at −80 °C. The 30 μg of FrpB4 lysate were incubated with 1.5 μg of HtrA (wt or S221A) in 100 mM NaCl, 25 mM HEPES pH 6.2 (total 120 μl reaction mixture). Sample aliquots (20 μl) were incubated for 0, 2, 4, and 6 h at 37 °C; the reactions were stopped by adding 5 μl of 4x Laemmli buffer. The proteins were separated using SDS-PAGE in 8 % gel. The Western blot analysis was performed using anti-HisTag and anti- $HtrA_{Hp}$ antibodies. The levels of FrpB4 were analyzed densitometrically.

2.9. Quantitative real time PCR (qPCR) analysis

To evaluate gene expression under heat-shock and non-stressful conditions, bacteria (N6Δ*htrA*, N6Δ*htrA/htrA wt*, N6Δ*htrA/htrA SA*) were grown as described in Ref. [29] to OD 0.5–0.6. The cultures were divided into two parts (5 ml each); one was incubated at 37 °C, the second at 42 °C for 30 min. RNA isolation and reverse transcription were performed as described in Ref. [30]. The qPCR assays were done according to Ref. [29] with the use of specific primers for the genes *groEL, fecA1, fecA2, fecA3, frpB2, frpB4, sodB, amiE, ureA, ureF, ceuEI, ceuEII, nikR* or *fur* (S_Table 2). Data analysis was done using the ΔΔCt method, in which the 16S rRNA gene was used as internal reference for data normalization, according to Ref. [31].

To evaluate *cagA*, *slt*, *omp19* and *homC* gene expression, the liquid cultures of *H. pylori* strains (N6Δ*htrA*, N6Δ*htrA/htrA wt*, N6Δ*htrA/htrA SA*) were prepared as described in 2.2.2 to obtain OD_{595nm} of 0.5–0.6. RNA isolation, reverse transcription and quantitative real-time PCR were performed as described in Ref. [32]. The characteristics of the primers are shown in S_Table 2. To determine the level of the genes of interest, the stability of seven reference genes (seven biological replicates for each gene) validated for different bacterial species was examined: *era*, *gyrA*, *gyrB*, *dnaG*, *ftsZ*, *adk*, and *gmk* [33]. The stability of the reference genes was evaluated using geNorm (version 2020). The following gene pairs that showed the highest stability among the tested strains were selected for normalization: for N6Δ*htrA* and N6Δ*htrA/htrA wt*: *adk/gyrB*, for *N6ΔhtrA/htrA SA* and N6Δ*htrA/htrA wt*: *adk/dnaG*. The Pfaffl ΔΔCT method with correction for PCR efficiency was used to assess the relative expression of target genes. Statistical analysis was conducted using the REST2009 software (v. 2009, Qiagen, Hilden, Germany).

2.10. Surface hydrophobicity test

2.10 Surface hydrophobicity test on N6Δ*htrA*, N6Δ*htrA/htrA wt*, N6Δ*htrA/htrA SA* was performed exactly as described in Ref. [34].

2.11. Susceptibility to low concentrations of sodium dodecyl sulfate (SDS)

The *H. pylori* cultures in logarithmic growth phase were centrifuged 2370×*g*, 10 min. The bacterial pellets were washed twice with PBS and settled by centrifugation 10 min at 2370×*g*. Bacteria were resuspended in PBS buffer so that of the suspension (OD_{595nm}) was 0.8. To 937.5 μl of each bacterial suspension, 62.5 μl of 0.1 % SDS was added. OD_{595nm} measurements were performed for 10 min at 1-min intervals. The degree of detergent-induced cell lysis was determined by the decreasing optical density (OD_{595nm}).

To test the ability to grow on solid medium in the presence of SDS, liquid cultures of *H. pylori* strains in the logarithmic phase of growth were brought to OD595nm = 0.35 using BHI liquid medium. Serial dilutions of 10^{-1} to 10^{-5} were prepared. 5 μl each of the bacterial suspension were spotted onto GC agar solid medium supplemented with 0.01 % SDS. The cultures were incubated under controlled atmospheric conditions (6 % oxygen, 7.1 % carbon dioxide, 3.6 % hydrogen, 83.3 % nitrogen) in jars at 37 °C for 72 h and the number of grown colonies (CFU) was counted.

3. Results

3.1. HtrA degrades H. pylori cell lysate proteins under both stressful and non-stressful conditions

To get preliminary information about which proteins can be degraded by HtrA, we treated the *H. pylori* lysate with recombinant HtrA protein. In the electropherogram shown in Fig. 1, there are several protein bands that disappeared after incubation with HtrA. As expected, the most intense degradation occurred under stressful conditions (low pH or elevated temperature). However, under non-stress conditions, a group of proteins was also digested efficiently. We selected 10 protein bands whose intensity changed under all conditions used and identified their content by LC-MS; the results are presented in Table 3. Since most of the bands contained more than one protein, only these that were represented by the largest number of peptides and the highest coverage of sequence are included in the list. The full list of identified proteins can be found in the supplementary data (Supplementary file mmc3).

The majority of identified proteins are cytoplasmic enzymes involved in basic metabolic processes. These include, among others: proteins related to translation (Tuf, Efp, RpsD, LeuS, FusA), central carbon metabolism and cell energy (AcnB, PorB, AtpD, FixO), and other cytoplasmic metabolic processes (HyuA, AcxA, HugZ). However, some of the identified proteins are directly related to bacterial virulence. Among them CagA, one of the main virulence factors of *H. pylori*, deserves special attention. The presence of CagA was detected mainly in two bands (1, 2), representing proteins with masses of approximately 135

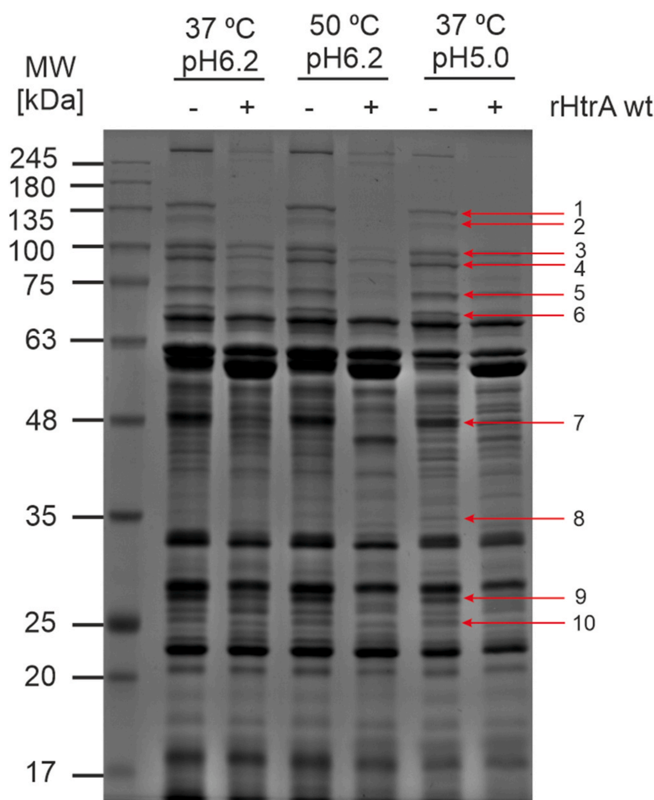

Fig. 1. HtrA degrades selected proteins in the *H. pylori* cell lysate. The bacterial lysates derived from the N6 Δ*htrA* strain were incubated with or without recombinant proteolytically active HtrA (rHtrAwt) under conditions marked at the top of the figure. The arrows indicate protein bands whose intensity decreased or disappeared in the presence of HtrA.

kDa. CagA was also detected in bands 4 and 5 (Sup_File 1) but in this case contribution of other proteins was more pronounced. Furthermore, the excised bands contained also extracytoplasmic proteins, including several OM proteins (FrpB4, Omp31, MetQ, HpaA).

3.2. *The mutations in the htrA gene cause alterations in the H. pylori proteome*

To verify these findings and identify additional substrates, we used two-dimensional difference gel electrophoresis (2D DIGE) to further analyze the impacts of the absence of HtrA or the lack of its proteolytic activity.

A total of 76 protein spots that had protein abundance ratios above a 1.5-fold cutoff were identified (SFig. 2 and supplementary file mmc4). With the exception of 5 spots (231, 377, 407, 592 and 619) with very low protein content, spots of interest were excised and subjected to LC-MS identification. As shown in the supplementary files mmc5 and mmc6, in many cases the spots contained more than one protein, and the contribution of individual proteins to the spots appeared to be comparable. Therefore, it was difficult to assess which specific protein was more or less abundant in the *htrA* mutant. In some cases, the identified peptides provided very low coverage of the protein sequence (or only one peptide was identified). For these reasons, further analyses were performed only for those spots that could be clearly assigned to a protein/polypeptide or the proteins identified in a single spot were closely functionally related (as in the case of FlaA and FlaB) (Table 4). The full list of the identified proteins is present in the supplementary data (mmc6).

As presented in Table 4 and Fig. 2, the majority of proteins with altered levels were cytoplasmic components involved in housekeeping and basic metabolic functions. Chaperones (GroEL-GroES system) and proteins protecting against the effects of oxidative stress (KatA, AhpC, MsrA, Tpx, Trx2, FtnA, SodB) were significant contributors. Further, the proteins associated with the basic processes of the cell should be distinguished, such as central carbon metabolism, nitrogen metabolism, translation, transcription. Some extracytoplasmic proteins were identified as well, including OMPs (Omp11, Omp21). Interestingly, proteins associated with *H. pylori* virulence were also present: CagA, proteins related to motility and chemotaxis, Ggt and Tipα. However, there was a large dispersion of values for measurements relating to the latter protein (see supplementary file mmc4). We therefore verified the Tipα protein content of the tested strains using the western blotting method. As shown in SFig. 3, the absence of HtrA or its proteolytic activity did not significantly affect Tipα levels in *H. pylori* cells (neither in the total protein fraction nor in the membrane-enriched fraction). The protein was also not degraded by HtrA in an *in vitro* assay.

In the case of *H. pylori* N6 Δ*htrA* mutant cells, levels of most of the identified proteins were reduced compared to the control strain. In the whole cell protein fractions only the levels of UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase (LpxD) and CagA increased, while in the MEF – of Omp20, KatA, RpsQ, Ndk, and CagA. In the Δ*htrA/htrA S221A* mutant, no such tendency was observed and many extracytoplasmic proteins seemed to be stabilized in the presence of the proteolytically inactive variant of HtrA (in particular the OM proteins: Omp11, Omp20 and Omp21, as well as CagA). At least some spots contained truncated CagA protein variants. These include spots 22, 32, and 80 with positions in the gel corresponding to masses ranging between 75 and 100 kDa (while the MW of the full length CagA is approximately 130 kDa). The peptides identified by LC-MS were localized in the N-terminal and central part of the protein, while no peptide from the C-terminal part of CagA was found (SFig. 4). This implies that CagA becomes truncated at the C-terminus and HtrA degrades the remaining truncated forms of the protein.

Since membrane proteins, especially OMPs, are not well separated by two-dimensional electrophoresis, we compared the proteomes of the insoluble fractions using SWATH LC-MS. As can be seen in Fig. 3 and S_table 3, the contents of many proteins with known extracytoplasmic localization, and therefore potentially colocalizing with HtrA, were found to be altered. These include typical integral β-barrel OMPs (Omp2, Omp4, Omp10, Omp11, Omp15, Omp19, Omp29, Omp31, OmpP1, HofE, HomC, FecA3, BamA), as well as OM-associated proteins (HP_0746, HpaA, HP_0657), type 4 secretion system proteins (CagT, Cag8 and Cag16), flagellin proteins (FlaA and FlaB) and periplasmic proteins potentially associating with membranes or the cell wall (e. g. ComH, Ggt, HP_0977, HP_0657, Slt). In the samples from the Δ*htrA* cells, the OM proteins were downregulated in most cases compared to the control. The proteins with the most reduced levels were Omp19 (more than tenfold), FecA3 and FlaA. In contrast, a strongly increased abundance of several periplasmic proteins was observed: Slt (more than tenfold), ComH, HP_0657 and one OM protein, HomC.

3.3. *CagA is efficiently degraded by HtrA in vitro*

The results of the experiments presented so far indicate that CagA may be a substrate for the HtrA protease. To verify this possibility, we performed an experiment in which the whole cell extract of *H. pylori* N6 Δ*htrA* was incubated with purified recombinant HtrA variants. Indeed, CagA was efficiently degraded by wt HtrA, but not by proteolytically inactive variant HtrAS221A (Fig. 4). CagA degradation occurred with similar efficiency at pH = 6.2 and acidic pH (pH = 5.0) (Fig. 4A and B). Interestingly, at pH = 6.2, the presence of HtrAS221A seemed to slightly stabilize CagA in *H. pylori* cell lysates, which may indicate that CagA is also slowly degraded by another cellular protease. Cleavage efficiency of CagA clearly correlated with the amount of HtrA used. Moreover, use of two types of anti- CagA antibodies (anti-N-terminal part and anti-C-terminal part) indicated that CagA degradation occurred mainly from

Table 3
List of proteins identified in the bands selected in the Fig. 1.

Protein	UniProt accession number	Localization	Function/activity	Sequence coverage (%)
Band nr 1				
CagA	A0A0N9H5Q1	Cytoplasm;	T4SS oncogenic effector	11.32
Band nr 2				
CagA	A0A223ZIG6	Cytoplasm;	T4SS oncogenic effector	12.0
CarB	D7FDD0	Cytoplasm	Carbamoyl phosphate synthesis	11.6
Band nr 3				
AcnB	I0ENW9	Cytoplasm	tricarboxylic acid cycle	16.76
SecA	T2SHS9	Cytoplasm	Protein export	16.7
CheA	I9VRV5	Cytoplasm	Chemotaxis	9.29
Band nr 4				
FrpB4, HP_1512	J0IQT0	OM	Iron uptake	32.79
ClpB	P71404	Cytoplasm	Chaperone	28.5
LeuS	K2KIC5	Cytoplasm,	Translation	27.9
Band nr 5				
FusA	J0L5Z5	Cytoplasm	Translation	57.23
HyuA, HP_0695	O25402	Cytoplasm	Amino acid metabolism	46.84
AcxA	N4TBT0	Cytoplasm	Acetone carboxylase	45.84
FlgE	A0A4Y4XI34	Membrane	Flagellar hook protein	39.37
Band nr 6				
MetQ	A0AAD1D9Q0	OM	Lipoprotein; unknown	55.7
HpaA	Q9ZL47	OM	Flagellar sheath lipoprotein	46.54
DnaK	A0A438ZKJ7	Cytoplasm	Chaperone	46.29
HP_1334	O25892	unknown	unknown	44.20
SpoOJ, ParA	HPG27_1084	Cytoplasm	chromosome partition regulation	43.56
FixO, HP_0145	O24956	Membrane	Cytochrome *c* oxidase	43.53
Band nr 7				
Tuf	G2MFE7	Cytoplasm	Translation, elongation factor Tu	84.0
AtpD	M7SV05	Membrane	ATP synthase subunit beta	76.2
GroEL	Q70NT7	Cytoplasm	Chaperonin	68.9
FtsZ	K2K8S2	Cytoplasm	Cell division	68.6
PorA	K2KVM8	Cytoplasm	Pyruvate synthesis	60.7
GroEL	Q0PXQ6	Cytoplasm	Chaperonin	55.86
Band nr 8				
Tuf	T2T3D9	Cytoplasm	Translation, elongation factor Tu	16.6
Band nr 9				
HugZ	A0A2A6U8C4	Cytoplasm	Heme iron utilization protein	59.76
HpaA	Q9ZL47	OM	Flagellar sheath lipoprotein	46.54
MetQ	A0A086RRV8	OM	Lipoprotein; unknown	46.13
FixO, HP_0145	N4TRE0	Membrane	Cytochrome *c* oxidase subunit	43.53
Band nr 10				
UreG	K2LMU0	Cytoplasm	Regulation of pH	48.74
RpsD	K2KRU9	Cytoplasm	Translation	46.15
Efp	M3P857	Cytoplasm	Translation	41.18
Omp31; HP_1469	A0AAW9KQN2	OM	Unknown	36.69

The protein names (HP_XXXX) are according to nomenclature of the *H. pylori* 26695 strain.

the C-terminus (as judged from the number and intensity of the degradation products bands) (Fig. 4C and D).

When compared to the complemented strain, the expression level of the *cagA* gene was increased approximately 2.5 fold and 2 fold in the Δ*htrA* and *htrAS221A* cells, respectively (Fig. 5A). The observed differences in *cagA* expression were in agreement with a slightly increased protein content in the mutant and control *H. pylori* cells (Fig. 5B and C). Therefore, it cannot be unequivocally concluded that it is the lack of proteolytic activity that triggers the accumulation of full-length CagA in *htrA* knockouts; rather, it is a consequence of elevated *cagA* gene expression. Nevertheless, some effect of CagA stabilization by HtrAS221A is apparent, as in CagA digestion assays shown above (Fig. 4B).

3.4. HtrA affects the level/stability of some proteins involved in metal homeostasis, peptidoglycan metabolism, and the OM components in H. pylori

The results shown in this work suggest that HtrA may be involved in regulation of some proteins engaged in uptake of metal ions. First, we found that HtrA possibly can degrade FrpB4. However, the DIGE and MS data did not indicate the altered amounts of this protein in membranes of the mutant cells. To clarify these doubts, we examined the digestion of FrpB4 in an *in vitro* system. Indeed, we found that the recombinant FrpB4 protein produced in *E. coli* cells is partially degraded by HtrA (Fig. 6A and B). To test whether the unaltered level of FrpB4 protein could be the result of a compensatory regulation at the transcriptional level of its gene *HP_1512*, we examined the *HP*_1512 mRNA level. As shown in Fig. 6E, the mRNA level of the *HP_1512* gene was not altered by mutations in the *htrA* gene under both physiological and stress conditions (heat-shock, where the HtrA proteolytic activity is particularly important). The expression level of the GroEL gene (Fig. 6C) was used in these experiments to confirm stress conditions at elevated temperatures. Thus, it can be concluded that in the case of FrpB4, the most likely role of HtrA is to remove denatured forms of this protein that cannot be incorporated into the OM -.

Next, the MS analysis showed a significant decrease in the FecA3 (HP_1400) level in the MEFs derived from the *H. pylori* Δ*htrA* cells. In this case, we expected that the observed effect was rather indirect and perhaps related to a change in the *HP_1400* gene expression levels. Indeed, the quantitative qPCR experiment unambiguously showed a large decrease in the mRNA level of the *HP_1400* gene in the Δ*htrA* cells (Fig. 6D). To test whether the lack of HtrA function could affect the expression levels of other genes related to metal transport and metalloregulation, we examined the levels of selected genes whose products are involved in these processes: *ureA*, *ureB*, *fecA1*, *fecA2*, *frpB2*, *frpB3*,

Table 4
Proteins with altered levels in the *H. pylori* cells deprived of the *htrA* gene (Δ*htrA*) or expressing the proteolytically inactive variant HtrAS221A (*htrA S221A*).

Uniprot accession number	Protein name	Spot number	Fold change			
			Δ*htrA* vs contr	*htrA S221A* vs contr	Δ*htrA* vs contr MEF	*htrA S221A* vs contr MEF
Omps and periplasmic proteins						
A0A438UEP5	Neuraminyllactose-binding hemagglutinin; HpaA-like (HP0492)	**412**				0.68
K2L579	HP1173	**575**		0.657		
A0AAD1DBL6	Omp11; HP0472	**605**		1.74	0.49	
C7BXG8	Omp20; HP0912	**260**			1.96	1.64
A0A496H542	Omp21; HP0913	**230**		2.2		
A0AAW9KR34	Omp21; HP0913	**222**		2.61		
A0A438XPP2	Gamma-glutamyltransferase; Ggt	**524**		1.47		
A0A2T6UZZ4	Tipα; Tumor necrosis factor alpha-inducing protein HP0596;	**569**	0.609			2.11
Motility						
Q17W42 T2T144	FlaA; Flagellin A (HP0601) and FlaB; Flagellin B (HP0115)	**174**	0.558		0.4	
Q17W42 T2T144	FlaA; Flagellin A (HP0601) and FlaB; Flagellin B (HP0115)	**169**	0.595		0.38	
Q17W42 T2T144	FlaA; Flagellin A (HP0601) and FlaB; Flagellin B (HP0115)	**136**			0.56	
Q17W42 T2T144	FlaA; Flagellin A (HP0601) and FlaB; Flagellin B (HP0115) HP0115 flaB	**164**	0.645		0.43	
Q17W42 T2T144	FlaA; Flagellin A (HP0601) and FlaB; Flagellin B (HP0115)	**165**			0.41	
Q17W42 T2T144	FlaA; Flagellin A (HP0601) and FlaB; Flagellin B (HP0115)	**174**			0.4	0.54
T2SCD3	Flagellar motor switch protein FliY	**375**	0.349	0.357		
Chaperones						
A0A2L2I3X9	GroL; 60 kDa chaperonin	**147**		0.64		
A0A2L2I3X9	GroL; 60 kDa chaperonin	**155**		0.65		
A0A2L2I3X9	GroL; 60 kDa chaperonin	**715**		0.65		
Q6A0V6	GroS; 10 kD chaperonin HspA	**639**	0.446	0.57		

Q6A0V6	GroS;10 kD chaperonin HspA	**652**	0.469	0.48		
Q6A0V6	GroS;10 kD chaperonin HspA	**634**		0.67		1.77
Protection against the effects of oxidative stress						
A0A439CBM7	KatA; Catalase	**213**	0.645	1.56	0.49	1.67
A0A439CBM7	KatA; Catalase	**221**		1.53	0,56	
K2KZC4	AhpC; Alkyl hydroperoxide reductase; HP_1563	**483**	0.429	0.45		
C7BY63	Peptide methionine sulfoxide reductase MsrA	**280**	0.67	0.57		
C7BY63	Peptide methionine sulfoxide reductase MsrA	**283**	0.68	0.55		
A0A438QHH8	Thiol peroxidase Tpx HP0390	**561**	0.67	0.65		
A0A438QHH8	Thiol peroxidase Tpx HP0390	**588**	0.66	0.67		
K2KQD4	Thioredoxin-2; Trx2; HP1458	**688**				1.49
A0A5M8VV77	TrxB; HP1164; Thioredoxin reductase	**343**			0.63	
T2SV99	FtnA; Pfr; Ferritin (HP0653)	**544**	0.609		2.82	5.44
A0A439C9A2	SodB; Superoxide dismutase (HP0389)	**484**	0.375	0.52		
Amino acid metabolism						
T2SNK7	MetK; S-adenosylmethionine synthase	**711**	0.623	0.64		
A0A2T6VA74	Aspartate ammonia-lyase AspA	**244**	0.565	0.63		
Translation						
T2SB68	RpsQ; 30S ribosomal protein S17	**614**		0.67	3.19	1.89
K7Y954	Ndk; nucleoside diphosphate kinase; HP0198	**630**		0.67	2.56	1.93
T2SB68	RpsQ; 30S ribosomal protein S17	**648**		1.54		
T2SB68	RpsQ; 30S ribosomal protein S17	**661**		1.49		
Central carbon metabolism						
Q8VL20	Fba; Fructose-bisphosphate aldolase	**394**	0.57	0.39		
Q8VL20	Fba; Fructose-bisphosphate aldolase	**404**	0.473	0.39		
A0A1V3AZ93	FldA; Flavodoxin	**624**		2.38		
V6L8C1	Pgk; Phosphoglycerate kinase	**300**	0.565	0.42		
T2SKF3	Icd; Isocitrate dehydrogenase [NADP]	**276**	0.311	0.46		
Other						

A0A438VMW6	CagA	**32**	1.52	1.56	1.62	
A0A438VMW6	CagA	**22**	1.67			
A0A438VMW6	CagA	**80**			1.88	2.27
A0A438Y1I0	HslV; ATP-dependent protease subunit;	**533**		1.54		
Q1CS63	NusG; Transcription termination/ antitermination protein; HP1203	**518**		1.5		
A0A496G630	AmiE; Aliphatic amidase	**310**	0.54		0.56	
A0A7K1P0I1	FabF; 3-oxoacyl-[acyl-carrier-protein] synthase 2; HP0558 – fatty acid biosynthesis	**274**		1.55		
T2SDU6	LpxD; UDP-3-O-(3-hydroxymyristoyl) glucosamine N-acyltransferase	**308**	2.06	2.08		

As a control (contr) Δ*htrA/htrA wt* strain was used. Protein abundance ratio was calculated relative to the complementation strain. The membrane enriched fractions are denoted with "MEF". A decrease in protein relative abundance is marked in green, an increase in red.

ceuE1, ceuE2, amiE, sodB, fur, and nikR. Of all these genes, only *fur* showed slightly decreased expression in the Δ*htrA* cells but only at elevated temperature (SFig. 5). Therefore, the downregulation of the *HP_1400* gene expression in the Δ*htrA* mutant probably occurs in a manner that is independent of the levels of the master regulators of metal metabolism, Fur and NikR.

To test whether the significantly altered abundance of other extracytoplasmic proteins correlates with the expression levels of the genes encoding them, we performed qPCR analysis for *HP_0896, HP_0645* and *HP_0373,* encoding Omp19, Slt and HomC proteins, respectively. While the decrease in expression of the *HP_0896* gene was in agreement with the very low levels of Omp19 in the Δ*htrA* mutant, the results were different for Slt and HomC. We observed that *HP_0645* gene expression remained unchanged, while it decreased in the case of *HP_0373* (Fig. 7). This result suggests that Slt and HomC proteins are stabilized in the *H. pylori htrA* mutants.

3.5. The lack of HtrA affects the properties of the outer membrane

Since the results presented above show significant differences in the periplasmic and OM protein composition in the *H. pylori htrA* mutant strains versus those of the complementation strain, it is reasonable to assume that the membranes of the mutant cells exhibit properties different from those in the wild-type strain. To confirm this hypothesis and compare the *H. pylori* cell surface properties, surface hydrophobicity and susceptibility to ionic detergents were tested. Surface hydrophobicity is a parameter, which is dependent on the composition and structure of the OM. It can be determined by measuring bacterial adherence to hydrocarbons (BATH), which results in the formation of cell aggregates. In this work, the adhesion ability of *H. pylori* Δ*htrA* or *H. pylori* Δ*htrA/htrA S221A* cells to n-hexadecane was tested. Bacteria of the parental strain N6 and complementation strain were the controls. In addition, a control experiment without hexadecane was performed to account for autoagglutination and cell lysis due to centrifugation [34]. A reduction in the ability to adhere to hexadecane was observed only for bacteria of the strain lacking the *htrA* gene. In contrast, no differences were found between the other strains (Fig. 8A). This suggests that it is the chaperone activity and not the proteolytic activity of HtrA that plays a more important role in ensuring the appropriate cell surface properties of *H. pylori*.

Low concentrations of the ionic detergent SDS affected differently the ability of individual bacterial strains to form colonies on solid media. A statistically significant reduction in the number of colony-forming units of the *H. pylori N6* Δ*htrA* strain was observed relative to the *H. pylori* Δ*htrA/htrA SA* strain and the complementation strain in the presence of 0.01 % SDS (Fig. 8B). Experiments evaluating SDS-induced cell lysis showed a significant difference between the strain lacking the *htrA* gene and the wild-type and complement strains (Fig. 8C).

3.6. Content of some periplasmic proteases is elevated in the H. pylori htrA mutant cells

The HtrA protein is regarded as one of the most important components of EPQCS. Therefore, it can be expected that there will be an increase in the levels of other proteins in the *htrA* mutants that can potentially replace or compensate for the lack of HtrA function.

The SWATH LC-MS data indicated that the periplasmic protein HP_0657 content was significantly increased in the MEFs. This protein is a component of the proteolytic complex with HP_1012 metalloprotease [35]. The HP_1012 protein and the other periplasmic protease HP_1350 were not detected in our proteomic analyses. Therefore, we performed western blotting analysis of the whole cell extracts and MEFs of the *H. pylori htrA* mutants and the control strains using anti-HP_0657, anti-HP_1012 and anti- HP_1350 antibodies. We detected that the MEFs derived from the mutant bacteria indeed had higher levels of both components of the proteolytic complex, namely HP_0657 and HP_1012. In contrast, the content of the protease HP_1350 did not change in the absence of active HtrA. The picture was slightly different in the whole-cell lysate; there were higher levels of HP_1012 and HP_1350 proteins in the *htrA* mutants, while the content of HP_0657 was unchanged (Fig. 9).

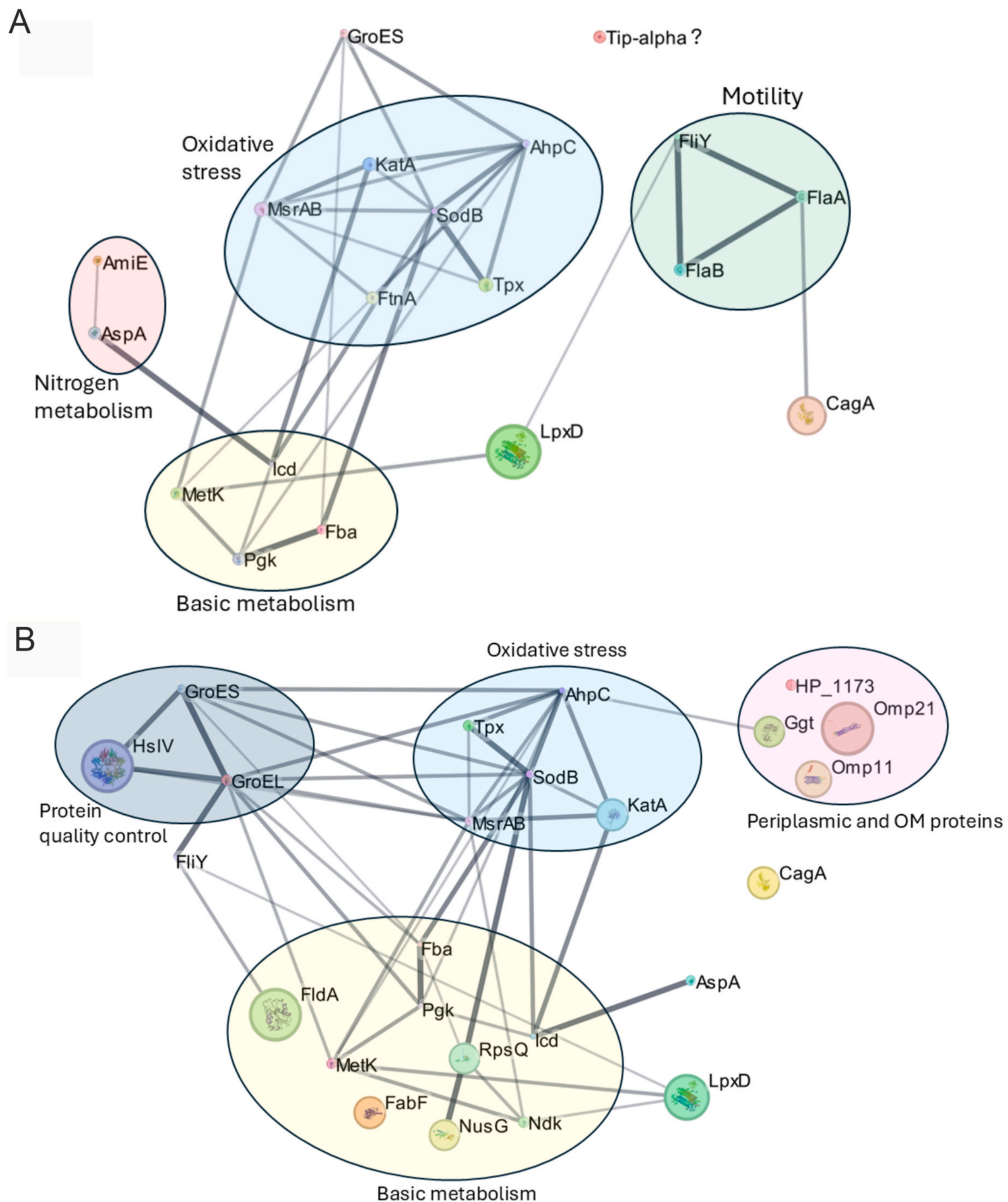

Fig. 2. Possible protein networks affected by a lack of the HtrA function according to DIGE analysis, analyzed in STRING and Cytoscape (version 3.10.3). A: proteins differently populated in the *H. pylori* N6 Δ*htrA* strain (Δ*htrA*/complemented protein ratios); B: panel: protein differently populated in the *H. pylori* N6 Δ*htrA*/*htrA S221A* strain (*htrAS221A*/complemented protein ratios). The increase or decrease in individual protein content is depicted by the larger or smaller diameter of the protein circles. The question tag at the Tip-alpha denotes a lack of certainty regarding the increase in protein levels in the mutant cells.

4. Discussion

The success of a pathogen in colonizing a host organism is dependent on both production of appropriate virulence factors and maintenance of cell homeostasis in a hostile environment during infection. HtrA family proteins are involved in both processes. Experimental data indicate diverse functions of HtrA in the cellular envelope of Gram-negative bacteria, requiring both proteolytic and chaperone activity of this protein. As a protease, HtrA can degrade misfolded proteins and thus prevent the occurrence of inappropriate protein-protein interactions and/or the formation of protein aggregates. In addition, it may facilitate the maturation of extracytoplasmic proteins [36–38]. The chaperone activity of HtrA prevents the formation of large aggregates of denatured proteins (holdase function) under stress conditions, while under physiological conditions HtrA can assist in the periplasmic transit of proteins into the OM and out of the cell [36,39]. Of all these potential functions, only a role of holdase has been demonstrated for *H. pylori* HtrA, albeit only in an *in vitro* system against a model substrate, lysozyme [14,40].

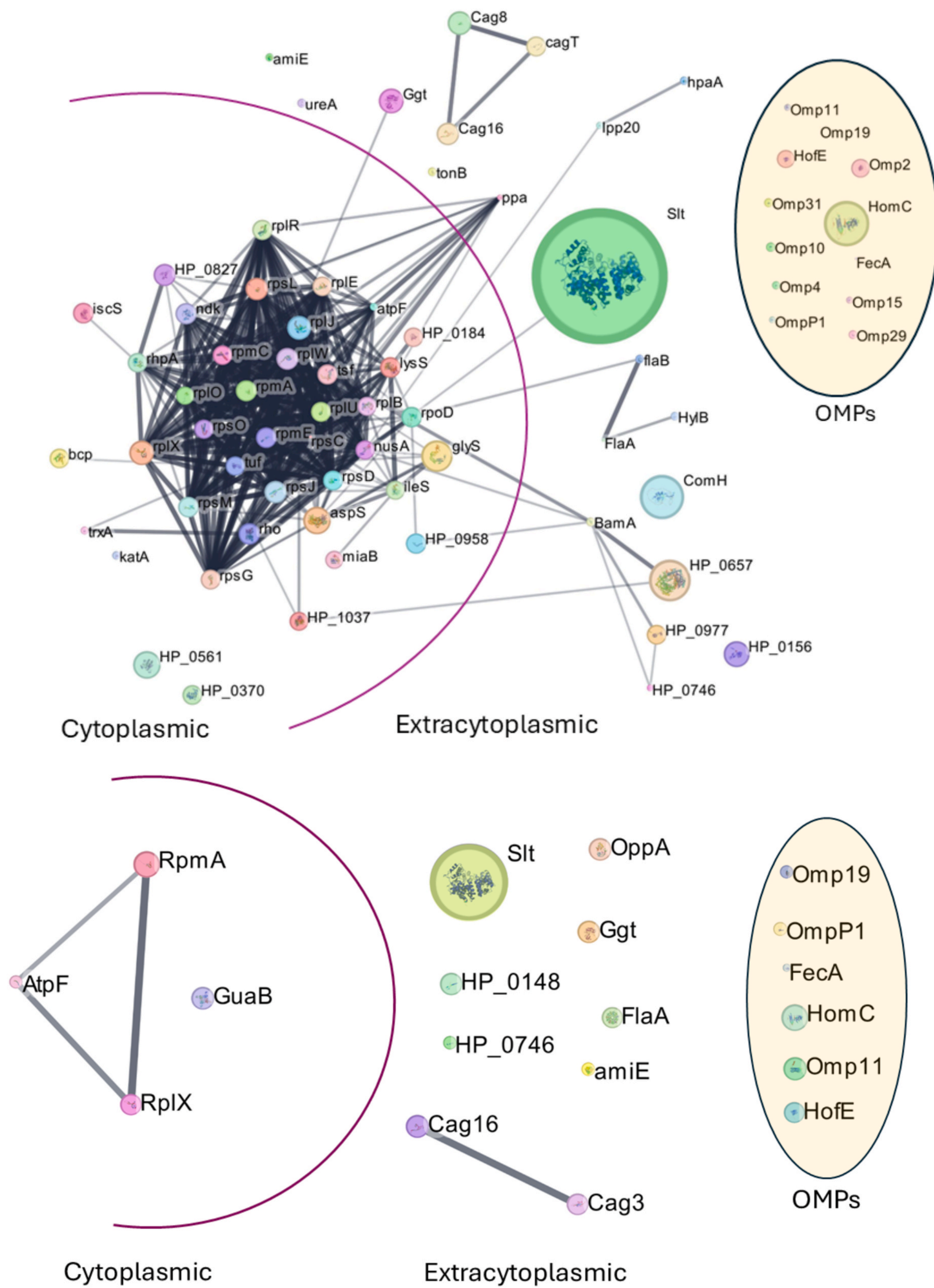

Fig. 3. Proteins differentially populated in the membrane enriched fraction of the *H. pylori N6 ΔhtrA* (upper panel) or *H. pylori N6 ΔhtrA/htrA S221A* (lower panel) compared to the complemented *H. pylori* strain. The analysis was performed using STRING (version 12) and Cytoscape (version 3.10.3). The outer membrane proteins are grouped in yellowish ellipse. Proteins with established cytoplasmic or extra-cytoplasmic localization have been separated by a purple line. The increase or decrease in individual protein content is depicted by the larger or smaller diameter of the protein circles.

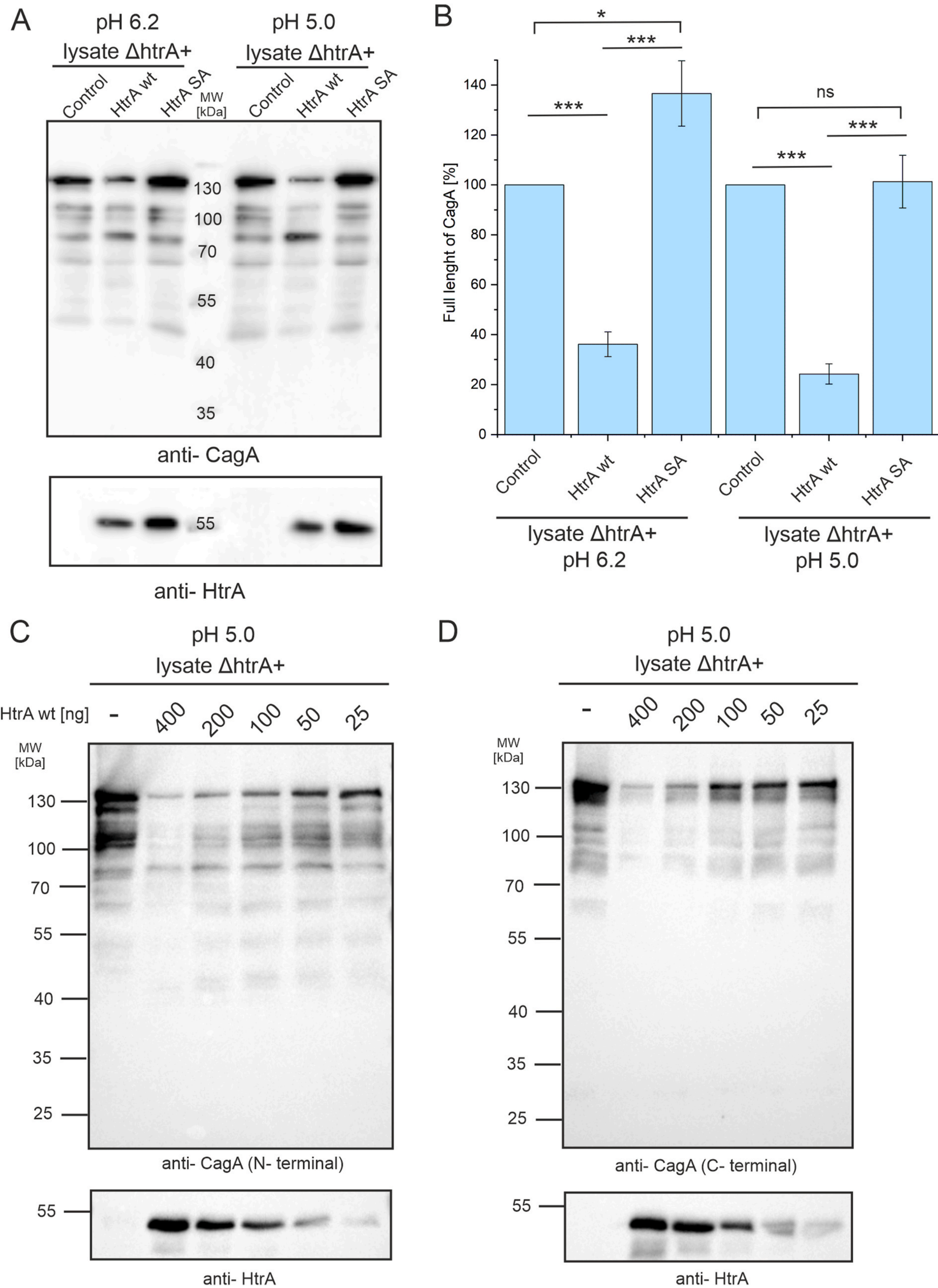

Fig. 4. CagA cleavage by recombinant HtrA. The lysates of N6Δ*htrA* were incubated with the proteolytically active (wt) or inactive (SA) HtrA variants at 37 °C, pH 5.0 or 6.2 for 16 h. (A) A representative Western blot analysis using anti-N-terminal part of CagA antibodies. (B) The graph shows the full length CagA content after HtrA cleavage. The levels of CagA without HtrA were set to 100 %. Differences were analyzed using Tukey test (*$p < 0.05$; ***$p < 0.001$; ns - not significant differences). The standard error of mean (SEM) was calculated using five independent repetitions. (C, D) HtrA dose dependent cleavage of CagA in the N6Δ*htrA* lysates. The loss of the full-length CagA protein bands and the appearance of degradation products were monitored by Western blotting using anti-N-terminal part of CagA (C) or anti-C-terminal CagA antibodies (D).

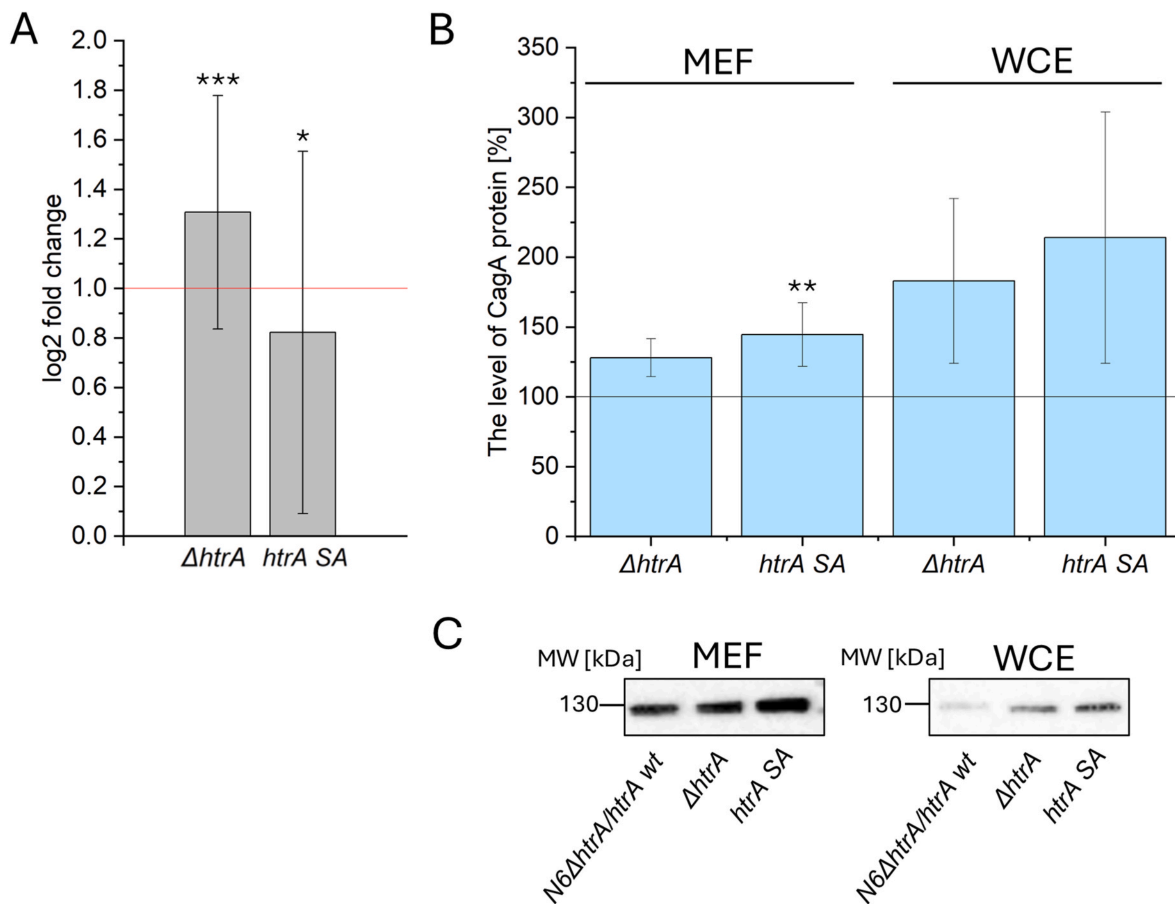

Fig. 5. Effects of the *htrA* mutations on the *cagA* gene expression and CagA gene content. (A) The relative log2 fold change of the expression levels of *cagA* gene in *H. pylori* Δ*htrA* (Δ*htrA*) and *H. pylori* Δ*htrA/htrAS221A* (*htrA SA*) compared to Δ*htrA/htrA* wt analyzed by qPCR. The data correspond to the means ± S.D. of five different samples, including three technical replicates. A red horizontal line indicates a relative two-fold increase in expression level. * indicates statistically significant (95 % confidence interval) fold change in expression level according to the REST 2009 software. The CagA protein level was analyzed by western blotting in the whole cell extracts (WCE) and membrane enriched fractions (MEF). Densitometric analysis of CagA content (B) is presented as mean values with standard deviation from 3 independent replicates. The horizontal line on the graph represents the reference value, set at 100 %, for the protein level in the complementation strain. Statistical analysis was performed by Tukey's test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.The representative results of western blotting are shown in C.

Therefore, our work aimed to add to the knowledge of how HtrA functions within the *H. pylori* cell.

First, identification of proteins that may be substrates for the proteolytic activity of HtrA was attempted. The proteomic and biochemical studies have identified two proteins that can be degraded by HtrA: CagA and FrpB4. The effector protein CagA is secreted out of the cell via type four secretion system [12], thus bypassing the periplasmic transit. However, there are some papers indicating the association of CagA with the cell surface of *H. pylori* [41] and its presence on the outer membrane vesicles (OMVs) [42–44]. In addition to periplasmic localization, HtrA is also detected in the culture medium [14,45,46] and OMVs [43]. Therefore, it can be speculated that both proteins may colocalize on the bacterial surface and probably in OMVs. In the *in vitro* system, CagA was efficiently digested by HtrA. Western blotting analysis using antibodies against the N- or C-terminal regions of CagA indicated that HtrA mainly cuts peptides from the carboxyterminal part of CagA. Proteomic studies of the MEFs showed that *htrA* knockouts contained elevated levels of the CagA protein. This result suggested stabilization of CagA in the absence of wt HtrA. Also, the CagA content in *htrA* mutant cell lysates was slightly higher. However, analysis of the *cagA* gene expression in *H. pylori* Δ*htrA* and *H. pylori* Δ*htrA/htrA S221A* cells showed that the mutant cells produced more *cagA* mRNA compared to the control strain. This fact may therefore be the reason for the generally higher content of CagA detected in the *htrA* knockouts. Nevertheless, DIGE analysis clearly showed the accumulation of certain C-terminally truncated forms of CagA (100 kDa and lower) in MEFs derived from the *htrA* mutants. We also demonstrated that CagA fragments of similar masses were efficiently digested by HtrA in *H. pylori* lysates *in vitro*. It was demonstrated that CagA translocation depends on the presence of its 20 C-terminal amino acids [47]. Therefore, HtrA may have an important function in the removal of C-terminally truncated CagA, unable to translocate into host cells.

In the case of FrpB4, HtrA degraded this protein both in *H. pylori* Δ*htrA* lysate and in the lysate of *E. coli* expressing recombinant His-tagged FrpB4. Since MS analysis of MEFs did not indicate changes in the content of this protein in the membranes, and mutations of the *htrA* gene did not affect *frpB4* gene expression, we can assume that HtrA removes non-native forms of this protein and is unlikely to participate in the periplasmic transit and incorporation process of FrpB4 into the OM. FrpB4 is an OM protein involved in the transport of metals, mainly nickel ions. Analysis of the MEF proteome of *htrA* mutants showed a significant decrease in the content of another OM receptor for nickel, FecA3 (*HP_1400* gene product). Reduced levels of FecA3 protein correlated with reduced expression of the *fecA3* gene, ruling out a direct involvement of HtrA in the regulation of this protein. The *fecA3* gene is under the control of both NikR and Fur regulators. Metal-bound holoforms of Ni-NikR and Fe-Fur lead to repression of this gene. However, we did not observe changes in the expression of other genes under the control of Ni-NikR in the *htrA* mutants (e.g. *frpB2, frpB4, ureA*) or Fe-Fur (*fecA1, fecA2, amiE*) under either physiological or stress conditions.

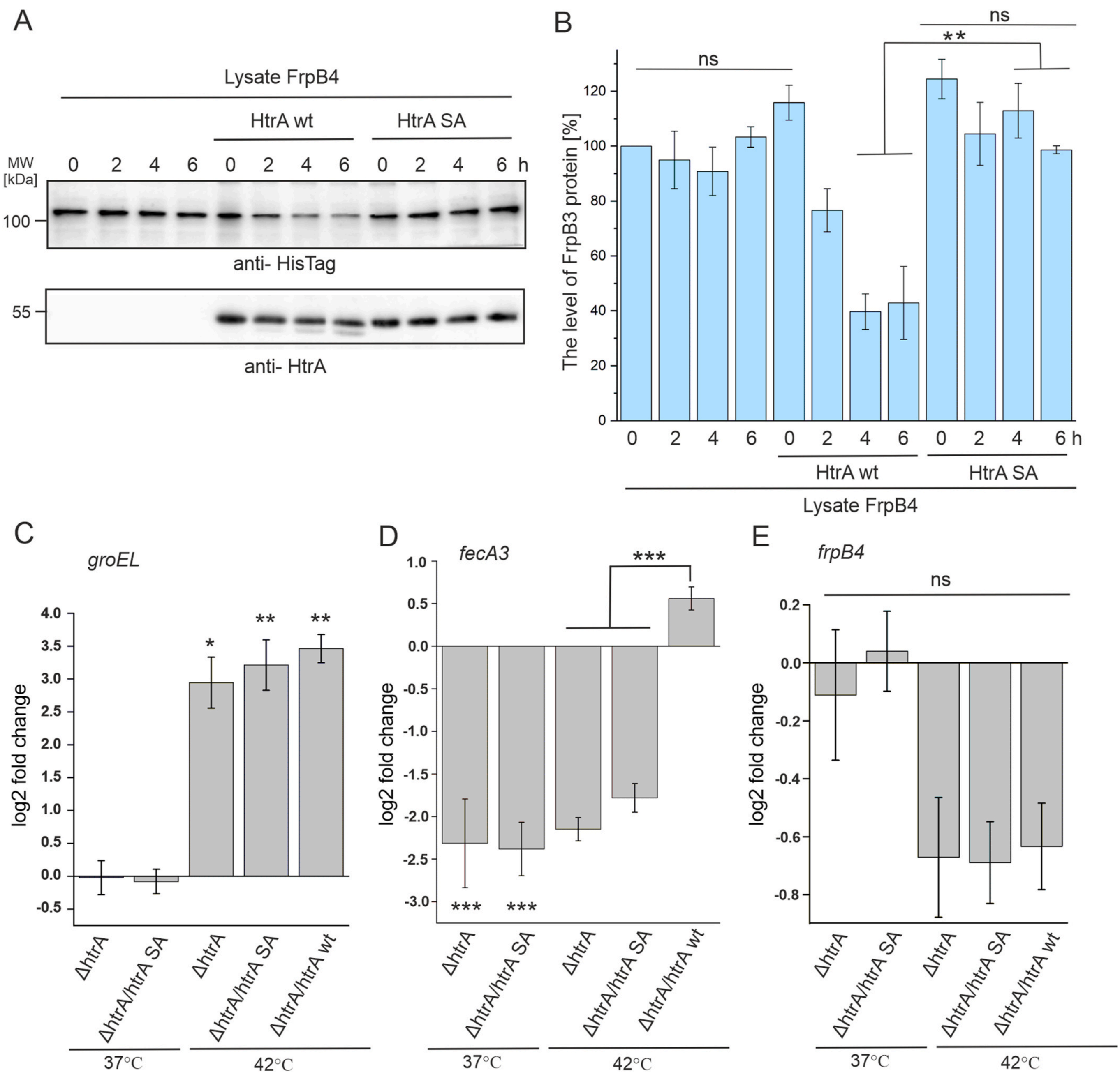

Fig. 6. Involvement of HtrA in metal homeostasis. Upper panel: FrpB4 cleavage by recombinant HtrA. The lysates of *E. coli* FrpB4 were incubated with the proteolytically active (wt) or inactive (SA) HtrA variants at 37 °C for 0, 2, 4, and 6 h. (A) A representative Western blot analysis using anti-HisTag or anti-HtrA antibodies. (B) The graph shows the full length FrpB4 content after HtrA cleavage. The levels of FrpB4 without HtrA at the time point "0" were set to 100 %. Differences were analyzed using Tukey test ($p < 0.01$ **; ns - not significant differences). The standard error of mean (SEM) was calculated using four independent repetitions. Lower panel: *fecA* (C) and *frpB* (D) gene transcription levels at non-stressful and stressful conditions. Bacteria were incubated at 37 °C or exposed to heat shock conditions (42 °C) for 30 min. (C) The *groEL* level was used as a control for stress conditions (42 °C). Strain *H. pylori* N6Δ*htrA/htrA* wt (cultured at 37 °C) was used as a control. Differences were analyzed using Tukey test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns - not significant differences). The standard error of mean (SEM) was calculated.

Also, the expression levels of the regulators themselves remained unchanged. Thus, the selective inhibition of *fecA3* expression appears to be a result of an additional mechanism, not yet identified, dependent on the proteolytic activity of HtrA.

Other proteins degraded in the *H. pylori* Δ*htrA* lysate are components of the cytoplasm; thus, they are unlikely to be natural substrates for HtrA. However, there is growing evidence that some of proteins associated with basic metabolic processes, in addition to their canonical functions in the bacterial cytoplasm, may perform important functions on the bacterial cell surface or as secreted proteins. This phenomenon is called "moonlighting". The moonlighting proteins include Tuf (EF-Tu), a translation elongation factor. A fraction of this protein is attached to the bacterial surface where it can interact with membrane receptors and with extracellular matrix on the surface of plant and animal cells (reviewed in Ref. [48]). In case of *H. pylori,* it was shown that Tuf is a potential adherence factor of *H. pylori* during pathogenesis [49]. Moreover, in *Campylobacter jejuni,* a bacterium closely related to *H. pylori*, Tuf (Cj0470) was identified in the OM fraction [50]. Therefore, secreted HtrA potentially may regulate the level of surface attached Tuf or eventually process it to modify its functionality, as shown in other

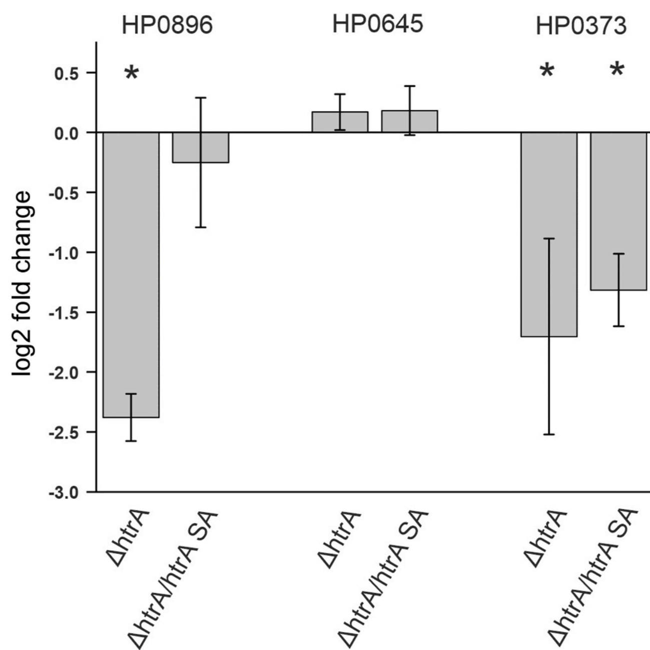

Fig. 7. Expression levels of genes *HP_0896, HP_0645* and *HP_0373*. The relative log2 fold change of the expression levels of selected genes compared to N6Δ*htrA/htrA* wt analyzed by qPCR. The data correspond to the means ± S.D. of five different samples with three technical replicates. * indicates statistically significant (95 % confidence interval) fold change in expression level according to the REST 2009 software.

bacteria [51]. The full length Tuf is a 43.6 kDa protein, hence Tuf polypeptides identified in the band 8 (Fig. 1) may represent a truncated form.

Attempts to select additional substrates for the proteolytic activity of HtrA using the quantitative proteomic analysis SWATH-MS of MEF combined with qPCR analysis of selected genes' expression indicated two more possible substrates for the HtrA protease: soluble lytic transglycosylase Slt and the OM protein HomC. The expression levels of genes encoding these proteins were unchanged or decreased in the *htrA* mutant cells; hence, the accumulation of Slt and HomC in MEFs of the *htrA* knockouts may be due to the lack of proteolytic activity of HtrA. This hypothesis is a subject of our current investigation.

The lack of apparent accumulation of other HtrA protease substrates may at least in part be due to upregulation of other extracytoplasmic proteases. This possibility seems to be supported by overall increased content of HP_1012 and HP_1350 in the cells and a marked increase in the level of the proteolytic complex HP_0657/HP_1012 in the MEF. Perhaps these proteases are able to partially replace HtrA in substrate degradation. The role of the proteases HP_0657/HP_1012 and HP_1350 in the *H. pylori* cell is unknown. However, it was shown that these proteins are secreted outside the bacterial cell [52], and HP_1012/HP_0657 complex degrade host cell components [35].

Quantitative SWATCH-MS analyses of MEFs indicate a very important role of the HtrA chaperone activity for maintaining cellular envelope homeostasis. While MEF from the Δ*htrA* cells showed altered levels of more than 80 proteins, less than 20 proteins were affected in the fraction derived from the Δ*htrA/htrA S221A* strain. Among the Δ*htrA* strain proteins with altered levels, the most abundant were those related to translation: ribosome components, tRNA ligases, and elongation factors Tuf and Tsf. This may indicate the intense processes of co-translational export of proteins from the cytoplasm, involving the association of ribosomes to membranes. Since MEF represents the total cellular insoluble fraction (not just membranes), we cannot exclude the possibility that these proteins represent aggregates of ribosomes that are formed when cells are exposed to stress conditions. Still, we did not observe an increase in the level of cytoplasmic stress induced protein markers, such as chaperonins GroE, nor an increase in *groEL* gene expression. In contrast, some observations point to the presence of stress in the cellular envelope. First, this is evidenced by a decrease in some motility-related proteins, especially those belonging to the late flagellar gene complex (class 3), controlled mainly by the alternative RNA polymerase sigma subunit FliA (sigma 28). These include genes encoding flagellin (FlaA) and the Omp11 protein of unknown function. In addition, MEFs have reduced levels of many other OM proteins: β-barrel OMPs and lipoproteins. Finally, the expression of two OMP encoding genes (Omp19 and HomC) was significantly reduced. The extracytoplasmic stress has not been intensively studied to date in *H. pylori*, and the response systems to cellular envelope damaging agents in this

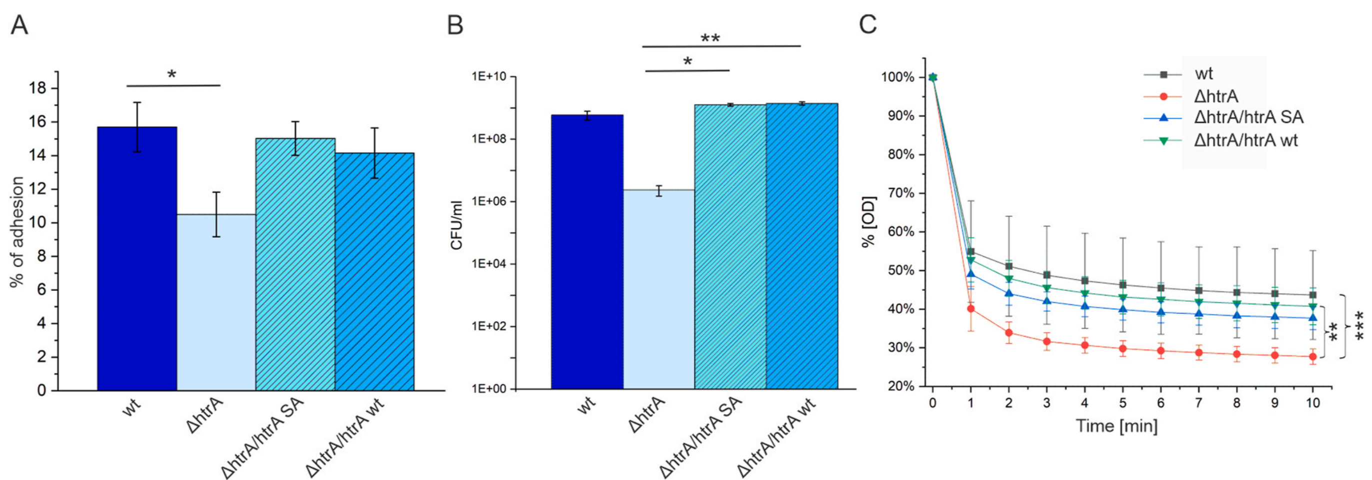

Fig. 8. Effects of the *htrA* mutations on the *H. pylori* cell surface properties: surface hydrophobicity (A) and susceptibility to SDS treatment (B, C). The percentage of adhesion to hexadecane was expressed as the mean of at least three independent experiments and three technical repetitions of each experiment (n = 9). Statistical analysis was performed using Tukey test, $^{**}p < 0.01$. (B) Effect of SDS on bacterial growth on solid medium. CFU counts data are based on 2 biological repetitions (with 2 or 3 technical repetitions, total n = 5). Statistical analysis performed by Kruskal-Wallis test, $^{**}p < 0.01$. (C) Susceptibility of bacterial cells to lysis in the presence of SDS. Bacteria in logarithmic phase were suspended in phosphate-buffered saline (PBS) to OD = 0.8 and were treated with 0.00625 % sodium dodecyl sulfate. The change in OD was analyzed at 1 min intervals. Results are presented as percentage of OD relative to OD at time 0. Median and Q3-Q1 quartile range from 2 or 3 independent replicates conducted on 3 different cell cultures (total n = 8). Statistical analysis performed at time point 10′ by Kruskal-Wallis test,$^{**}p < 0.01$, $^{***}p < 0.001$.

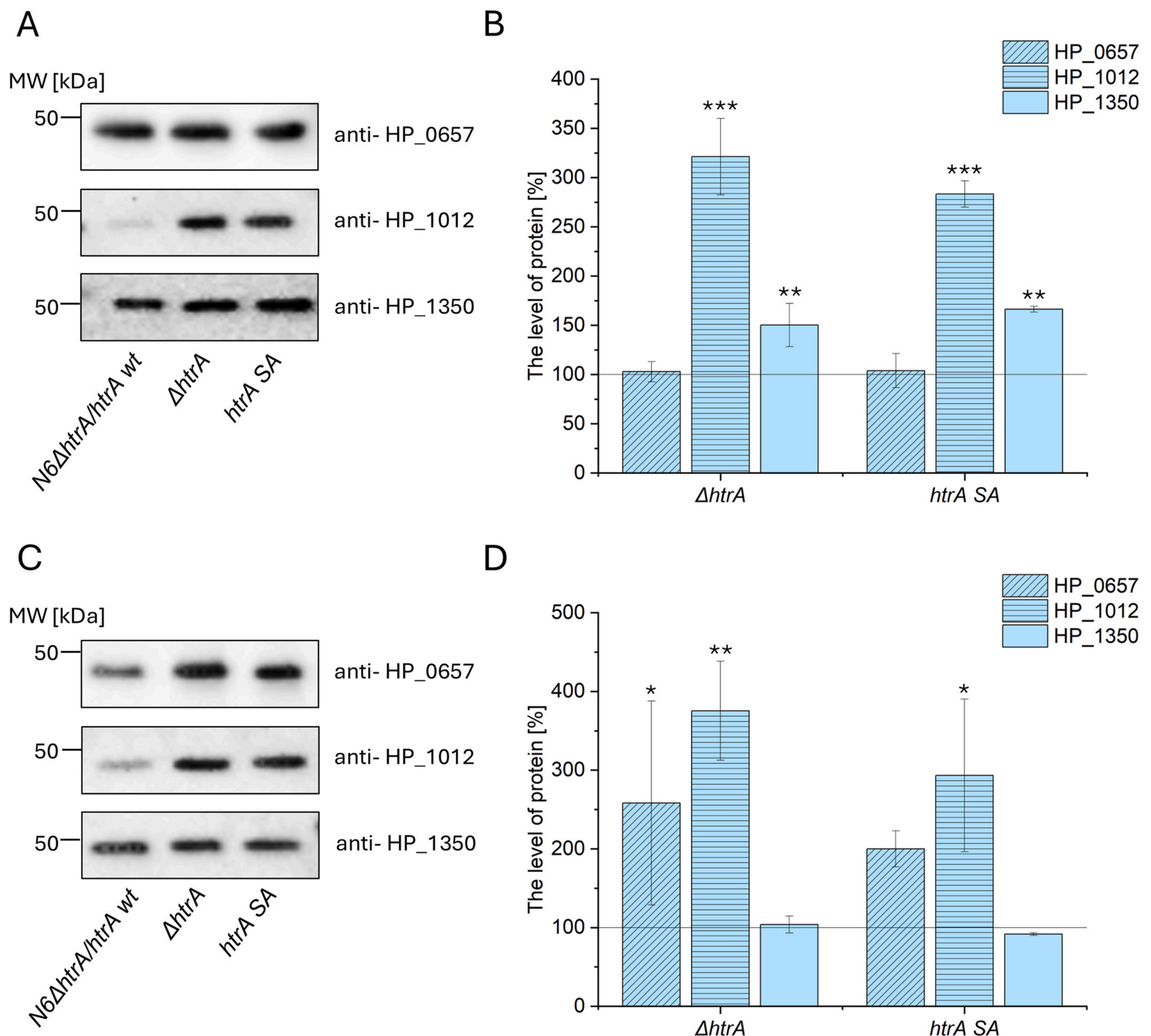

Fig. 9. Content of HP_0657, HP_1012 and HP_1350 proteases in the whole cell extracts (A, B) and membrane enriched fractions (C, D) of the *H. pylori htrA* mutants. (A, C) Representative western blotting images of MEFs isolated from: *H. pylori* N6 Δ*htrA* (Δ*htrA*), N6 Δ*htrA/htrA S221A* (*htrA SA*), N6 Δ*htrA/htrA wt* complemented strain using antibodies anti-HP_0657, anti-HP_1012, anti-HP_1350, (B, D) Densitometric analysis of HP_0657, HP_1012, HP_1350 content presented as mean values with standard deviation from 3 independent replicates. The horizontal line on the graph represents the reference value, set at 100 %, for the protein level in the complementation strain. Statistical analysis was performed by Tukey's test, **$p < 0.01$, ***$p < 0.001$.

bacterium are not known. Nevertheless, inhibition of flagella biosynthesis and a decrease in the level of OMPs under perturbed conditions of cellular envelope homeostasis, including OM damage, have been observed in other Gram negative bacteria [53,54]. Finally, increased levels of the LpxD protein, engaged in the lipid A biosynthesis, and Slt, responsible for peptidoglycan rearrangements, in the mutant cells points to possible aberrations within LPS and peptidoglycan, respectively.

The proper functioning of cell membranes that ensure selective and tightly regulated exchange of molecules with the environment is crucial for maintaining cell homeostasis. Disorders in membrane biogenesis resulting from, inter alia, malfunctioning EPQCS can lead to changes in membrane structure, negatively affecting membrane function as a barrier. Potential cell membrane disturbances due to lack of HtrA were confirmed by changed surface hydrophobicity and increased susceptibility to the ionic detergent SDS observed in the Δ*htrA* mutants. Both parameters are indicative of changes in the composition and/or structure of the OM. Mutations in the *htrA* gene do not affect the adhesion ability of *H. pylori* [17,55]. Therefore, we can assume that the changed properties of the OM are not linked with the altered adhesion ability of bacteria to the host cells. The proteomic analyses of the MEFs do not indicate changes in the adhesin content in the Δ*htrA* strain compared to the control strain. Although, the major adhesins of *H. pylori*, BabA (Omp28; HP_1243) and SabA (Omp17, HP_0725) were not detected in these studies, some other known *H. pylori* adhesins [56], such as Omp13 (OipA; HP_0638), Omp27 (HopQ; HP_1177) and Omp2 (LabA, HopD; HP_0025) were found and their levels were unchanged or even slightly elevated (Omp2) in *H. pylori* Δ*htrA*. Probably, the altered cell surface properties of the Δ*htrA* mutant strain are associated with changed levels of the other OM proteins that rather do not function as adhesins.

A fully functional OM provides protection against the destructive

effects of low concentrations of anionic detergents (e.g., SDS) [57]. The action of SDS is associated with direct destruction of the lipid bilayer, leading to cell lysis. Due to the presence of lipopolysaccharide, Gram-negative bacteria show significant resistance to low concentrations of this detergent, which is due to the negative charge of membrane saccharides [58,59]. When disruption of the lipopolysaccharide structure occurs, increased sensitivity of cells to SDS is observed [59]. Our study showed a difference in susceptibility to SDS treatment between the strain lacking the *htrA* gene and the parental and complementation strains. Interestingly, differences were observed between a strain lacking *htrA* and one containing the mutated *htrAS221A* gene. It turned out that the presence of *htrAS221A* provides resistance to the detergent comparable to that found in the control strain. This indicates that the proteolytic function of HtrA does not play a key role in maintaining the integrity of the OM, and that the chaperone activity of HtrA is sufficient to provide a protective barrier function by the OM against the ionic detergent.

Given the importance of the functions performed by the OM, it would be expected that disturbances in its composition and integrity would affect not only the physiology of the periplasm, but also the metabolism of the entire Δ*htrA* cell. In this regard, it is not surprising that proteomic studies have revealed altered profiles of cytoplasmic proteins involved in basal metabolism, including central carbon metabolism, amino acid metabolism and translation.

5. Conclusions

The experimental results presented in this work confirm that HtrA plays a very important role in maintaining cellular envelope homeostasis in *H. pylori*. The absence of a functional *htrA* gene causes changes within the OM, resulting in reduced OM integrity and altered surface properties, which presumably causes stress in the cellular envelope. It also appears that the chaperoning activity of HtrA considerably contributes to the maintenance of the OM integrity. On the other hand, HtrA proteolytic activity is indispensable for viability of *H. pylori*, as the wild-type gene could not be replaced by *htrAS221A* in all but one (N6 strain) *H. pylori* strains. Moreover, even in the cells of the N6 strain, suppressor mutations in the *secA* gene were present in both Δ*htrA* and Δ*htrA*/*htrA S221A* mutants. The mechanism of this suppression is not understood, and further studies are required to reveal the functional relationship between Sec translocon and HtrA in maintaining homeostasis of the cellular envelope in *H. pylori*. Nevertheless, the proposed housekeeping functions of proteolytic activity towards abnormally folded proteins (e.g. FrpB4) or truncated non-functional proteins (CagA) indicate important roles for both HtrA activities, chaperone and protease, in maintaining homeostasis of cellular envelope in *H. pylori*.

CRediT authorship contribution statement

Urszula Zarzecka: Writing – review & editing, Writing – original draft, Visualization, Investigation, Funding acquisition, Conceptualization. **Ombretta Repetto:** Writing – review & editing, Visualization, Investigation. **Patrycja Ambroziak:** Writing – original draft, Visualization, Investigation. **Małgorzata Bielecka:** Investigation. **Paulina Czaplewska:** Investigation. **Inez Mruk:** Investigation. **Natalia Musiał:** Investigation. **Donata Figaj:** Visualization, Investigation. **Davide Roncarati:** Writing – review & editing, Supervision. **Sebastian Diechler:** Investigation. **Valli De Re:** Writing – review & editing. **Silja Wessler:** Writing – review & editing, Conceptualization. **Renata Godlewska:** Writing – review & editing, Resources. **Joanna Skorko- Glonek:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2025.107979.

Data availability

Data are available via ProteomeXchange with identifier PXD062402 and PXD062510).

The raw data are available in Supplementary Data. The remaining data will be made available upon request.

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