Effects of Helicobacter pylori on the glutathione-related pathway in gastric epithelial cells

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Highlights

- The glutathione cycle in AGS cells were downregulated by *H. pylori* infection.
- The GSH level was reduced in AGS cells infected with *H. pylori*.
- The declined GSH level was greater in *H. pylori* gastric cancer strain infection.

Effects of *Helicobacter pylori* on the glutathione-related pathway in gastric epithelial cells

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Abstract

Virulence factors of *Helicobacter pylori* (*H. pylori*) are diverse, so various biological responses happen in a host infected with *H. pylori*. The aim of this study is to conduct the metabolomics-based evaluation on *H. pylori* infection. AGS human gastric carcinoma cells were infected with *H. pylori* strain 26695, and then the altered metabolite pathways in the infected AGS cells were analyzed by metabolomics. Metabolites related to the glutathione (GSH) cycle were downregulated by *H. pylori* infection. Next, we evaluated the effects of *H. pylori* on the GSH-related pathway in AGS cells infected with *H. pylori* isolated from patients with atrophic gastritis (AG), duodenal ulcer (DU) and gastric cancer (GC). We found that the declined degree of GSH levels and oxidative stress were greater in AGS cells infected with GC strains than DU and AG-derived strains. There were no significant differences in almost mRNA expressions of GSH-related factors among different clinical strains, but the protein expression of glutathione synthetase was lower in AGS cells infected with GC-derived strains than DU and AG-derived strains. Our data demonstrates that GC-derived *H. pylori*-induced oxidative stress in a host is stronger and GC-derived strains may have suppressive influences on the host's GSH-related defense systems.

Keywords: *Helicobacter pylori*; gastric epithelial cells; metabolomics; oxidative stress; glutathione.

1. Introduction

Helicobacter pylori (*H. pylori*) is the causes of the fatal diseases such as gastric cancer (GC) and gastric mucosa-associated lymphoid tissue lymphoma and the common

diseases such as atrophic gastritis or duodenal ulcer [1,2]. Almost more than half of the world's population is infected with *H. pylori* [3], and the number of these patients is very large. In addition, *H. pylori* is classified as a definite carcinogen by the International Agency for Research on Cancer of the World Health Organization in 1994 [4]. When the *H. pylori* infection is detected, eradication therapy is chosen in almost all of cases in Japan. A previous multicenter retrospective observational study in Japan showed that the cumulative incidence of metachronous GC 5 years after successful *H. pylori* eradication was 15% [5]. Even after *H. pylori* eradication, the risk to suffer from GC remains, and a study to elucidate what kind of thing happens is necessary for *H. pylori* infection.

Regarding the associations between *H. pylori* and diseases, *H. pylori* virulence factors such as cytotoxin-associated protein A (CagA) or vacuolating cytotoxin A (VacA) have been usually focused. CagA is a typical pathogenic factor of *H. pylori*, and infection with CagA-positive strains of *H. pylori* increases the risk for GC [6]. VacA has been also extensively examined, and *H. pylori* infection to produce s1/i1/m1-type VacA raises a risk of GC [7]. Virulence factors of *H. pylori* are diverse. Generally, a disease occurs in a host as results that various virulence factors act on a host, so it is important to evaluate the host's reactions induced by various virulence factors. In addition, there is a possibility that diseases may develop even after eradication of *H. pylori*, so it is important to clarify what happens in the host with *H. pylori* infection from various viewpoints.

The aim of this research is to conduct a new evaluation from different viewpoints on *H. pylori* infection. Metabolomics/metabolome analysis is a strategy for studying comprehensive analysis of low molecular weight metabolites, which are affected by both genetic and environmental factors, and moreover may represent the closest molecular

phenotype. Previously, we found the *H. pylori*-induced metabolite alterations in the host by metabolomics [8], so in current study we performed metabolome analysis to assess the altered metabolic pathways in *H. pylori*-infected gastric epithelial cells and moreover to evaluate the different metabolic changes between various *H. pylori* strains derived from different clinical disease patients.

2. Materials and methods

2.1. Bacteria and infection

A standard strain (26695) of *H. pylori* was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Six *H. pylori* strains, which were isolated from two each patient with atrophic gastritis (AG) (F13, F36), duodenal ulcer (DU) (F21, F52) and GC (F32, MKM1) in Japan, were used in this study [9,10]. The types of CagA (east Asian ABD-type) and VacA (s1m1-type) of six *H. pylori* strains are the same. *H. pylori* was cultured on trypticase soy agar with 5% sheep blood (Nippon Becton Dickinson, Tokyo, Japan) under microaerobic conditions (5% O_2, 5% CO_2, and 90% N_2) at 37℃. Prior to infection to AGS human gastric cancer cells, *H. pylori* was grown in Brucella broth (Becton Dickinson and Company Sparks, France) with 10% fetal bovine serum (FBS) overnight. For the infection, *H. pylori* was added to AGS cells at a multiplicity of infection (MOI) of 100:1 for the indicated times.

2.2. Cell culture

AGS cells were grown in RPMI-1640 supplemented with 10% FBS and 50 mg/mL penicillin-streptomycin in an atmosphere of 5% CO_2 at 37℃. AGS cells were serum-starved for 16 hr before *H. pylori* infection. AGS cells were collected at indicated times after *H. pylori* infection, leading to RNA, protein and metabolite preparations.

2.3. LC/MS analysis

Extraction of low molecular weight metabolites from AGS cells, LC/MS measurement and the data analysis for the semi-quantitative evaluation were performed as described in our previous report [11].

2.4. siRNA experiment

The knockdown of glutathione synthetase (GSS) was performed by using GSS siRNA (Thermo Fisher Scientific, CA, USA) and the control siRNA (Thermo Fisher Scientific), Lipofectamine RNAiMAX (Thermo Fisher Scientific), and Opti-MEM (Life Technologies Corporation, Carlsbad, CA, USA). AGS cells were cultured on the 6-well plate at a density of 2×10^5 cells/well for 24 hr, and then were treated with the siRNA mixture for 48 hr. The cells were infected with *H. pylori* for the last 6 hr of 48 hr at a MOI of 100:1.

2.5. RNA extraction and real-time RT-PCR

RNA was extracted with TRIzol reagent (Life Technologies Corporation), and cDNA was synthesized with RT2 first standard kit (Qiagen, Germantown, MD, USA) according to the manufacturer instructions. Relative quantitation of mRNA was evaluated by real-time RT-PCR using power SYBR green PCR master mix (Applied Biosystems, Tokyo, Japan).

2.6. Western blotting

For protein extraction, AGS cells infected with *H. pylori* at a MOI of 100:1 were harvested with RIPA buffer (50 mM Tris-HCl (pH 8.0), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with phosphatase inhibitors and protease inhibitors. Lysates from cells were separated by SDS-PAGE, and then immunoblotting was performed with primary antibodies against β-actin and GSS. The band images were

visualized with LAS-4000mini (FUJIFILM, Tokyo, Japan).

2.7. Statistical analysis

One-way ANOVA combined with Steel-Dwass test or Tukey test was used to compare the variances of each group. The statistical significance was defined as the P-values < 0.05. The statistical analyses were operated by JMP version 10 software (SAS Institute Inc., Cary, NC).

3. Results

First, AGS cells were infected with *H. pylori* strain 26695 for 6 or 24 hr, and then we measured the levels of metabolites, which consist of various metabolic pathways including the nucleotide biosynthesis, glycolysis pathway, pentose phosphate cycle, urea cycle, TCA cycle, amino acids metabolism, and methionine and cysteine metabolism with glutathione (GSH) cycle, in AGS cells by LC/MS analysis. These results are shown in Supplemental Table 1, and the metabolite map was also made based on the metabolite data (Figure 1). We could not find characteristic metabolite changes about the nucleotide biosynthesis, glycolysis pathway, pentose phosphate cycle, urea cycle and TCA cycle. In amino acid metabolism, we could confirm the decreased and increased tendency for 6 and 24 hr *H. pylori* infections, respectively. Regarding the GSH cycle, the decreased tendency was observed in both 6 and 24 hr *H. pylori* infections. Based on the metabolite map, we focused on GSH and its related enzymes as the following experiments.

GSH is generated from γ-glutamylcysteine and glycine by GSS. To investigate the influences of GSH lowering condition in *H. pylori*-related biological responses, we knocked down GSS by the siRNA method, and then evaluated whether GSH downregulation affects *H. pylori* infection-induced inflammatory responses. We could

confirm GSS downregulation of in AGS cells treated with both 400 and 1200 pM GSS siRNA (Figure 2A). In the conditions with GSS knockdown, *H. pylori* infection-induced IL-8 upregulation in AGS cells was enhanced (Figure 2B).

Next, we analyzed GSH and glutathione disulfide (GSSG) levels in AGS cells infected with six *H. pylori* strains, which were isolated from two each patient with AG, DU and GC in Japan, and then evaluated whether the change of GSH levels varied according to the differences in origin diseases. We could confirm decreased levels of GSH in all six *H. pylori* strains, and the degree of declined GSH levels was greater in *H. pylori* strains obtained from GC patients compared with other strains (Figure 3, Supplemental Figure 1). The ratio of GSSG to GSH indicates the degree of oxidative stress, so the GSSG/GSH ratios were evaluated. The GSSG/GSH ratios were higher in *H. pylori* strains obtained from GC patients compared with other strains (Figure 3, Supplemental Figure 1). In AGS cells infected with *H. pylori* isolated from six different disease strains, expression levels of IL-8 mRNA were evaluated (Supplemental Figure 2 and 3). As a result, 6 hr infection groups had the significant differences in IL-8 upregulation, although 24 hr infection groups had the increased tendency without the significant differences (Supplemental Figure 2). The results summarized in 3 clinical diseases categories showed the significant differences at both 6 and 24 hr infections, but we could not find any differences between these clinical diseases (Supplemental Figure 3).

Regarding GSH synthesis, γ-glutamylcysteine, which is a dipeptide, is synthesized from cysteine and glutamic acid by glutamate-cysteine ligase (GCLC). GSH, which is a tripeptide thiol, is synthesized from γ-glutamylcysteine and glycine by GSS [12]. Regarding GSH degradation, cation transport regulator 1 (CHAC1) can catalyze the cleavage of GSH into 5-oxo-L-proline, and CHAC1 overexpression can deplete GSH [13].

Therefore, we investigated whether decreased levels of GSH seen in *H. pylori*-infected AGS cells may be associated with inactivation of GCLC and GSS, or activation of CHAC1 (Supplemental Figure 4 and 5). In CHAC1, GCLC and GSS mRNA expressions at 6 hr infection with six different disease strains, the downregulated tendency was observed, but there were no significant differences. As for 24 hr infection, there were no characteristic changes of CHAC1 and GCLC mRNA expressions between different diseases strains. GSS mRNA expression tended to be downregulated, especially in *H. pylori* strains derived from GC, but there were no significant differences between each group. The results summarized in 3 clinical diseases categories presented no prominent alterations among clinical diseases categories at both 6 and 24 hr infections.

Nuclear factor erythroid-2-related factor 2 (Nrf2), which is a transcription factor activated by oxidative stress such as reactive oxygen species and electrophilicity materials, is known to protect cells in various situations. Nrf2 regulates the induction of genes encoding several antioxidants such as GCLC, GSS and heme oxygenase-1 (HO-1) [14]. Kelch-like ECH-associated protein 1 (Keap1) suppresses the Nrf2 signaling in normal conditions, and oxidative stress results in the detachment of Nrf2 from Keap1 leading to its translocation into the nucleus [15]. Therefore, expression levels of Nrf2, Keap1 and HO-1 mRNAs were evaluated (Supplemental Figure 4 and 5). Levels of Nrf2, Keap1 and HO-1 tended to be downregulated at 6 hr infection with six *H. pylori* strains, but there were no significant differences among different clinical diseases. The HO-1 level at 24 hr infection tended to be downregulated in six *H. pylori* strains, but the Nrf2 level were upregulated. The Keap1 level in six *H. pylori* strains at 24 hr infection were almost the same as the control. Summarized results of HO-1 at 6 hr infection in 3 clinical diseases categories indicated significant differences only in DU compared with the non-

infection control. In results of Nrf2 at 6 hr infection, the significant decreases were observed in DU and GC. As for the results of Keap1 at 6 hr infection, the significant decreases were seen in AG, DU and GC. There were no significant differences at 24 hr infection in 3 summarized different disease categories (Supplemental Figure 5).

To further investigate the reason why the degree of the reduced GSH level and GSSG/GSH ratio were greater in *H. pylori* strains obtained from GC patients, we assessed the differences in expressions of GSS protein among six *H. pylori* strains isolated from different clinical disease patients (Figure 4). GSS protein expression was downregulated in AGS cells infected with GC strains, but not other strains.

4. Discussion

The purpose of the present study is to evaluate effects of *H. pylori* on a host by metabolomics. In our previous study, we analyzed the metabolites in stomach tissues of mice that had been infected with *H. pylori*, and some metabolites were changed by *H. pylori* in an infection period dependent manner [11]. The metabolite alterations of AGS cells infected with *H. pylori* were also confirmed in our study [8]. The current study showed that the GSH cycle was downregulated in *H. pylori*-infected AGS cells, and that GC-derived *H. pylori* strains highly induced oxidative stress compared with AG and DU-derived strains. Normally, when oxidative stress is induced in a cell, reactions to return to normal are caused by homeostasis, but the current study exhibited the possibility that this homeostasis responses may be suppressed in the cells infected with GC-derived *H. pylori* strains.

The continuous infection with *H. pylori* may be caused by lowering the GSH level in the host, which means declined protective functions in the host. In *H. pylori*-

infected AGS cells, the intracellular GSH level were decreased [16]. In our study, *H. pylori* infection also reduced the GSH level in AGS cells, and moreover the decreased tendency of the GSH cycle was observed. CagA transferred to host cells via *H. pylori* infection causes abnormal cell proliferation and disrupts epithelial cell polarity through the interaction with SHP-2 and PAR-1 [17,18], leading to collapse of bacterial attachment to gastric epithelial cells. Therefore, autophagy induced by VacA reduces CagA in gastric epithelial cells for the persistent infection. It was reported that m1-type VacA lowers the GSH level in host cells via binding to LRP1, resulting in induction of autophagy for CagA degradation depending on ROS accumulation [19]. The 26695 and clinical isolated strains used in our study have m1-type VacA, so CagA-degrading autophagy might be induced through GSH reduction, and next the host's reactions to remove *H. pylori* might be suppressed leading to the continuous infection. Possibly, the degree of GSH reduction based on *H. pylori* infection is involved in different types of clinical diseases. GSH also increases the lymphocyte proliferation and NK cell activity [20-22]. The immune response to *H. pylori* is the Th1-type but not the Th2-type, which is effective against extracellular parasitic bacteria, so this is also considered to be one of major reasons for the continuous infection [23]. Therefore, there is several associations among GSH and *H. pylori* infection, and the *H. pylori*-induced decrease in the GSH level may be involved in suppression of the host's innate immunity accompanied by the continuous infection.

The results from our study suggested the possibility that ROS production was higher in AGS cells infected with GC-derived *H. pylori* strains, because the decreased degree of GSH level and ratio of GSSG to GSH was greater in the GC-derived strains. A previous report showed that glutamine deficiency decreased the intracellular GSH level and increased the level of hydrogen peroxide, and moreover the GSH treatment cancelled

these responses [24]. These results indicates that GSH worked to reduce ROS. In our study, the GSH level in AGS cells infected with GC-derived *H. pylori* strains was significantly lower compared with AG and DU-derived strains, so oxidative stress caused by GC-derived *H. pylori* strains might be stronger than AG and DU-derived strains. When AGS cells were incubated with outer membrane vesicles derived from *H. pylori* with s1m1-type VacA, the percentage of micronuclei formation was increased, and the GSH administration decreased it [25]. In degradation of hydrogen peroxide in the cells, glutathione peroxidase converts GSH to GSSG. The *H. pylori*-derived outer membrane vesicles-induced decline in the GSH level in the host resulted in the increased endogenous hydrogen peroxide, leading to DNA damage [25]. DNA damage caused by chronic inflammation was linked to GC development in mice [26], and it was also suggested that the accumulation of DNA damage based on *H. pylori*-induced chronic inflammation triggered human GC [27,28]. In addition, an administration of S-allylcysteine, which is one of components derived from garlic and has anti-inflammatory and antioxidant effects, significantly increased the GSH level in gastric tissues, liver tissues and serum of GC model rats, and reduced the GC risk [29]. These results suggest that GSH reduction is related to the pathogenesis of GC, and the greater decreased degree of GSH level and GSSG/GSH ratio in AGS cells infected with GC-derived *H. pylori* strains may be associated with carcinogenesis. Previously, to investigate the relationship between *H. pylori* virulence factors and disease specificity, AGS cells were infected with *H. pylori* isolated from patients with GC, DU and gastritis. As a result, IL-8 concentrations were similar among 3 groups [30]. These findings were consistent with our results, so it seems difficult to judge the disease specificity based on IL-8 as an indicator for *H. pylori* infection.

In AGS cells infected with GC-derived *H. pylori* strains, the protein synthesis process from GSS mRNA might be regulated, because expression of GSS protein but not mRNA was reduced in AGS cells infected with GC-derived *H. pylori* strains. miRNAs are endogenous non-coding RNAs that are involved in many biological processes through the regulation of target gene expressions [31], and can affect *H. pylori*-associated pathology via transcriptional regulations and expressions of various genes. miR-27a, which is known as an oncogenic miRNA in GC, increased oxidative stress by suppressing factors that act protectively against oxidative stress, such as prohibitin and FOXO1 [32]. These findings may be associated with our results that the infection with GC-derived *H. pylori*-strains had higher oxidative stress than DU and AG-derived strains. In addition, *H. pylori* infection accelerated ubiquitination and proteasome-mediated degradation of p53 protein [33], so expressions of various proteins may be also regulated. In the rats with alcoholic liver injury, an administration of PS-341 as a proteasome inhibitor upregulated expressions of GCLC and GSS [34], suggesting that the proteasome system is involved in GSS protein regulation. Possibly, the proteasome system via the infection with GC-derived *H. pylori*-strains led to degradation of GSS protein.

Our study has some limitations. To assess disease specific differences, the genotypes of representative virulence factors, CagA and VacA, were matched in our study, but other virulence factors were not taken into account. Therefore, our study assessed the host's reactions induced by *H. pylori* possessing several virulence factors. In addition, our study selected an *in vitro* experiment to investigate metabolome responses in gastric epithelial cells, so it is important to evaluate associations of GSH and clinical diseases in the future's human study. Moreover, it is necessary to find virulence factors linked to GSH degradation in the host. In conclusion, our study has the novelty in

evaluations of differences in the diseases based on host's metabolome responses but not pathogenetic genes, and suggests that *H. pylori* infection causes oxidative stress in the host, and that GC-derived *H. pylori* strains show more suppressive effects on the host's GSH-related defense systems.

Conflict of interests

The authors have no conflict of interest to declare.

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Figure legends

Figure 1. Alterations in metabolic pathways of *H. pylori*-infected AGS cells observed at 6 and 24 hr infections.

AGS cells were infected with *H. pylori* 26695 strain for 6 and 24 hrs, and then we measured levels of their metabolites based on the nucleotide biosynthesis, glycolysis pathway, pentose phosphate cycle, urea cycle, TCA cycle, amino acids metabolism, and methionine and cysteine metabolism with glutathione cycle by LC/MS analysis. The red, yellow, blue, and green bars indicate the results of 6 hr non-infection, 6 hr *H. pylori* infection, 24 hr non-infection, and 24 hr *H. pylori* infection, respectively. Data are shown as the mean ± SD (n=5 at most, because some metabolites were not detected in all samples).

Figure 2. The effects of GSS downregulation on IL-8 mRNA expression in *H. pylori*-infected AGS cells.

(A) AGS cells were treated with 400 and 1200 pM GSS siRNA or the corresponding control siRNA as a vehicle control for 48 hr. GSS and β-actin proteins

were detected by Western blotting. (B) AGS cells were treated with 400 pM GSS siRNA or the corresponding control siRNA for 48 hr. AGS cells were infected with *H. pylori* 26695 strain for the last 6 hr of 48 hr. IL-8 mRNA expressions were evaluated by the real-time RT-PCR. Data are shown as the mean ± SEM (n=4). One-way ANOVA combined with Tukey post hoc test was used for data comparisons. $p<0.05$ was considered statistically significant. Different letters indicate significant differences.

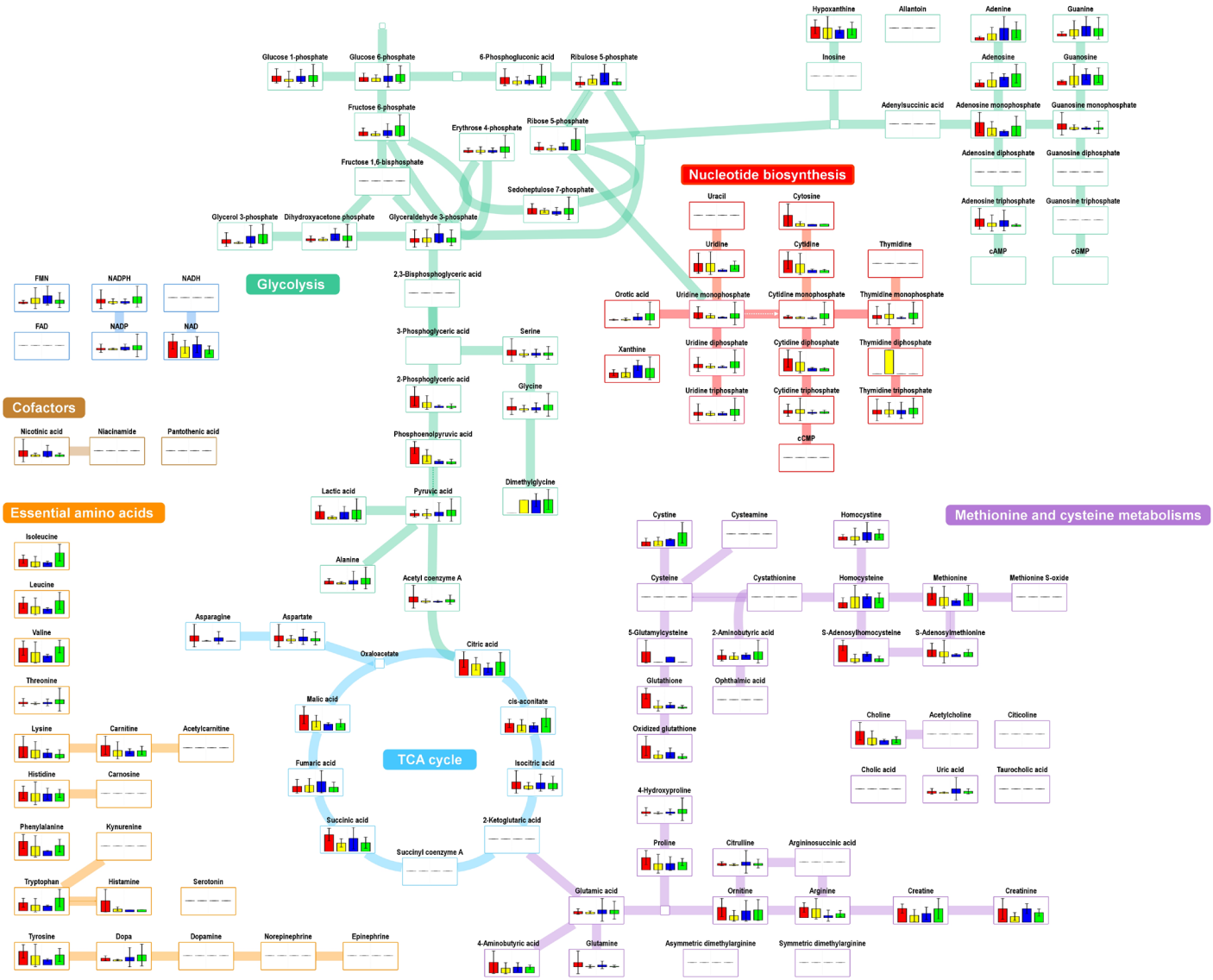
Figure 3. GSH levels and the GSSG/GSH ratio in AGS cells infected with *H. pylori* isolated from clinical diseases patients.

AGS cells were infected with *H. pylori*, which was isolated from two each patient with AG, DU and GC, for 6 hr. Then, GSH levels and the ratios of GSSG against GSH were evaluated using LC/MS analysis. Data are shown as the mean ± SEM (n=6). One-way ANOVA combined with Steel-Dwass and Tukey post hoc tests were used for GSH and GSSG/GSH comparisons, respectively. $p<0.05$ was considered statistically significant. Different letters indicate significant differences.

Figure 4. The protein expressions of GSS in AGS cells infected with *H. pylori* isolated from clinical diseases patients.

AGS cells were infected with *H. pylori*, which was isolated from two each patient with AG, DU and GC, for 12 hr. GSS and β-actin proteins were detected by Western blotting.

Figure 1


Glycolysis
Glucose 1-phosphate
Glucose 6-phosphate
6-Phosphogluconic acid
Ribulose 5-phosphate
Fructose 6-phosphate
Erythrose 4-phosphate
Ribose 5-phosphate
Fructose 1,6-bisphosphate
Sedoheptulose 7-phosphate
Glycerol 3-phosphate
Dihydroxyacetone phosphate
Glyceraldehyde 3-phosphate
2,3-Bisphosphoglyceric acid
3-Phosphoglyceric acid
Serine
2-Phosphoglyceric acid
Glycine
Phosphoenolpyruvic acid
Dimethylglycine
Lactic acid
Pyruvic acid
Alanine
Acetyl coenzyme A
Nucleotide biosynthesis
Hypoxanthine
Allantoin
Adenine
Guanine
Inosine
Adenosine
Guanosine
Adenylsuccinic acid
Adenosine monophosphate
Guanosine monophosphate
Adenosine diphosphate
Guanosine diphosphate
Adenosine triphosphate
Guanosine triphosphate
cAMP
cGMP
Uracil
Cytosine
Uridine
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Orotic acid
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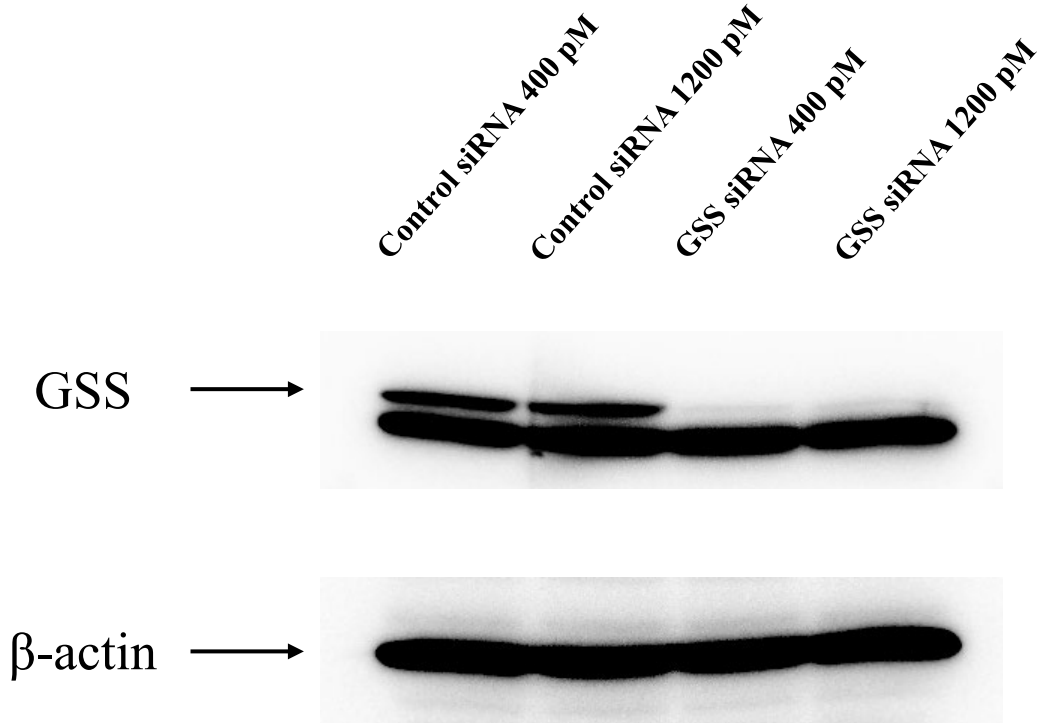
Thymidine triphosphate
cCMP
Cofactors
FMN
NADPH
NADH
FAD
NADP
NAD
Nicotinic acid
Niacinamide
Pantothenic acid
Essential amino acids
Isoleucine
Leucine
Valine
Threonine
Lysine
Carnitine
Acetylcarnitine
Histidine
Carnosine
Phenylalanine
Kynurenine
Tryptophan
Histamine
Serotonin
Tyrosine
Dopa
Dopamine
Norepinephrine
Epinephrine
TCA cycle
Asparagine
Aspartate
Oxaloacetate
Citric acid
Malic acid
cis-aconitate
Fumaric acid
Isocitric acid
Succinic acid
2-Ketoglutaric acid
Succinyl coenzyme A
Methionine and cysteine metabolisms
Cystine
Cysteamine
Homocystine
Cysteine
Cystathionine
Homocysteine
Methionine
Methionine S-oxide
5-Glutamylcysteine
2-Aminobutyric acid
S-Adenosylhomocysteine
S-Adenosylmethionine
Glutathione
Ophthalmic acid
Oxidized glutathione
Choline
Acetylcholine
Citicoline
Cholic acid
Uric acid
Taurocholic acid
4-Hydroxyproline
Proline
Citrulline
Argininosuccinic acid
Glutamic acid
Ornithine
Arginine
Creatine
Creatinine
4-Aminobutyric acid
Glutamine
Asymmetric dimethylarginine
Symmetric dimethylarginine

Figure 2

(A)


Control siRNA 400 pM
Control siRNA 1200 pM
GSS siRNA 400 pM
GSS siRNA 1200 pM
GSS
β-actin

(B)

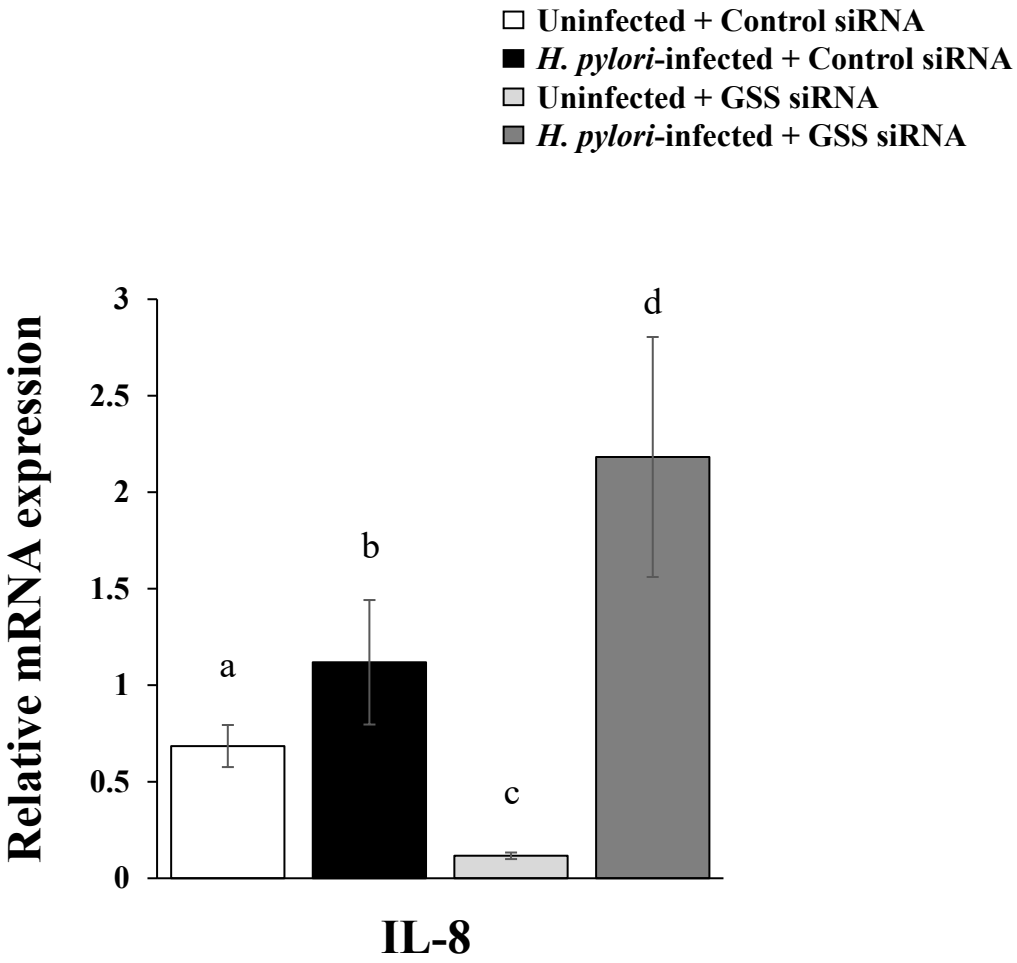

Uninfected + Control siRNA
H. pylori-infected + Control siRNA
Uninfected + GSS siRNA
H. pylori-infected + GSS siRNA
Relative mRNA expression
3
2.5
2
1.5
1
0.5
0
a
b
c
d
IL-8

Figure 3

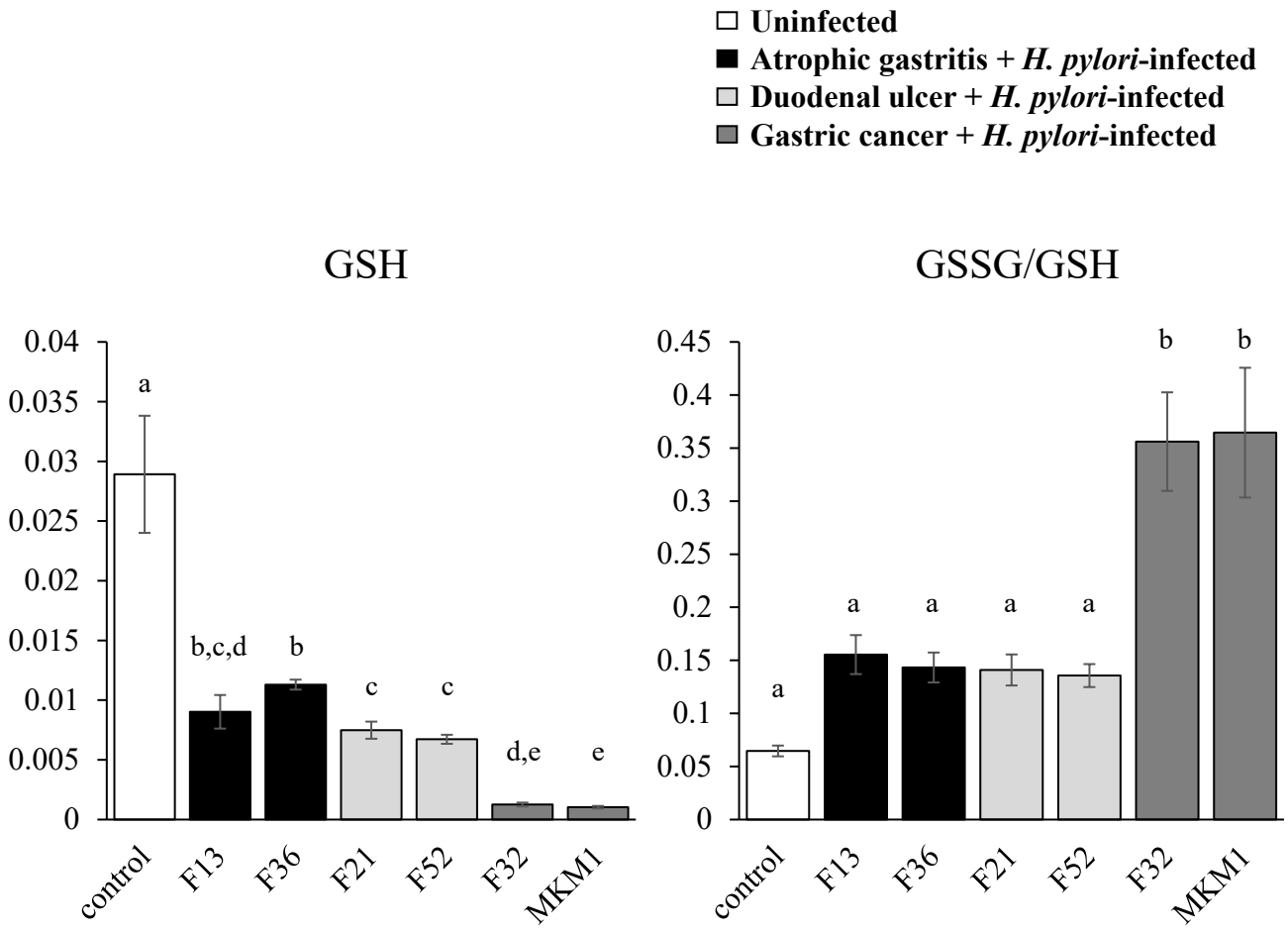
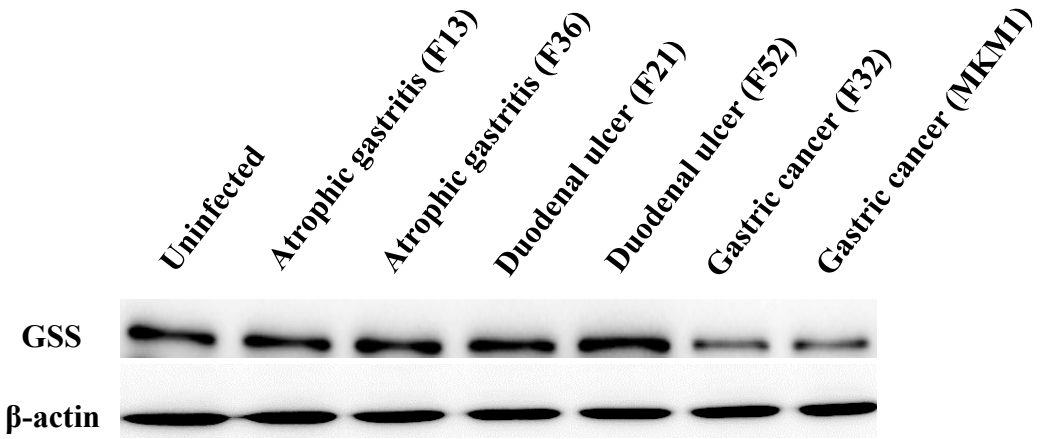

Uninfected
Atrophic gastritis + H. pylori-infected
Duodenal ulcer + H. pylori-infected
Gastric cancer + H. pylori-infected
GSH
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
a
b,c,d
b
c
c
d,e
e
control
F13
F36
F21
F52
F32
MKM1
GSSG/GSH
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a
a
a
b
b
control
F13
F36
F21
F52
F32
MKM1

Figure 4


Uninfected
Atrophic gastritis (F13)
Atrophic gastritis (F36)
Duodenal ulcer (F21)
Duodenal ulcer (F52)
Gastric cancer (F32)
Gastric cancer (MKM1)
GSS
β-actin

Supplemental Materials

Supplemental Figure legends

Supplemental Figure 1. GSH levels and the GSSG/GSH ratio in AGS cells infected with *H. pylori* isolated from clinical diseases patients.

Results of six different diseases strains shown in Figure 3 were summarized in three clinical diseases categories, such as atrophic gastritis (AG), duodenal ulcer (DU), and gastric cancer (GC). Data are shown as the mean ± SEM (n=7-17). One-way ANOVA combined with Steel-Dwass post hoc test was used for data comparisons. $p<0.05$ was considered statistically significant. Different letters indicate significant differences.

Supplemental Figure 2. Relative IL-8 mRNA expressions in AGS cells infected with *H. pylori* isolated from clinical diseases patients at 6 and 24 hr infections.

AGS cells were infected with *H. pylori*, which was isolated from two each patient with AG, DU, and GC, for 6 or 24 hr. IL-8 mRNA expression levels were evaluated using the real-time RT-PCR. Data are shown as the mean ± SEM (n=4-7). One-way ANOVA combined with Steel-Dwass post hoc test was used for data comparisons. $p<0.05$ was considered statistically significant. Different letters indicate significant differences.

Supplemental Figure 3. Relative IL-8 mRNA expressions in AGS cells infected with *H. pylori* isolated from clinical diseases patients at 6 and 24 hr infections.

Results of six different diseases strains shown in Supplemental Figure 2 were summarized in three clinical diseases categories, such as AG, DU, and GC. Data are

shown as the mean ± SEM (n=4-14). One-way ANOVA combined with Steel-Dwass post hoc test was used for data comparisons. $p<0.05$ was considered statistically significant. Different letters indicate significant differences.

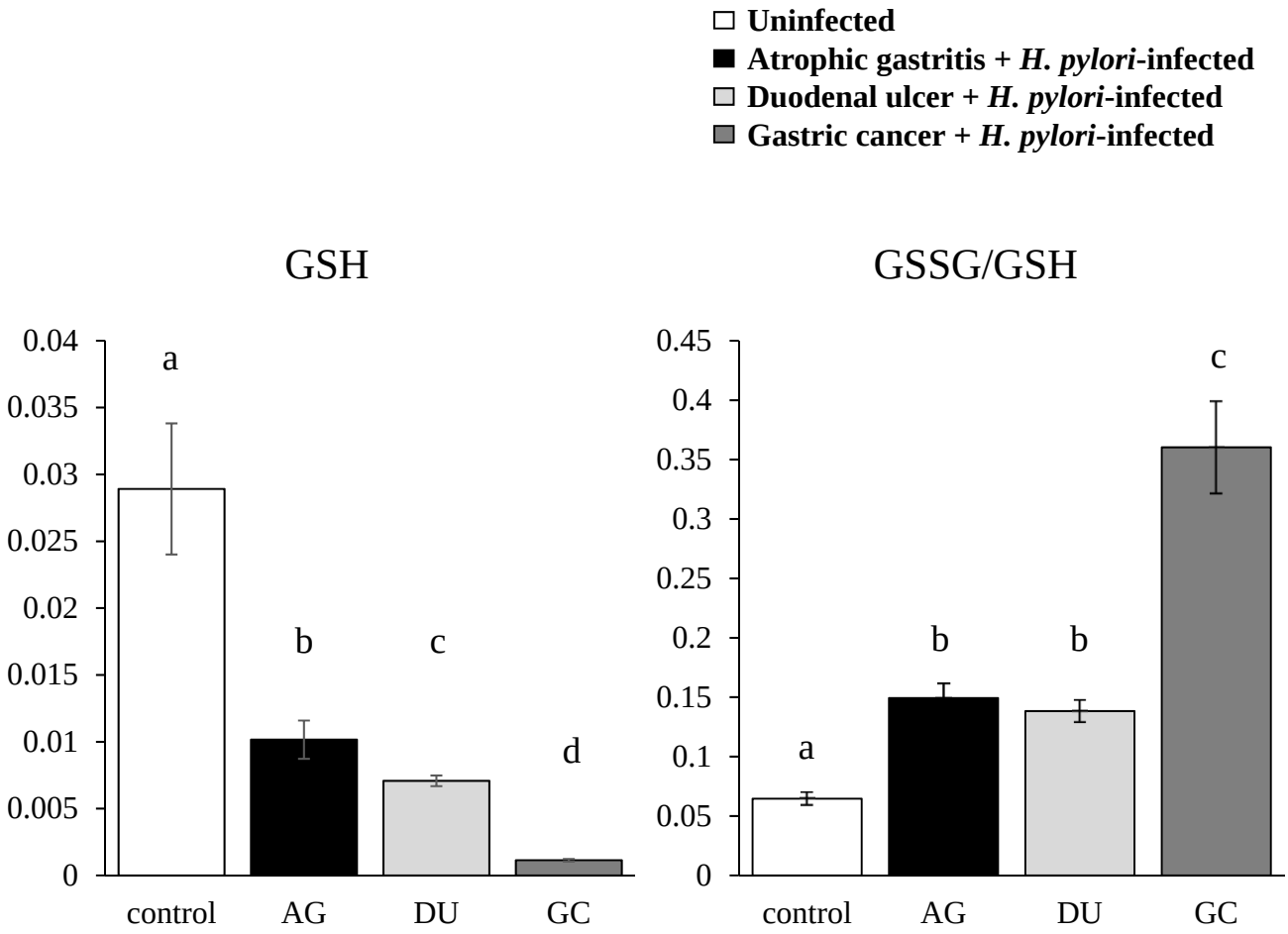
Supplemental Figure 4. Relative mRNA expressions in AGS cells infected with *H. pylori* isolated from clinical diseases patients at 6 and 24 hr infections.

AGS cells were infected with *H. pylori*, which was isolated from two each patient with AG, DU, and GC, for 6 or 24 hr. CHAC1, GCLC, GSS, HO-1, Nrf2 and Keap1 mRNA expression levels were evaluated using the real-time RT-PCR. Data are shown as the mean ± SEM (n=4-6). One-way ANOVA combined with Steel-Dwass post hoc test was used for data comparisons. $p<0.05$ was considered statistically significant. There were no significant differences between each group.

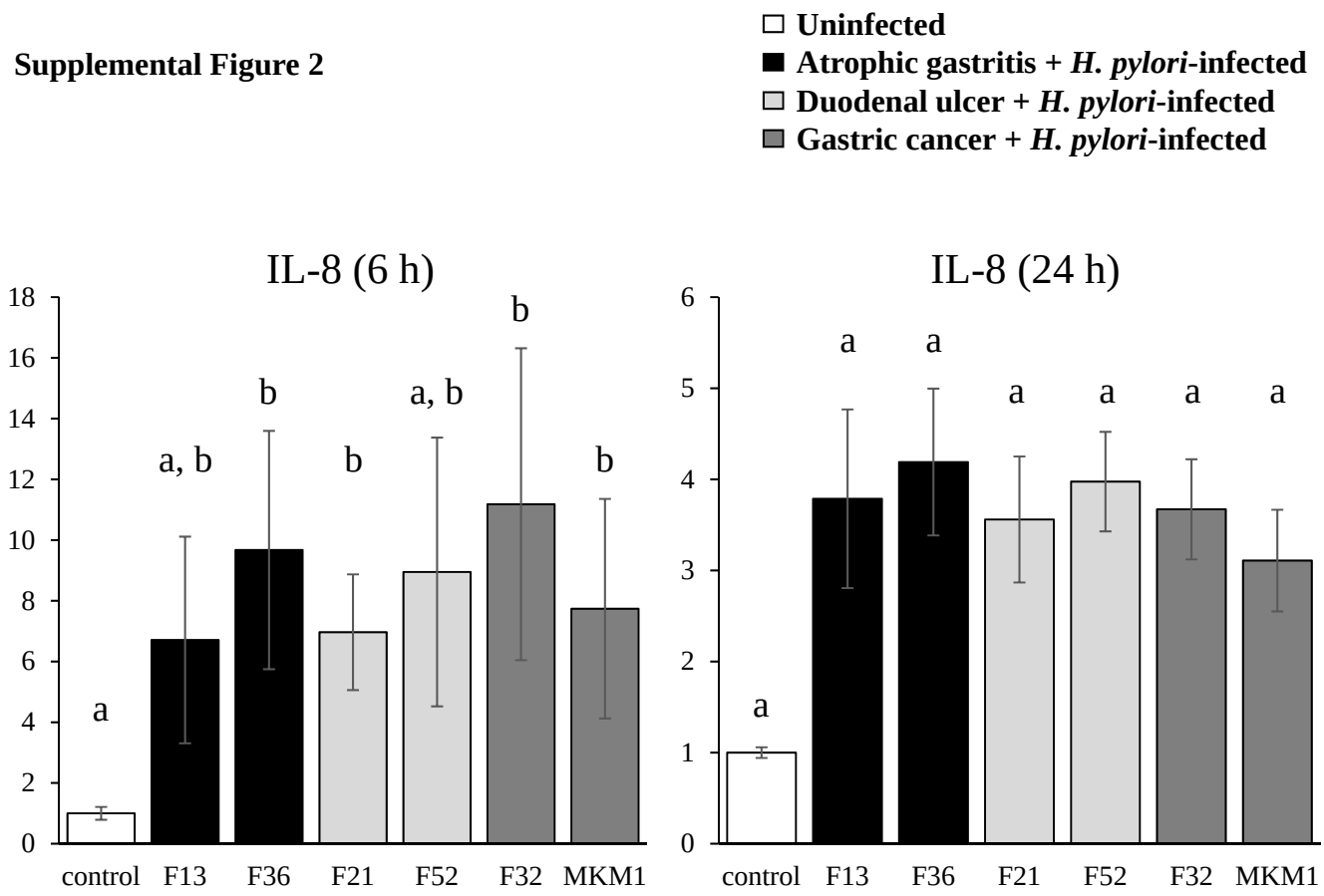
Supplemental Figure 5. Relative mRNA expressions in AGS cells infected with *H. pylori* isolated from clinical diseases patients at 6 and 24 hr infections.

Results of six different diseases strains shown in Supplemental Figure 4 were summarized in three clinical diseases categories, such as AG, DU, and GC. Data are shown as the mean ± SEM (n=4-12). One-way ANOVA combined with Steel-Dwass post hoc test was used for data comparisons. $p<0.05$ was considered statistically significant. Different letters indicated significant differences.

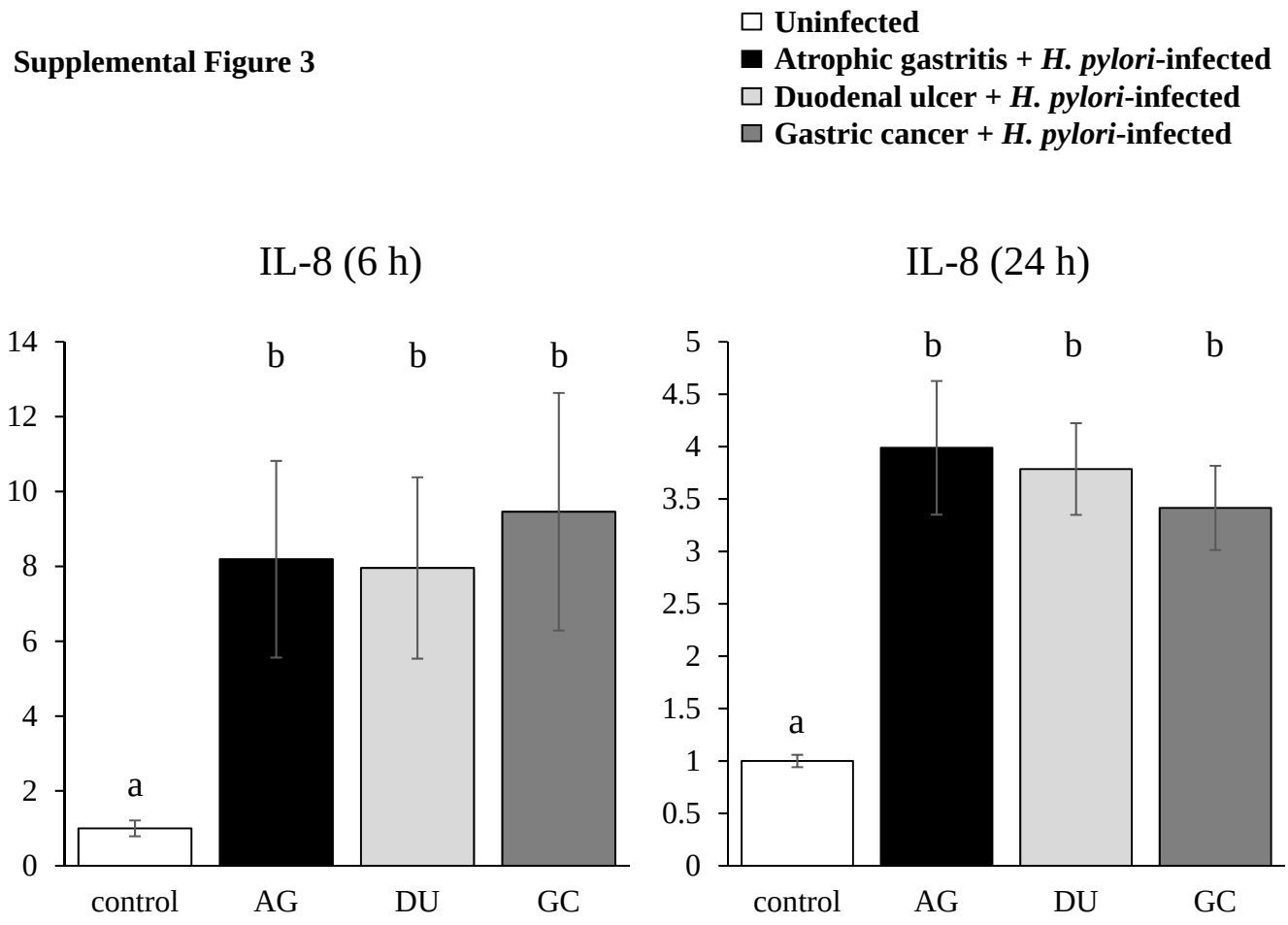
Supplemental Figure 1



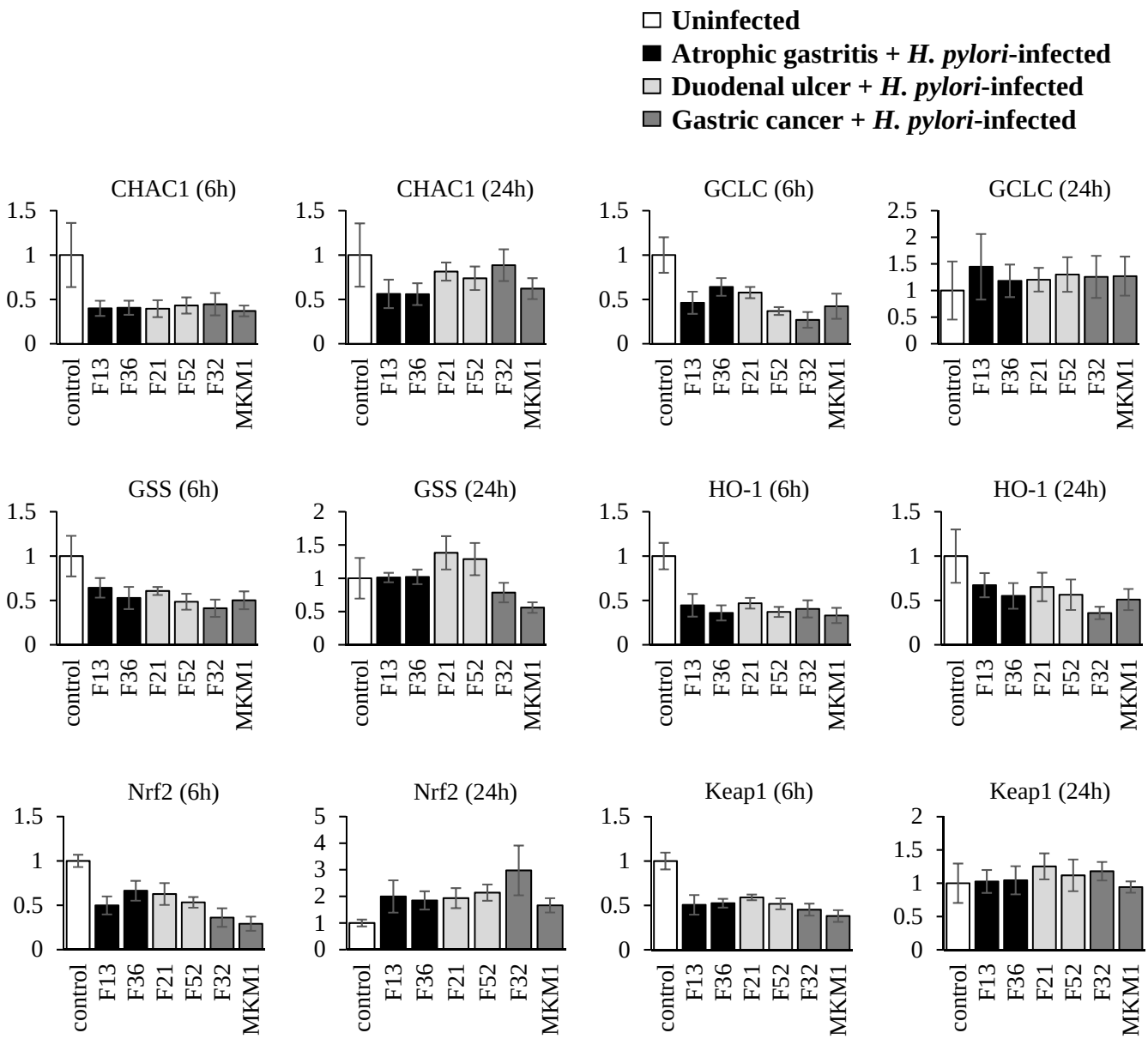
Supplemental Figure 2



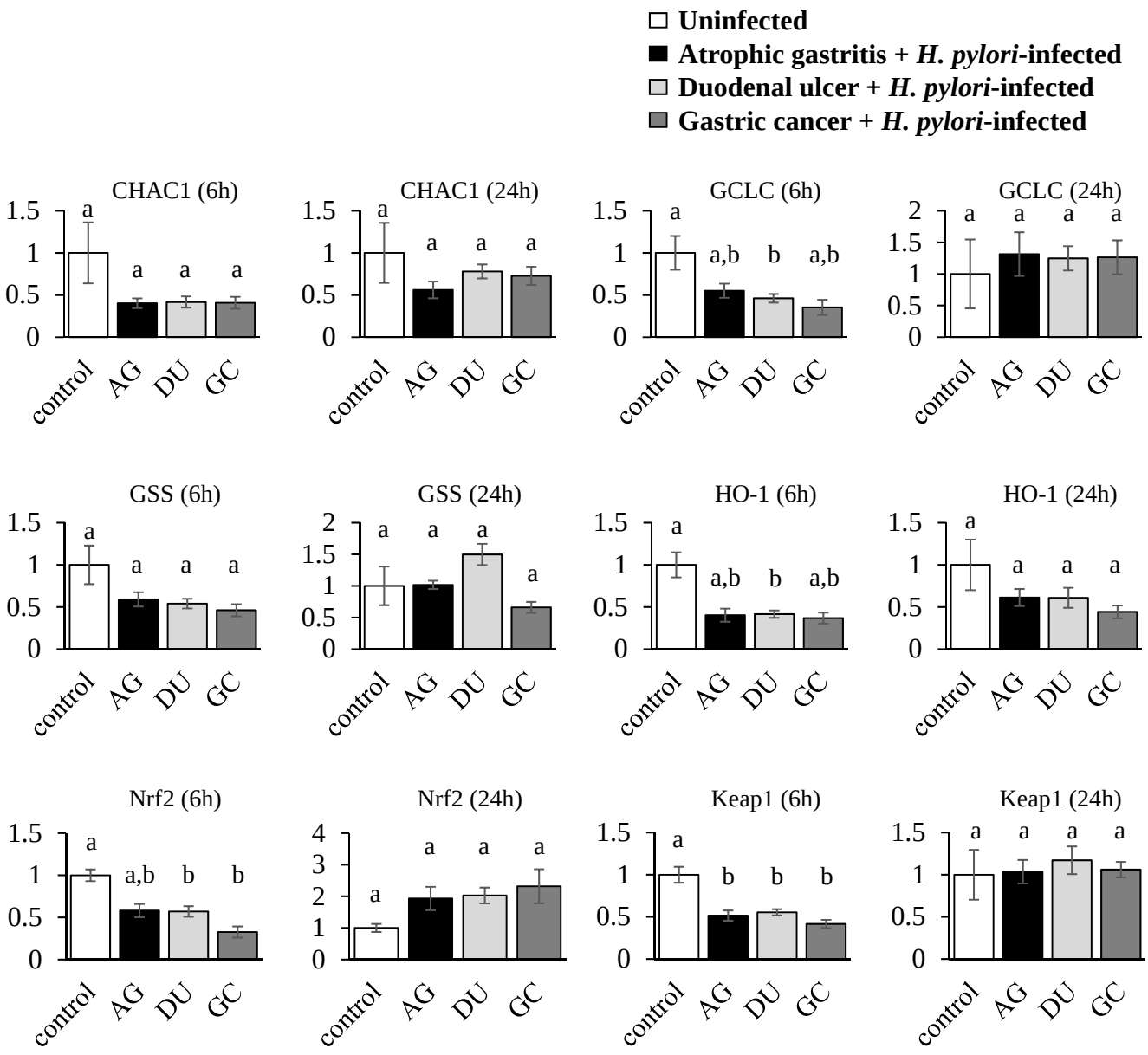
Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5



Supplemental Table 1. Metabolite profiles of *H. pylori* -infected AGS cells at 6 and 24 hr infections

Compound name	6 hr infection		24 hr infection	
	Fold induction *H. pylori* -infected/uninfected	P-value	Fold induction *H. pylori* -infected/uninfected	P-value
(6R,S)-5-Formyl-5,6,7,8-tetrahydrofolic acid	0.48	0.06	0.43	-
1-Hydroxy-2-methyl-2-buten-4-yl 4-diphosphate	0.53	0.51	2.56	0.17
2-Ethylhexanoic acid	0.63	0.13	1.35	0.34
2-Hydroxy-3-methyl-butyric acid (2-Hydroxyisovaleric acid)	1.03	0.94	0.96	0.95
2-Hydroxy-glutaric acid	0.42	<0.01 *	1.50	0.08
2-Hydroxy-isobutyric acid	0.85	0.67	1.24	0.66
2-Hydroxy-isocaproic acid	1.07	0.92	1.16	0.69
2-Oxoadipic acid	0.74	0.56	2.61	0.47
2-Phospho-gleceric acid	0.45	0.04 *	1.00	0.99
2-Propylglutaric acid	0.35	0.32	3.04	0.15
3-Ethoxypropionic acid	1.34	0.79	0.45	0.41
3-Hydroxy-2-methyl-butanoic acid (2-Methyl-3-Hydroxybutyric acid)	0.51	0.19	1.98	0.19
3-Hydroxy-3-methyl-butanoic acid (3-Hydroxyisovaleric acid)	0.68	0.42	1.25	0.76
3-Hydroxy-3-methyl-glutaric acid	0.77	0.50	1.22	0.74
3-Hydroxy-benzoic acid	0.73	0.57	2.77	0.28
3-Hydroxy-butyric acid	0.76	0.46	1.67	0.02 *
3-Hydroxy-isobutyric acid	1.20	0.81	3.27	0.15
3-Hydroxy-phenylacetic acid	0.55	0.24	0.73	0.61
3-Hydroxy-propionic acid	1.27	0.53	1.88	0.37
4-Hydroxy-3-methoxy-benzoic acid	1.33	0.45	3.07	0.13
4-Hydroxy-3-methoxy-mandelic acid	0.67	0.56	0.76	0.69
4-Hydroxy-L-proline	0.27	0.24	2.75	0.40
4-Hydroxyphenylacetic acid	0.62	0.48	1.29	0.80
4-Hydroxy-phenyl-lactic acid	1.19	0.67	0.97	0.96
4-Methyl-2-oxovaleric acid	1.33	0.66	4.21	<0.01 *
5,10-Methylene-5,6,7,8-tetrahydro-folic acid	1.41	0.43	1.71	0.34
5-Hydroxy-anthranilic acid	0.39	0.22	1.10	0.88
6-Phospho-gluconic acid	0.45	0.12	2.19	0.20
Acetyl-L-glutamine	1.00	0.99	0.48	0.31
Adenine	2.44	0.02 *	0.87	0.67
Adenosine	1.69	0.13	1.31	0.25
ADP-D-glucose	0.54	0.55	3.27	0.20
ADP-D-ribose	1.16	0.85	1.06	0.93
AMP	0.59	0.07	2.09	0.06
ATP	0.46	0.19	0.28	0.14
Azelaic acid	0.75	0.23	1.30	0.60
Benzoic acid	0.17	0.19	1.93	0.12
beta-Alanine	0.40	0.02 *	1.58	0.56
cAMP	1.91	0.35	0.57	0.52
Carnitine	0.54	0.11	1.11	0.65
CDP	0.42	0.25	2.89	0.23
cGMP	0.31	0.23	0.78	0.59
Choline	0.50	0.03 *	1.21	0.28
cis-Aconitic acid	0.81	0.31	2.10	0.01 *
Citraconic acid	0.63	0.05	1.61	0.12
Citric acid	0.72	0.08	1.73	0.07
CMP	0.77	0.23	6.03	0.17
CoA	0.21	0.14	3.36	0.19
Creatine	0.49	<0.01 *	1.54	0.16
CTP	1.31	0.75	1.36	0.55
Cytidine	0.71	0.27	0.90	0.48
dATP	0.29	0.37	5.07	0.26
DHAP	0.91	0.84	0.71	0.61
DL-Homocysteine	2.12	0.06	0.88	0.34
DL-Homocystine	1.05	0.94	0.82	0.63
dTMP	1.35	0.72	5.90	0.14
dTTP	1.03	0.97	1.61	0.51
E4P	1.09	0.88	3.00	0.25
Ethyl-malonic acid	0.68	0.28	2.92	0.08
F1P	1.35	0.54	3.57	0.18
F6P	0.54	0.12	1.96	0.18
FMN	3.50	0.16	0.45	0.20
Fumaric acid	1.17	0.67	0.46	0.11
G1P	0.40	0.19	1.13	0.84
G6P	0.77	0.39	1.24	0.71
GABA	0.45	0.07	0.99	-
Gallic acid	0.40	0.40	0.43	0.17
GAP	1.12	0.86	0.52	0.21
Gluconic acid	0.96	0.94	0.93	0.94
Glucuronic acid	2.19	0.34	0.63	0.45
Glutaconic acid	0.56	0.16	1.24	0.45
Glutaric acid	0.40	<0.01 *	2.00	0.06
Glyceric acid	0.56	0.57	7.39	0.18
Glycerol-P	0.19	0.08	1.30	0.58
Glycine	0.45	0.33	1.98	0.42
Glycolic acid	1.49	0.39	4.88	0.23
Glyoxylic acid	0.34	0.02 *	0.74	0.66
GMP	0.36	0.20	1.39	0.74
GSH	0.16	<0.01 *	0.39	0.04 *
GSSG	0.29	<0.01 *	0.33	0.02 *
Guanine	2.82	0.08	0.80	0.61
Guanosine	2.36	0.07	0.92	0.73
Hypoxanthine	0.91	0.74	1.15	0.51
IMP	1.48	0.65	1.35	0.57
Indol-3-acetic acid	0.75	0.45	1.57	0.42
Isocitric acid	0.42	0.17	0.90	0.83
L-2-Aminobutyric acid	0.86	0.71	1.71	0.30
Lactic acid	0.30	<0.01 *	1.29	0.53

L-Alanine & Sarcosine	0.48	0.03 *	1.68	0.52
L-allo-Isoleucine	0.68	0.16	2.70	<0.01 *
L-Arginine	0.87	0.62	1.98	0.24
L-Asparagine	0.04	0.07	0.03	0.12
L-Asparate	0.33	0.22	0.62	0.56
L-Citrulline	0.41	0.08	0.52	0.59
L-Cystine	1.17	0.53	1.99	0.04 *
Levulinic acid	1.82	0.33	3.80	0.06
L-Glutamate	0.64	0.25	0.91	0.92
L-Glutamine	0.10	0.16	0.26	0.20
L-Histidine	0.87	0.68	1.06	0.80
L-Homoserine & D-Homoserine	0.27	0.26	3.49	0.34
L-Isoleucine	0.66	0.13	3.51	<0.01 *
L-Leucine	0.66	0.14	2.76	<0.01 *
L-Lysine	0.68	0.27	0.74	0.42
L-Methionine	0.68	0.19	2.43	<0.01 *
L-Norvaline	0.68	0.13	2.49	<0.01 *
L-Ornithine	0.36	<0.01 *	1.09	0.87
L-Phenylalanine	0.65	0.12	2.09	0.01 *
L-Proline	0.50	0.04 *	1.22	0.49
L-Pyroglutamic acid	0.47	<0.01 *	1.29	0.19
L-Serine	0.33	0.22	1.16	0.87
L-Threonine	0.25	0.24	3.06	0.38
L-Tryptophan	0.69	0.20	2.63	<0.01 *
L-Tyrosine	0.69	0.19	1.92	0.02 *
L-Valine	0.70	0.17	2.43	<0.01 *
Maleic acid	0.57	0.42	0.46	0.13
Malic acid	0.60	0.03 *	1.15	0.40
Mesaconic acid	0.62	0.15	1.67	0.04 *
N-Acetyl-DL-alanine	0.54	0.03 *	1.21	0.50
N-Acetyl-neuraminic acid	0.35	0.37	0.54	0.44
NAD	0.68	0.04 *	0.61	0.06
NADP	0.70	0.65	1.72	0.56
NADPH	0.44	0.35	3.82	0.12
Nicotinic acid	0.30	0.14	0.32	0.05
O-Acetyl-L-homo serine	0.56	0.08	1.06	0.84
Octanoic acid (Caprylic acid)	0.65	0.42	2.00	0.04 *
Orotic acid	1.88	0.34	2.10	0.28
Oxalic acid	0.99	0.99	2.79	0.42
Oxaloacetic acid	0.43	0.23	1.02	0.97
PEP	0.51	<0.01 *	0.81	0.50
Phosphocholine	0.33	0.16	1.21	0.81
Phosphoric acid	0.72	0.21	1.62	0.04 *
Phthalic acid (benzene-1,2-dicarboxylic acid)	0.70	0.13	1.26	0.27
p-Hydroxybenzoic acid	0.48	0.21	0.58	0.44
Pimelic acid	0.34	0.28	1.37	0.42
PRPP	0.17	0.29	1.41	0.56
Putrescine	0.49	0.30	0.89	0.89
Pyruvic acid	0.95	0.92	1.53	0.59
Quinic acid	1.11	0.82	1.58	0.51
R5P	0.56	0.22	2.92	0.04 *
Ru5P	1.94	0.08	0.26	<0.01 *
RuBP	2.09	0.31	3.04	0.03 *
S7P	0.67	0.15	2.06	0.36
Saccharic acid	0.84	0.83	1.67	0.42
SAH	0.19	<0.01 *	0.38	<0.01 *
SAM	0.76	0.59	1.77	0.06
Shikimic acid	1.10	0.80	0.99	0.99
S-Methyl-L-cysteine	0.67	0.16	1.64	0.08
Suberic acid	0.73	0.23	1.66	0.04 *
Succinic acid	0.51	<0.01 *	0.66	0.18
T6P	1.45	0.43	1.97	0.39
Tartaric acid	3.78	0.21	0.81	0.66
ThPP	2.82	0.19	4.93	0.24
Threonic acid	0.37	0.08	2.62	0.30
Tricarballylic acid	0.94	0.90	1.73	0.50
UDP	0.53	0.34	5.50	0.11
UDP-D-glucose	0.49	<0.01 *	5.87	0.28
UMP	0.48	0.08	4.30	0.23
Uric acid	0.40	0.10	0.44	0.40
Uridine	1.01	0.98	3.68	<0.01 *
UTP	0.57	0.54	3.54	0.19
Xanthine	1.05	0.84	0.77	0.40
Xu5P	2.26	0.51	2.09	0.25

AGS cells were serum-starved for 16 hr, and the cells were collected at 6 or 24 hr after being infected. Data are characterized as fold-induction values of the normalized peak intensity for the *H. pylori* -infected AGS cells (n=5) against that of the uninfected controls (n=4 or 5). P-values for comparisons between the *H. pylori* -infected AGS cells and the corresponding control cells were estimated using the Student's t-test in each infection period, and asterisks indicate P-values of <0.05. The metabolties without P-values indicate less than three of numbers of detected samples in each group. Abbreviation: ADP-D-glucose, Adenosine diphosphate glucose; ADP-D-ribose, Adenosine diphosphate ribose; AMP, Adenosine 5'-monophosphate; ATP, Adenosine 5'-triphosphate; cAMP, Adenosine 3',5'-cyclic phosphate; CDP, Cytidine 5'-diphosphate; cGMP, Guanosine 3',5'-cyclic monophosphate; CMP, Cytidine-5'-monophosphate; CoA, Coenzyme A; CTP, Cytidine 5'-triphosphate; dATP, Deoxyadenosine 5'-triphosphate; DHAP, Dihydroxyacetone phosphate; dTMP, Deoxythymidine 5'-phosphate; dTTP, Deoxythymidine 5'-triphosphate; E4P, D-Erythrose 4-phosphate; F1P, Fructose 1-phosphate; F6P, D-Fructose 6-phosphate; FMN, Flavin mononucleotide; G1P, D-Glucose 1-phosphate; G6P, alpha-D-Glucose 6-phosphate; GABA, gamma-Aminobutyric acid; GAP, GTPase activating protein; GMP, Guanosine 5'-monophosphate; GSH, Reduced glutathione; GSSG, Oxidized glutathione; IMP, Inosine 5'-monophosphate; NAD, Nicotinamide adenine dinucleotide; NADP, Nicotinamide adenine dinucleotide phosphate; NADPH, Reduced nicotinamide adenine dinucleotide phosphate; PEP, Phosphoenolpyruvate; PRPP, 5-Phosphoribosyl 1-pyrophosphate; R5P, Ribose 5-phosphate; Ru5P, L-ribulose-5-phosphate 4-epimerase; RuBP, D-ribulose-1,5-bisphosphate; S7P, Sedoheptulose 7-phosphate; SAH, S-Adenosylhomocysteine; SAM, S-Adenosylmethionine; T6P, Trehalose 6-phosphate; ThPP, Thiamin pyrophosphate; UDP, Uridine 5'-diphosphate; UMP, Uridine 5'-monophosphate; UTP, Uridine 5'-triphosphate; Xu5P, D-Xylulose 5-phosphate.