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Acid regulated gene expression of *Helicobacter pylori*: insight into acid protection and gastric colonization

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Abstract

Background—The pathogen *Helicobacter pylori* encounters many stressors as it transits to and infects the gastric epithelium. Gastric acidity is the predominate stressor encountered by the bacterium during initial infection and establishment of persistent infection. *H. pylori* initiates a rapid response to acid to maintain intracellular pH and proton motive force appropriate for a neutrophile. However, acid sensing by *H. pylori* may also serve as a transcriptional trigger to increase the levels of other pathogenic factors needed to subvert host defenses such as acid acclimation, antioxidants, flagellar synthesis and assembly, and CagA secretion.

Materials and Methods—*H. pylori* were acid-challenged at pH 3.0, 4.5, 6.0 vs non-acidic pH for four hours in the presence of urea, followed by RNAseq analysis and qPCR. Cytoplasmic pH was monitored under the same conditions.

Results—About 250 genes were induced and an equal number were repressed at acidic pHs. Genes encoding for antioxidant proteins, flagellar structural proteins, particularly class 2 genes, T4SS/CagPAI, F₀F₁-ATPase and proteins involved in acid acclimation were highly expressed at acidic pH. Cytoplasmic pH decreased from 7.8 at pH_{out} of 8.0 to 6.0 at pH_{out} of 3.0.

Conclusions—These results suggest that increasing extracellular or intracellular acidity or both are detected by the bacterium and serve as a signal to initiate increased production of protective and pathogenic factors needed to counter host defenses for persistent infection. These changes are dependent on degree of acidity and time of acid exposure, triggering a coordinated response to the environment required for colonization.

Introduction

H. pylori colonizes the normal acid-secreting stomach of about 50% of the world's population, leading to gastritis, gastric and duodenal ulcers, gastric carcinoma, and MALT lymphoma (1–5). Persistent infection with *H. pylori* causes immune activation and increases the risk of developing gastric cancer about 20-fold (1). Initial infection often occurs early in

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life probably due to lower acid secretion {Agunod, 1969 #558} and typically evades clearance by the immune system (6). While bioenergetically a neutralophile, *H. pylori* is the only organism capable of colonizing the normal human stomach (7). As such, it is continually exposed to a variable range of acidity from pH 1.0 to pH 6.0 in the gastric environment (8).

Most neutralophilic bacteria have developed strategies to maintain cytoplasmic pH (pH_{in}) between pH 7.5 and pH 8.0 over a range of external pH (pH_{out}) between pH 4.0 and pH 8.0. The relatively constant pH_{in} is due mainly to a proton barrier afforded by the inner membrane and internal buffering by cytoplasmic proteins. These passive acid resistance mechanisms can maintain a constant pH_{in} when exposed to moderate acidity (pH 4.0–7.0) but not to the pHs encountered in the gastric environment (pH 1.0 to pH 6.0) (9).

In addition to the passive acid responses, neutralophilic bacteria respond to mild acidity via the acid tolerance response (ATR). ATR is the concept that prior exposure to mild acidity prepares bacteria for exposure to high acidity through a series of mechanisms involving transcriptional and translational responses. These mechanisms include the export of protons by amino acid decarboxylation coupled to amino acid/base counter-transport, by oxidases and by the F_0F_1 -ATPase, the generation of NH_3 by amino acid deiminases, deaminases and urease, decreasing proton permeability of the cell envelope and protection and repair of acid damaged proteins, purines and pyrimidines.

The *H. pylori* acid transcriptome has been analyzed in at least 4 microarray studies (10–13). Unfortunately, there is very little agreement in gene expression levels among the various transcriptome analyses. The lack of reproducibility is likely due to differences in the experimental design and array technology (Table 1). Each study used a different strain of *H. pylori* (G-27, 26695, NTU-D1 and SS1). The media used differed among the four studies (BHI, Brucella broth, Columbia agar) and only one study included urea in the incubation medium (10). The level of acidity varied from 4.5 to 6.2 and the acid exposure time was 30 minutes to 48 hours. Different microarray platforms were used including fluorescent slide arrays and colorimetric and radiolabeled microarray membranes.

The hybridization-based microarray approaches used to define the *H. pylori* acid transcriptome have several disadvantages that are overcome using NGS RNAsequencing (RNAseq). Hybridization-based approaches require synthesizing labeled cDNA that is then hybridized to custom-made microarrays or commercial high-density oligo microarrays. Hybridization-based approaches require knowledge about genome sequence to synthesize probes. Cross hybridization and limited dynamic range (~100 fold) increase background levels and signal saturation. In contrast, NGS RNAseq directly determines the cDNA sequence, abrogating the need for labeled probes and hybridization biases and resulting in a dynamic range of >8000 fold.

In this work, our aim is to more accurately define the *Helicobacter pylori* acid transcriptome and use this information to better understand the cascade of events that allow *H. pylori* to colonize the stomach. We performed RNA-seq analysis at pH values of 3.0, 4.5, 6.0 compared with non-acidic pH in the presence of 5 mM urea after 4 hours of incubation to

approximate *in vivo* conditions. We used a novel incubation system that maintains the urea concentration and pH at a constant level. Acid exposure induced the transcription of about 250 genes at all acidic pHs studied and about the same number were repressed. These data show the improvement in analysis by RNA-seq compared with microarray methods. Under this more physiologic incubation system, the exposure to acidity triggers not only the acid acclimation response, but also a cascade of other critical pathogenic factors that aide in gastric colonization.

Materials and Methods

Bacterial strains and culture conditions

H. pylori strain G27 was used (14, 15). Bacteria were grown under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) in a mixed gas incubator on Trypticase Soy Agar (TSA) plates supplemented with 5% sheep blood (Gibco). For broth culture, *H. pylori* strain G27 was grown in brain heart infusion (BHI) medium (Difco) supplemented with 7% horse serum (Gibco), 0.25% yeast extract (Difco) and Dent selective supplement (Oxoid). The pH of the liquid culture was adjusted by the addition of HCl (pHs 3.0, 4.5, and 6.0) or NaOH as appropriate. Non-acidic pH used for all experiments was either 7.4 or 8.0. pH 8.0 is the upper limit of the range of survival and growth of a neutralophile.

RNA isolation

Bacteria from three plates were suspended in 3.5 mL BHI, and 0.5 mL of the bacterial suspension was added to each of four dialysis cassettes. Dialysis cassettes (Slide-A-Lyzer, MWCO 10kD) were suspended in 750 mL media at pH 3.0, 4.5, 6.0, 7.4, or 8.0 with 5 mM urea. Following incubation at each pH for 4 hours, RNA was isolated from *H. pylori* using a combination of the TRIzol[®] method and the RNeasy kit (Qiagen) as described previously (10). RNA quality was determined using an Agilent 2100 bioanalyzer. Only RNA with an RNA integrity number greater than 8 was used for analysis. Biologic replicates (3X) were completed for all experiments. For qPCR experiments, the starting material was scaled up proportionally to allow for filling of 4 cassettes with bacteria at each pH. One cassette at each pH was removed at 30 minutes, 1, 2, and 4 hours, and the RNA was isolated and quality confirmed as above. The pH of the incubation media was unchanged after 4 hours for all experiments.

RNAseq analysis

Total RNA was extracted and stored at -80 °C until use. All RNA-sequencing and alignment procedures were conducted by ChunLab (Seoul, South Korea). The Ribo-Zero rRNA removal kit (Epicentre, USA) was used for ribosomal RNA depletion according to manufacturer instructions. Libraries for Illumina sequencing were made with the TruSeq Stranded mRNA sample prep kit (Illumina, USA) following the manufacturer's protocol. RNA sequencing was performed on the Illumina HiSeq 2500 platform using single-end 50bp sequencing.

The sequence data for the reference genome (G27) was retrieved from the NCBI database. Quality-filtered reads were aligned to the reference-genome sequence using Bowtie2. The

relative transcript abundance was measured in fragments in reads per kilobase of exon sequence per million mapped sequence reads (FPKM). Visualization of mapping results and differentially expressed gene (DEG) analysis were performed using the CLRNaseqTM program (ChunLab, South Korea). RNAseq experiments were completed at two non-acidic pHs, pH 8.0 and 7.4. pH 7.4 data was used for all ratios shown in the results section, but there was no significant difference in expression between pH 8.0 and pH 7.4 (data not shown).

Quantitative PCR

One step qRT-PCR was completed from RNA with the qScript One-Step SYBR® Green qRT-PCR Kit (Quanta Biosciences), using a Bio-Rad iCycler CFX-96 machine. Primer design was aided by the Primer3 software available at <http://www-genome.wi.mit.edu/genomesoftware/other/primer3.html> (16). Unique primers were designed for 100–300 base pair regions of each gene in the urease gene cluster and a housekeeping gene (Table 2).

Standard PCR performed with genomic DNA from *H. pylori* strain G27 as the template was used to assure that all primer pairs resulted in amplification of a single product (data not shown). qPCR was completed using 10 QL reactions in a 96 well plate using the standard cycling protocol, conditions were 50o for 10 minutes, 95° for 5 minutes, followed by 45 cycles of 95o for 10 seconds, 60o for 30 seconds (data collection). Threshold cycle was calculated using the Bio-Rad software. A melting curve was used at the end of the run to confirm that there was only one peak and only one product for each primer. Results were analyzed using the comparative C_T method (16, 17) with *gyrB* used as a housekeeping gene. RNA isolation was completed two times for each condition, and each RNA sample was run in triplicate. Non-acidic pH (pH 7.4) was used as the denominator for comparison with acidic pHs (pH 3.0, 4.5, and 6.0).

SDS-PAGE and Western blotting

Two plates of *H. pylori* strain G27 were suspended in 7 mL BHI and 3 mL of suspension was added to two dialysis cassettes as described above. Bacteria were incubated in the dialysis cassettes suspended in 750 mL of media at pH 6.0 or 8.0 with 5 mM urea for 4 hours. Bacteria were then pelleted at 10,000 rcf, 4°C for 5 minutes. Bacterial pellets were resuspended in 2 mL ice cold lysis buffer (ProNET Live!, ESI Source Solutions, LLC) containing 150 QL bacterial protease inhibitor (Sigma). Bacteria were lysed by 3 passes through a French Press at 20,000 p.s.i. Unbroken bacteria were harvested by a 10-minute spin at 4°C, 1500 rcf. The supernate fraction was collected as total bacterial lysate. Protein concentration was determined by the BCA method (Pierce/Thermo).

The bacterial lysates were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–12% NuPAGE bis-tris gradient gels (Life Technologies) and transferred onto nitrocellulose (Bio-Rad). Western blot analysis was performed using antibodies against the periplasmic loops of UreI, GS1 and GS2, which were combined and used at a dilution of 1:500 (18). Secondary antibodies were horseradish peroxidase linked goat anti-rabbit (1:10,000, American Qualex). Protein bands were

detected using SuperSignal West Pico Chemiluminescence Kit (Thermo). Immunoblots were quantified by densitometry using Image Studio Software (LI-COR Inc.).

Measurement of internal pH (pH_{in})

Internal (cytoplasmic) pH was measured using a fluorimeter as described previously (18). *H. pylori* grown overnight on TSA plates supplemented with 5% sheep blood (BD) were resuspended in BHI then added to each of four dialysis cassettes. Dialysis cassettes (Slide-A-Lyzer, MWCO 10kD) were suspended in 750 mL media at pH 3.0, 4.5, 6.0 or 8.0 with 5 mM urea and incubated for 4 hours as described above. After 3.5 hours, the membrane-permeant, pH-sensitive fluorescent probe 2,7-bis-(2-carboxyethyl)-5-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was added to each of the bacterial suspensions to a final concentration of 10 QM. After four hours, the bacteria were pelleted by gentle centrifugation (2,000 × g, 5 minutes) and resuspended in 300 QL of HP medium (140 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 0.5 mM MgSO₄, 10 mM glucose, 1 mM glutamine, 5 mM urea) at pH 3.0, 4.5, 6.0 or 8.0. To measure pH_{in}, 100 QL of BCECF-loaded bacteria at each pH was added to 3 mL of 100 mM phosphate buffered HP medium of the same pH and fluorescence was monitored using a dual-excitation (Ex1; 502 nm, Ex2; 436 nm), single-emission (Em; 530 nm) fluorimeter (Jobin Yvon Horiba Fluorolog). Each experiment was calibrated independently. Once the fluorescence of the internal BCECF had been measured, 150 nM of the protonophore 3,3,4,5-tetrachlorosalicylanilide (TCS) was added to equilibrate the internal pH with that of the medium. HCl was then added to obtain minimum fluorescence of the dye, followed by the addition of NaOH to obtain maximum fluorescence of the dye. The internal pH was calculated using the equation:

$$\text{pH}_{\text{in}} = \text{pK}_a + \log \left(\frac{(R - R_A)/(R_B - R)}{(F_A(\lambda_2)/F_B(\lambda_2))} \right)$$

where pK_a = pK_a of BCECF = 6.98, *R* = value at 502 nm/value at 436 nm for each data point, *R_A* = ratio at minimum fluorescence, *R_B* = ratio at maximum fluorescence, *F_A*(λ₂) = minimum fluorescence at 436 nm, and *F_B*(λ₂) = maximum fluorescence at 436 nm.

To determine if the F₀F₁-ATPase or oxidases contribute to proton extrusion in acid, pH_{in} was monitored using BCECF in the presence and absence of the F₀F₁-atpase inhibitor N, N'-dicyclohexylcarbodiimide (DCCD) or the oxidation inhibitor (cytochrome bc), antimycin A. We hypothesized that if the F₀F₁-ATPase reverses in acid and extrudes protons, the cytoplasmic pH would increase and this increase would be abolished in the presence of DCCD. Bacteria grown overnight on TSA plates were resuspended in 5 ml BHI medium (Difco). BCECF-AM was added to the bacterial suspension to a final concentration of 5 QM. The bacterial suspension was then incubated in a microaerobic (5% O₂, 10% CO₂, 85% N₂) environment at 37°C for 30 minutes with shaking. The bacteria were pelleted by gentle centrifugation (2,000 × g, 5 minutes) and resuspended in 300 QL of HP medium (140 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 0.5 mM MgSO₄, 10 mM glucose, 1 mM glutamine) buffered with 1 mM phosphate buffer to pH 7.4. To measure pH_{in}, 20QL of BCECF-loaded bacteria was added to 3 mL 100 mM phosphate buffered HP medium at pH 4.5, and fluorescence was monitored in the presence and absence of 100 nM DCCD or 10 μM antimycin A.

Results and Discussion

NH₃ and H₂CO₃ generation

H. pylori is a neutrophile that encounters low pH at its site of infection, the gastric mucosa. Gastric colonization by *H. pylori* is facilitated by its ability to maintain periplasmic pH near neutrality in highly acidic environments through the mechanism of acid acclimation (19). Acid acclimation relies on the urease system (urease and its ancillary proteins including UreI, a proton gated urea channel), two carbonic anhydrases and perhaps other NH₃⁺ or urea producing proteins (RocF, AmiE and AspA). The periplasm is buffered to a pH of 6.1 by NH₃, produced through the hydrolysis of urea by urease, entering the periplasm from the cytoplasm and by HCO₃⁻ generation via carbonic anhydrase in the periplasm (20). However, measurement of cytoplasmic pH under identical conditions used for RNASeq analysis (pH 3.0, 4.5, 6.0 vs non-acidic pH for four hours) showed a progressive fall in cytoplasmic pH, reaching 6.2 at an external pH of 3.0 (Figure 1), suggesting that, in addition to sensing and responding to extracellular acidification, *H. pylori* gene expression is also dependent on cytoplasmic pH. The pH_{in} of 7.8 at pH 8.0 indicates an outward proton gradient (21) however, at the acidic pH values used in this study, there is always an inward pH gradient.

In the current study, RNA-seq analysis revealed that all the genes of the urease cluster increased transcription at pH 6.0 relative to pH 7.4, with the exception of *ureF*, which was not detected. The level of expression of these genes was maximal at pH 6.0, then declined with increasing acidity (Table 3). *ureB* and *ureA* transcripts were induced at pH 6.0 with respect to pH 7.4 by nearly 3 and 4-fold respectively. There was a threefold induction of the *ureI* transcript at pH 6.0 as compared to pH 7.4 (Table 3). Increased transcription of *ureI* was also reflected in the UreI protein abundance as determined by quantitative Western analysis (Figure 2). However, at pH 3, *ureI* transcription was not induced (1.48) and failed to reach statistical significance (p=0.16). Similarly, induction of *ureE*, *ureG* and *ureH* was maximal at pH 6.0 (Table 3). The pattern of expression of the urease genes is consistent with RNA degradation analysis in which the RNA was most stable at pH 6.0 or by acid induced auto-repression of the ArsRS two-component system (TCS) that regulates expression of the urease gene cluster (22, 23).

The pH dependent transcription of the urease gene cluster revealed by RNAseq was confirmed by qRT-PCR. Figure 3 shows the expression ratio of pH 3.0, 4.5 and 6.0 with pH 8.0 as the denominator for *ureB* and *ureI*, with greater than a 2-fold change at 4 hours. In addition to pH dependent transcription changes of the urease gene cluster, we also wanted to know if the discrepancies among the various acid transcriptome studies was a function of incubation time (10–13). *H. pylori* was incubated at pH 3.0, 4.5, 6.0 and 8.0 for 30, 60, 120 and 240 minutes followed by qPCR for the detection of genes in the urease operon. *ureB* and *ureI* gene expression was unchanged when acid challenged for up to two hours. However, *ureB* and *ureI* gene expression increased greater than 3-fold after 4 hours of incubation. This pattern of expression was similar for the other members of the urease gene cluster. These results explain that the differences in acid dependent gene transcription among the various transcriptomal studies is at least partially due to length of acid exposure. For example, the genes of the urease operons were highly induced *in vivo*, where *H. pylori* is continually

exposed to acid, while 30 minutes of acid exposure in the presence of urea resulted in no change in transcript levels (9, 10).

The urease gene cluster is composed of 2 operons, *PureA* and *PureI* (23). The *PureA* promoter regulates gene transcription of *ureA* and *ureB*, encoding the catalytic subunits of urease, while *PureI* regulates the transcription of the urease accessory genes (*ureE-ureH*), which encode proteins needed for nickel insertion into apoenzyme and *ureI*, encoding for a proton-gated urea channel. In turn, the *ure*-promoters are regulated by the ArsRS TCS. The ArsRS TCS responds to periplasmic acidity via protonation of His94 of ArsS (*HP0165*, gene numbers in the text refer to the ATCC 26695 reference strain (24)), resulting in auto-phosphorylation and subsequent phospho-transfer to Asp52 of its cognate response regulator ArsR (*HP0166*) (25). Phosphorylation of ArsR leads to increased transcription of the urease genes and decreased transcription of *arsS* and *arsR* genes encoding for the ArsRS TCS (Table 3).

The transcriptional analysis of the genes encoding α -carbonic anhydrase (*aca*) and arginase (*rocF*) in response to changes in pH was somewhat different from the response of the urease gene cluster. Transcription remained high at both pH 4.5 and 3.0, suggesting another level of regulation, possibly through the cytoplasmic histidine kinase, FlgS (26). α -carbonic anhydrase is required for buffering of the periplasm since the pK_a of the NH_4^+/NH_3 couple is too high (9.26) for effective buffering at pH of ~ 6.1 , the periplasmic pH in the presence of urea when the pH_{out} ranges from 3.0–6.0. Arginase produces urea used either as a nitrogen source or a safety measure in case gastric juice urea becomes too low. Arginine is not required for acid acclimation *in vivo* but is necessary *in vitro* in the absence of urea (27).

The ammonia producing enzyme, aspartate-ammonia lyase (*aspA*) was mildly induced (1.54 fold) as the pH decreased from non-acidic to pH 6.0 but not at pH 4.5 or 3.0. The ammonia producing formamidase (*amiF*) was repressed as acidity increased. Acid repression of *amiF* contrasts with previous acid transcriptome analyses by Merrell et al and Ang et al, which showed *amiF* induction (11, 13). In the current study, in contrast with prior work, 5 mM urea was included in the incubation medium, suggesting that urease activity produces sufficient ammonia to combat acidity and *AmiF* is only needed when urease activity is insufficient to raise periplasmic pH in acid due to low gastric urea concentrations. The other ammonia producing enzymes, peptidyl-arginine deiminase (*pad*), aliphatic amidase (*amiE*), L-asparaginase II, (*ansB*), and hydrogenase accessory protein (*hypA*), trended to no change or toward repression in the presence of increasing acidity without statistical significance.

F₀F₁-ATPase

These genes represent the mitochondrial complex responsible for either ATP synthesis or proton export as a function of ATP hydrolysis. In general, the genes that encode for the F₀F₁-ATPase were induced at pH 6.0, 4.5 and 3.0 when compared to pH 7.4 (Table 3). The F₁ epsilon subunit was induced at pH 6.0 and 4.5 when compared to pH 7.4 arguing for increased expression of the ATP synthase and a decline in ATPase activity (28). This subunit inhibits the ATPase activity of the F₀F₁-ATPase complex, leaving the ATP synthase activity intact (29). Transcription of the gene encoding the pore forming B-subunit was induced at

pH 3.0 while transcription of the B'-subunit was not affected by acid. Transcription of the A and C subunits was induced at all acidic pHs studied when compared to pH 7.4 (Table 3). There are at least two possible explanations for the increased transcription of several of the genes encoding the F₀F₁-ATPase in *H. pylori* in acid; acid exposure either increases the metabolic demands of the bacterium requiring increased ATP synthesis or reverses the pump to extrude protons to maintain cytoplasmic pH homeostasis at the expense of ATP. To distinguish between these two possibilities, cytoplasmic pH was monitored with the pH-sensitive fluorescent probe BCECF in the presence or absence of the F₀F₁-ATPase inhibitor DCCD. DCCD addition did not alter cytoplasmic pH, showing that the F₀F₁-ATPase does not reverse to expel protons from the cytoplasm during an acid challenge. DCCD is bactericidal at pHs > 6.0 but not below (30). The p*H*_{in} of *H. pylori* at p*H*_{out} 6.0 and 7.4 is ~7.8, resulting in an inward pH gradient with the F₀F₁-ATPase acting as an ATP synthase, not an ATPase. As the pH of the medium is raised, the F₀F₁-ATPase is required to allow proton entry to maintain a viable internal pH. Therefore, inhibition of the F₀ subunit by DCCD prevents proton entry, resulting in excessive alkalization and bacterial death. Thus, the F₀F₁-ATPase does not act as a proton exporting ATPase at high medium pH but as an ATP synthase (Figure 4A). In contrast, antimycin A inhibition of the cytochrome *bc*₁ complex activity resulted in a rapid decline in cytoplasmic pH due to loss of electron transfer and subsequent loss of proton translocation to the periplasm (Figure 4B). Therefore, the electron transport chain is mainly responsible for proton extrusion in acid, not the F₀F₁-ATPase. These results are in accord with previous data that found no loss of survival after a one hour acid challenge with pump inhibition by DCCD and is also consistent with the increase in the epsilon subunit that inhibits the F₀F₁-ATPase, sparing the ATP synthase activity of the complex (29–31). Thus, when *H. pylori* is exposed to an acidic medium pH, the F₀F₁-ATPase acts as an ATP synthase, not an ATPase. This is presumably due to the requirement for ATP for a variety of reactions in the organism that are not supplied by substrate metabolism and emphasizes the need for acid entry at high medium pH to allow survival. These data are substantiated by the high level of induction of the epsilon subunit that inhibits the ATPase activity of the F₀F₁-ATPase {Keis, 2006 #194} in the RNA-seq data (Table 3) that show a consistent induction of the F₀F₁-ATPase genes across the pH range from 6.0 to 3.0.

Metabolism

The *H. pylori* genome encodes for proteins that constitute the major metabolic pathways that include the TCA cycle, the Entner-Doudoroff pathway, the glycolytic pathway and the pentose phosphate pathway (24). Early on, there was controversy as to whether *Helicobacter pylori* had a complete or modified oxidative citric acid cycle because malate dehydrogenase, malate synthase and α-ketoglutarate open reading frames (ORFs) were not identified in genomic sequence of *H. pylori* (24). However, biochemical studies detected activity of these three enzymes, suggesting that *H. pylori* does indeed have a complete citric acid cycle (32). Many of the genes that comprise the TCA pathway were induced at pH 6.0 as compared to pH to pH 7.4 with the exception of ferredoxin, which was repressed by >2-fold (Table 3). Citrate synthase (*gltA*), located in the cytoplasm, catalyzes the reaction: Acetyl-CoA + H₂O + oxaloacetate = citrate + CoA, the first step of the TCA pathway that synthesizes citrate

from oxaloacetate. Citrate synthase was induced by nearly 3-fold at pH 6.0, ~2 fold at pH 4.5 and was not induced at pH 3.0. *acnB*, the gene that encodes for aconitase B, the enzyme that catalyzes the reversible isomerization of citrate to isocitrate via cis-aconitate, was not induced at pH 6.0 or pH 4.5 but was repressed at pH 3.0, showing the same pattern of expression as *glcA*, namely pH 6.0 > pH 4.5 > pH 3.0. This pattern of decreasing induction or increasing repression with increasing acidity (pH 6.0 > pH 4.5 > pH 3.0) is exemplified by many of the genes encoding for metabolic enzymes across all pathways. The only exception to this expression pattern in the TCA pathway is the expression of the OorD subunit of the 2-oxoglutarate oxidoreductase (*oorD*), whose expression pattern is pH 3.0 > pH 4.5 > pH 6.0. 2-Oxoglutarate oxidoreductase (OOR) of *H. pylori* is a heterotetramer consisting of four subunits, OorA, OorB, OorC and OorD, with their genes arranged in the order 5'-*oorD-oorA-oorB-oorC*-3' (33). Induced expression of *oorA* and *oorB* at pH 6.0 with no change in expression at pH 4.5 or 3.0 is indicative of the ATR that induces expression of genes at mild acidity, which is required to combat subsequent high acidity. It is expected that increased metabolism is required to energize the various acid resistant mechanisms expressed by *H. pylori*.

The expression of genes encoding for the enzymes of the Entner-Doudoroff pathway, glucose-6-phosphate 1-dehydrogenase (*zwf*, *g6pD*), phosphogluconate dehydratase (*edd*) and 2-keto-3-deoxy-6-phosphogluconate aldolase (*eda*) displayed the same expression pattern as many of the genes of the TCA cycle, pH 6.0 > pH 4.5 > 3.0. This expression pattern suggests the Entner-Doudoroff pathway is part of the ATR.

The expression pattern of genes of the glycolytic pathway decreased induction or increased repression with increasing acidity with the exception of phosphoglycerate kinase (*pgk*), which was nearly equally induced at pH 6.0, 4.5 and 3.0. Phosphoglycerate kinase catalyzes the reaction: ATP + 3-phospho-D-glycerate = ADP + 3-phospho-D-glyceroyl phosphate as step 2 of the pathway that synthesizes pyruvate from D-glyceraldehyde 3-phosphate. It is interesting that the genes encoding for the enzymes for steps 3 and 4 of the pathway, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (*pgm*) and enolase (*eno*), respectively, were repressed when acid challenged. At pH 3.0 most of the genes of the glycolytic pathway were either repressed or did not change expression, suggesting that glycolysis is reduced or inhibited at high acidity and therefore not needed as an energy source for acid resistance. At pH 6.0 about half of the genes of the glycolytic pathway were repressed or unchanged, indicating that it is unlikely glycolysis is part of the ATR. However, *gluP*, encoding the glucose/galactose transporter, was induced > six-fold at pH 6.0, perhaps in response to the absence or low abundance of medium glucose.

Only genes encoding for the non-oxidative phase of the pentose phosphate pathway changed expression in acid. *rpe* and *tktA*, the genes encoding for the enzymes ribulose-phosphate 3-epimerase and transketolase, respectively were repressed with the expression pattern of increasing repression with increasing acidity. *tal*, the gene encoding for transaldolase was induced in acid.

The pattern of expression of many genes encoding for the various metabolic pathways, namely induction, was greater at pH 6.0 than pH 4.5 and pH 3.0. This expression pattern

suggests that mild acidity (pH 6.0) induces the acid tolerance response in *H. pylori* by preparing the organism to resist stronger acidity and by meeting increased metabolic demand imposed by acid resistance mechanisms.

Antioxidant genes

H. pylori colonization of the stomach results in a robust ROS burst from inflammatory cells in the gastric mucosa to facilitate clearance of the bacterium. In response to ROS generation, *H. pylori* expresses at least 14 antioxidant proteins to combat ROS degradation of proteins and DNA (24). In addition, *H. pylori* also expresses DNA and protein repair enzymes to counter ROS induced damage but these are not discussed further, since they are not antioxidants per se. The mechanism of regulation of expression of the antioxidant genes is unknown.

All eight genes encoding for antioxidant proteins that attained statistical significance were induced at pH 6.0 as compared to pH 7.4 (Table 3.). As acidity increased (pH 4.5 and 3.0), the level of induction decreased. *kata*, the gene encoding for the enzyme catalase, was induced greater than 4-fold at pH 6.0 and greater than 2-fold at pH 4.5 and 3.0. It was also one of the most highly expressed genes in the genome, reflecting that it constitutes nearly 1.5% of total cellular protein (34) and catalyzes the reaction $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$. Catalase is localized to both the periplasm and cytoplasm (35). Transcription of the gene encoding the peroxiredoxin, alkylhydroperoxide reductase (*ahpC*) was neither induced nor repressed by an acid challenge. However, in one study, AhpC protein expression decreased in response to oxidative stress (36), while more recently it was shown to increase by 2-fold under oxidative stress conditions (37). This enzyme reduces a variety of peroxides at the same rate and can also inactivate peroxynitrite (38). The generation of peroxynitrite is hindered by the bacterial expression of arginase, removing the source of the nitrite in the host. Superoxide dismutase (*sodB*) gene expression was induced nearly five-fold with a mild acid challenge (pH 6.0) and 3-fold with greater acidity (pH 4.5 and 3.0). *H. pylori*/AGS cell co-culture gene expression analysis found that *sodB* gene transcription was down-regulated with attachment to host cells, suggesting that SOD is important for initial infection but not for persistence (39). This enzyme catalyzes the reaction $2\text{O} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$ and the resulting peroxide is inactivated by catalase. Thus, the enzymes SOD and catalase work in concert to counteract ROS.

H. pylori also expresses thioredoxin reductase (TrxR) and 2 thioredoxins (Trx) that also counteract oxidative stress (40). The organism does not express glutathione, making the expression of TrxR and Trx important for oxidative and nitrosative stress resistance (24, 41). Methionine sulfoxide reductase is also important and is Trx and TrxR dependent, reversing methionine oxidation and restoring protein activity (42).

The current study used neither host cells to generate ROS nor added ROS. Therefore, the acid-induced increased transcription of *H. pylori* genes that encode for antioxidant proteins suggests that increasing acidity is the significant driver for increased expression. Acid-induced transcription of these genes and subsequent protein expression would be advantageous for the bacterium to mount an effective defense against ROS when infecting

the gastric mucosa and to maintain persistence. The data here strongly suggest that pH is important in regulation of these genes and that the organism prepares itself for infection with the first appearance of acidic pH of 6.0.

Motility

Motility is essential for infection (43). Over 50 genes have been identified that contribute to *H. pylori* motility and chemotaxis (44). There are 3 structures that make up the flagellum, a basal body embedded in the inner membrane that determines rotation and chemotaxis, the helical filament and the hook connecting the basal body and the filament itself. In contrast to other bacteria, these genes in *H. pylori* are not contiguous but are scattered throughout the chromosome. Analysis of the genome shows that there are 3 sigma factors, σ^{80} , σ^{54} and σ^{28} , that regulate flagellar transcription.

Genes involved in motility and chemotaxis (and their encoded proteins) are classified into 4 groups based on their temporal and spatial expression. Class 1 genes encode many flagellar regulatory proteins such as FlgR (HP0703), FlgS (HP0244), RpoN (σ^{54} , HP0714), FlhA (HP1041), MotA (HP0815) and MotB (HP0816) and some proteins responsible for chemotaxis. The two-component system, FlgRS and the sigma factor, σ^{54} regulate the expression of the class 2 genes encoding for proteins that constitute the middle structural unit of the flagellar apparatus. In our RNA-seq analysis, the histidine kinase sensor encoded by *flgS* and its cognate response regulator, *flgR*, were only induced at pH 6.0. However, the gene encoding σ^{54} (*rpoN*) was repressed at pH 6.0 and pH 4.5, as were half of the other class 1 genes (Table 3). The genes encoding the flagellar motor proteins *motA* and *motB* were repressed by more than 2-fold at all acidic pHs as compared to pH 7.4. *cheA*, encoding the chemotactic histidine kinase, was induced with mild acidity (pH 6.0) but was unchanged at pHs 3.0 and 4.5 as compared to pH 7.4 while its cognate phospho-receptor, *cheY* was at repressed at all acidic pHs tested as compared to pH 7.4. Like *cheA*, the methyl-accepting chemotaxis protein encoding genes *tlpA* and *tlpB* were induced only with mild acidity (pH 6.0) while *tlpC* was induced only at pH 3.0.

The intermediate class of motility genes includes *flgM*, which was induced at pH 3.0 (Table 3). This protein is secreted, relieving suppression and allowing synthesis of the class 3 genes. These class 3 genes (Table 3) are regulated by FlhA/ σ^{28} and *flhA* is regulated by FlgM/anti- σ^{28} . Both genes changed in parallel with decreased transcription at pH 6.0 as compared to pH 7.4 and had a 2-fold increase at pH 3.0. The class 3 genes encode for proteins that constitute the late flagellar structure and all increased expression with increasing acidity with the exception of *flaA*, which failed to reach statistical significance but trended to no change in expression with an acid challenge.

FlaA and FlaB, encoded by the genes, *flaA* and *flaB* respectively, comprise the flagellar filament. *flaA* transcription was unaffected by acidity, while *flaB* was induced with decreasing pH. Merrell et al found an increase in *flaB* message at pH 5.0 after 30 minutes exposure but no longer (up to 2 hours) as compared to pH 7.0 but did not detect any changes in the transcription of *flaA* (11). Wen et al detected increased transcription of *flaB* and also *flaA* after a 30 minute acid challenge at pH 4.5, as did Dong et al after a prolonged acid

challenge (5 passages) (10, 45). The difference in expression of the *flaA* and *flaB* genes may be dependent on the growth phase. The previous acid transcriptome studies were of short duration (30 minutes – 2 hours), with the bacteria more likely to be in lag phase given the slow growth rate of *H. pylori*. In the current study, the bacteria were acid challenged for 4 hours, likely placing them at least in early growth phase. It has been shown that *flaB* expression occurs earlier in the growth phase than expression of *flaA*, thus accounting for the difference in expression of *flaA* and *flaB* (46).

The intermediate class of flagellar genes were mostly down-regulated or unchanged when acid challenged for 4 hours. In particular, a number of genes in this class were repressed at pH 6.0. Notable exceptions were *flhA*, *flhF*, *flgI*, and *flgM*, which were repressed or did not change at pH 6.0 but were induced at pHs 3.0 or 4.5. These genes are regulators of flagellar biosynthesis and their increased transcription suggests an impending increase in flagellar synthesis that perhaps would have been seen more clearly with a more prolonged acid challenge later in the bacterium's growth cycle. In general, it is clear that motility is positively pH regulated, requiring the cooperation of several genes that are translated in order to correctly assemble the flagellar system, generating the motility essential for infection (43).

Outer Membrane Proteins (OMPs)

Adherence of *H. pylori* to the gastric epithelium is a necessary step in establishing successful infection because it provides protection from clearance mechanisms such as bulk liquid flow, gastric peristalsis and the continuous shedding and replenishment of the mucus layer. *H. pylori* has evolved specific virulence determinants on their outer-membrane which recognize distinct protein, proteolipids or carbohydrates expressed on epithelial cells. These virulence factors are adhesins that bind to mucins in the gastric mucus and receptors on the surface of the gastric mucosa.

H. pylori expresses about 64 OMPs which can be organized into at least 5 paralogous gene families. Family 1 is composed of the Hop and Hor genes. These genes encode for adhesion proteins and include the well-studied BabA/B/C, SabA/B and AlpA/B. BabA is regulated by phase variation through a slipped strand mechanism (SSM). In the current study, although the *babA* and *babB* data failed to reach statistical significance, acid exposure had no effect on *babA/B* transcription (Table S1).

H. pylori infection of the gastric mucosa results in chronic active gastritis. This inflammation results in the replacement of the naturally produced Lewis antigens and the expression of sialylated glycans such as sialyl-Le^a and sialyl Le^x (47). In the absence of BabA, an adhesin was identified that binds to sialyl Le^x antigen and named SabA (48). As with *babA*, *sabA* expression is regulated by SSM and was also shown to be inversely related to gastric acidity (49). The acid transcriptomes performed by Merrell et al and Bury-Mone et al also found repression of both the *sabA* and *sabB* genes in acid. These results are in contrast with the results of the current RNAseq study, which found that the *sabA* and *sabB* genes were highly induced by acid exposure, 10 and 4-fold, respectively (Table 3). The differences in the experimental conditions used among the various studies may explain the

discrepancies in the results. The Merrell et al and Bury-Mone et al acid transcriptomes were made in the absence of urea and both groups did not control for changes in medium pH due to bacterial metabolism and urease activity. In the current study both these parameters were controlled for, suggesting that the presence of urea may induce *sabA* and *sabB* expression in acid. Hence, acid and urea may act as a signal to the bacterium that it is in the gastric environment and now requires robust expression of adhesins to maintain infection. There is evidence that *sabA* is a member of the ArsRS acid responsive regulon (22). Gene expression of SabA wild-type *H. pylori* and an isogenic ArsS histidine kinase mutant, found *sabA* was derepressed in the mutant (50). Since the ArsRS two component system negatively autoregulates, it is not surprising that *sabA* gene expression is induced in acid. Additionally, it was shown that ArsR binds to the *sabA* and *sabB* promoters, more evidence that their expression is acid induced (51).

H. pylori isogenic mutants lacking the OMP HopZ failed to adhere to the gastric carcinoma derived AGS cells (52), indicating that it is an adhesion, but its receptor is unknown. Like *sabA*, *hopZ* expression is regulated by SSM, however, in this study, transcription was acid induced. The acid transcriptome analysis of Merrell et al indicated that acid repressed transcription of *hopZ*. Two other OMPs, AlpA and AlpB, have been identified as putative adhesins due to loss of binding to gastric epithelium of their respective isogenic mutants (53) and, as with HopZ, the host receptors have not been identified. Like the adhesins discussed above, *alpA* and *alpB* were repressed in the acid transcriptome study by Merrell et al and induced in acid in this study.

The OMP family 2 genes encode for OMPs of unknown function. *hofA* and *hofE* were repressed in acid, while *hofB* was induced. Previous transcriptome studies found no change of expression of these genes. The only member of the OMP family 3 genes that changed expression in acid was *homA*. In this study, *homA* was induced in acid. The acid induction of *homA* contrasts with the work by Bury-Mone et al that showed acid repression of this gene (12). The difference may be due to the presence of urea in the current study.

The OMP family 5 genes encode for efflux pump proteins. The *hefA* gene encodes the outer membrane protein of the HefABC efflux system involved in resistance to antibiotics (54). The resistance-nodulation-division (RND) family of efflux systems is widespread among gram-negative bacteria. They are associated with bacterial resistance to antibiotics. *hefA*, *hefD* and *hefG* encode for a homolog of the *E. coli* TolC protein of the AcrAB-TolC system. In the current study, *hefA* was induced at pHs 4.5 and 3.0, while *hefD* and *hefG* were repressed with mild acidity (pH 6.0).

Several OMPs that are not part of the OMP family categorization changed expression in acid. Among these was HP0726, which was repressed in acid in this study but was induced at pH 4.0 in the absence of urea after 30 minutes incubation (55). HP0785, a periplasmic chaperone involved in membrane biogenesis, was induced in acid. The gene encoding an outer membrane protein, *plpA* (HP1564), belonging to the lipoprotein-28 superfamily and showing similarity to a *Pasteurella haemolytica* lipoprotein 1, was induced 3-fold in acid (56). The acid induction of these two OMPs is perhaps to respond to membrane damage or to increase the protective response of the membrane to protons. Recognizing that most

OMPs express porins (57), it will be important to identify the transport properties of these porins, with special attention to proton flux and the transport of urea.

Type 4 Secretion System (T4SS)/Cag pathogenicity island (PAI)

The bacterial type IV secretion systems move DNA or protein substrates into other bacteria or into eukaryotes. The T4SS is a nanomachine composed of 4 distinct complexes; (a) the type IV coupling protein, a hexameric ATPase (b) an inner membrane complex transferring substrate across the inner membrane (c) an outer membrane complex transferring substrate across the periplasm and outer membrane and (d) the conjugative pilus that contacts the host cell composed of several different subunits in *H. pylori* (58). The T4SS regulon of *H. pylori* G27 strain reflects the pathogenicity island obtained by horizontal transfer of DNA from other organisms and is controlled by 10 promoters (59). In *H. pylori*, the T4SS is SEC independent and is involved in DNA transfer and injection of CagA, a pathogenic protein, into gastric epithelial cells (60). In our analysis, for about 60% of the genes that comprise the T4SS regulon, transcription was acid induced and generally increased with increasing acidity (Table 9). Exceptions were *cag16* (CagM), which is unique to the *H. pylori* T4SS, and *cag26* (CagA), with the former being repressed at pH 6.0 and 4.5 while the latter was repressed at pH 3.0. Three of the genes of the $P_{Cag\zeta}$ operon, *cag2* through *cag4* (HP0521-HP0523), were acid induced at pH 3.0. The remaining member of this operon, *cag1* (HP05200), failed to attain statistical significance. Five of the 6 of the P_{CagV} (*cag5*, *cag6*, *cag9*, *cag10* and *VirB11*) regulated genes were induced at pH 3.0 with *cag6* being equally induced at all acidic pHs. *cag12*, *cag13* and *cag14* had induced transcription equally at all acid pHs when compared to pH 7.4. The genes regulated by the P_{CagF} promoter, *cag19* (CagI, HP0524), *cag20* (CagH, HP0541), *cag21* (CagG, HP0542) and *cag22* (CagF, HP0543), were acid induced at all three acid pHs studied, except for *cag19*, which failed to attain statistical significance although its transcription trended to be independent of acidity. *cag18* (CagL/VirB5, HP0539), also regulated by the P_{cagF} promoter, was acid induced at only pH 3.0. CagL (*cag18*) is a VirB5 homolog, while all the remaining members are unique to the *H. pylori* T4SS. CagL is an adhesin on the pilus surface, which binds to the integrin $\alpha 5\beta 1$ receptor on the gastric epithelial surface through an arginine–glycine–aspartate (RGD) motif (61). CagL binding initiates CagA translocation into gastric epithelial cells. Previous *in vitro*, target cell-free transcriptomal studies found no change in expression of *cagL* in acid (10, 11). It is possible that target cells are required for robust *cagL* expression, although transcriptome analyses of *in vitro* co-culture (39) or *in vivo* (9) did not detect changes in expression. *cag22* encodes for the CagA chaperone CagF (HP0543) (62) and may be the rate limiting step in CagA translocation due to its >50 fold less expression than CagA. Much less is known about the remaining members of the P_{CagF} operon and putative functions of the proteins encoded by these genes is based on protein-protein interactions and cellular localization studies (63, 64). *cag23* failed to attain statistical significance at all acid pHs but trended toward increased transcription with increasing acidity. *cag24*, a member of the P_{CagC} operon, was acid induced equally (2-fold) at all acidic pHs, while *cag25* only showed induction at pH 3.0. The proteins encoded by these genes are required for CagA translocation and IL-8 induction. Primer extension analysis of P_{CagC} showed an acid induced increase in transcription levels in accord with the increase seen in the genes of its regulon

(65). *cag25* encodes for CagC/VirB2 (*HP0546*) protein localized to the inner and outer membrane, forming the injection pilus (66). Its acid induction argues for a pH dependent formation of the pilus. *cag24* encodes CagD (*HP0545*), a periplasmic localized protein of unknown function unique to the *H. pylori* T4SS. CagD is secreted, associates with the pilus, and is required for maximum IL-8 secretion (67). *cag23* encodes for the VirB3/B4 homolog CagE (*HP0544*) and is an inner membrane associated NTPase that stabilizes pilus formation and T4SS assembly (68).

In our analysis, many of the genes that comprise the T4SS regulon displayed a transcription pattern of induction that increased with increasing acidity (Table 3). None of the proteins encoded by the genes of the CAG-PAI are necessary for acid survival/protection because CAG negative strains survive an acid challenge just as well as CAG positive strains. We suggest that acidity serves as a signal to the bacterium that it has reached its site of infection, the gastric mucosa, and the induction of the CAG-PAI genes prepares the T4SS for the injection of CagA into the host cells. It is interesting that an acid challenge had no effect on the transcription of CagA, suggesting that its regulation is dependent on *H. pylori* adherence to host cells or it is constitutively expressed at adequate levels.

Cell Division

The mechanism of bacterial cell division is a series of coordinated steps beginning with chromosome replication and segregation and ending in septation of the organism. Most of the cell division genes that were either induced or repressed in response to an acid challenge were from the *fts* and *min* loci. The genes of the *min* loci determine the division site and recruit *ftsZ* to the site to establish the Z ring. The *min* locus is composed of three genes, *minC*, *minD* and *minE*. The proper placement of the septal FtsZ ring is mediated by oscillation of a complex of these proteins and membrane placement of the complex requires all three proteins (69). MinE expression results in redistribution of MinC and D, eventually assembling into a ring like structure at mid-cell (the E ring). The average concentration of the division inhibitor, MinC is lowest in the middle of the cell. MinC deletion mutants form filamentous long cells but form short rods when complemented by wild type MinC (70). Here, all three genes were upregulated at pH 3.0. MinE, in particular, increased expression at pH 3.0 but declined at pH 6.0. In *E. coli*, the MinC and MinD proteins form a complex that inhibits cell division while MinE stimulates cell division. Competition between MinCD and MinE determines the rate of cell division (71).

Genes of the *fts* locus are involved in septum formation. After formation of the Z-ring, FtsZ recruits FtsA to the septum, and it is the FtsA and FtsZ ratio that determines initiation of cell division, with FtsA inhibiting cell division and FtsZ stimulating cell division. Transcription of *ftsA* was acid induced while *ftsZ* was acid repressed, indicating inhibition of cell division. *E. coli* regulates the expression of the *ftsA* and *ftsZ* genes via the two-component system, ResCB (72). Since *H. pylori* has only four complete TCSs of which two, ArsRS and FlgSR, sense and respond to acidity, it is tempting to speculate that the *ftsA* and *ftsZ* genes, in responding to acidity, are regulated through one of these acid sensing TCSs (26, 73).

Acid exposure repressed the transcription of three other genes in the *fts* locus, *ftsH*, *ftsK* and *ftsX*. *ftsH* is represented by two genes in the *H. pylori* genome, HP0286 and Hp1069, which is essential for cell division (74). Repression of HP0286 suggests that the bacterium is in a non-dividing state. On the other hand, HP1069 was induced in acid. In *E. coli*, *ftsH* encodes for an ATP-dependent zinc protease that has been shown to degrade SecY, the F₀ subunit, sigma 32 and EnvA (75, 76). Given that the two *ftsH* genes respond reciprocally to acid exposure, it is possible to speculate that the role of HP1069 is regulation of cell division while the role of HP0286 is mainly as a protease but is also involved with disassembling the septum. *ftsK* and *xerC* may be involved in the dimerization of the nascent chromosome and both were repressed when acid challenged (77).

The cell division acid transcriptome indicates that under the conditions used in this study, the bacterium is in a non-dividing state but poised to replicate, as evidenced by repression of most of the *fts* genes and induction of the *min* genes.

Conclusion

H. pylori has had to develop a unique response to acidity to facilitate acid acclimation and persistent gastric habitation. This response requires an ability to sense changes in environmental/periplasmic and cytoplasmic pH, and initiate a rapid and sustained change in transcription of relevant genes. Genes involved with acid acclimation, motility, F₀F₁-ATPase, reactive oxygen species, and T4SS/Cag pathogenicity island all showed increased expression at acidic pH, with some genes increasing more significantly at different extremes of acidity. Measurement of internal pH confirmed that, despite tight regulation by the bacteria, they do see some degree of pH change in response to external acidity, and even mild acidity can trigger important transcriptional alterations. As indicated by qPCR over several time points, many of these changes are more pronounced with longer time of exposure to acidic pH, consistent with the need for *H. pylori* to colonize the stomach rather than simply transit it on the way to less harsh environments in the more distal GI tract. The ability of *H. pylori* to sense acidity is critical not only to trigger acid acclimation, but also to stimulate transcription of virulence factors needed for colonization. Exposure to acid and urea are critical for all facets of gastric survival. With the current challenges in treating *H. pylori* infection and the worldwide disease burden, it is important to focus on development of non-antibiotic treatment targets for eradication of infection. The first step in this process is understanding how the bacteria can colonize their host. *H. pylori* relies on a wide range of transcriptional changes across different functional groups of genes to allow for persistence in the harsh gastric environment. Alteration of or interference with any of these adaptive responses, or with the ability to sense environmental pH, present attractive targets for eradication of infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BCA	bicinchoninic acid
BHI	brain heart infusion
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCS	two component system
TSA	Trypticase Soy Agar
pmf	proton motive force
DCCD	N, N'-dicyclohexylcarbodiimide
BCECF-AM2,7-bis-(2-carboxyethyl)-5-carboxyfluorescein acetoxymethyl ester	
PPI	proton pump inhibitor
ROS	reactive oxygen species
YE	yeast extract

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Figure 1. The effect of external pH on cytoplasmic pH

Measurement of cytoplasmic pH showed a progressive fall in cytoplasmic pH, reaching 6.1 at an external pH of 3.0. At this cytoplasmic pH (6.1), the pmf is almost entirely due to the ψ . *H. pylori* was incubated for 4 hours at pH 3.0, 4.5, 6.0 and 8.0 in the presence of 5 mM urea. Cytoplasmic pH was measured using the pH-sensitive fluorescent dye BCECF-AM.

Figure 2. UreI protein expression is pH dependent

The protein level of UreI increased at pH 6.0 as compared to pH 8.0, reflecting the pattern of gene expression determined by RNA-seq analysis. (A.) Representative Western blot (1 blot with intervening lanes removed) of pH-dependent UreI expression. (B.) Densitometry analysis of UreI expression. *H. pylori* was incubated for 4 hours at pH 6.0 and 8.0 in the presence of 5 mM urea. n=3, error bars represent standard deviation.

Figure 3. Expression of urease gene cluster genes increases over time at acidic pH

RT-qPCR was completed after incubation of *H. pylori* at pH 3.0, 4.5, 6.0, and 8.0 for the indicated time periods. *ureB* is shown in (A.) and *ureI* is shown in (B.). Both genes showed >2-fold increase in expression in acid at the four-hour time point, with minimal to no change prior to this time. Error bars represent standard deviation, n=3.

Figure 4. The F_0F_1 -ATPase does not reverse to translocate protons from the cytoplasm to the extracellular space to maintain internal pH in acid
(A.) Addition of the F_0F_1 -ATPase inhibitor DCCD did not change cytoplasmic pH in acidic medium. (B.) In contrast, antimycin A inhibition of the cytochrome *bc1* complex activity resulted in a rapid decline in cytoplasmic pH due to loss of electron transfer and subsequent loss of proton translocation to the periplasm.

Table 1

Comparison of experimental conditions of acid transcriptome studies in *H. pylori*.

	This study	Wen, etal 2003	Merrell, etal 2003	Ang, etal 2001	Bury-Mone, etal 2004
<i>H. pylori</i> strain	G-27	26695	G-27	NTU-D1	26695, SS1
Transcriptome analysis	RNAseq	Fluorescent microarray	Fluorescent microarray	colometric microarray membrane	radiolabeled microarray membrane
media	BHI, YE	BHI	Brucella broth + 10% FBS	Columbia agar, 5% sheep blood	BHI, 0.2% β -cyclodextrin, antibiotic/fungicide mix
pH	7.4, 6.0, 4.5, 3.0	7.4, 6.2, 5.5, 4.5	5.0	7.2, 5.5	7.0, 5.0
pH stat	yes	no	no	no	no
Urea concentration	5 mM	+/- 5 mM	0 mM	0 mM	0 mM
Time	4 hours	30 minutes	0,30, 60, 90, 120 minutes	48 hours	4 hrs (pH 7.0), 9 hrs (pH 5.0)
Atmosphere	10% CO ₂ , 5% O ₂ , 85% N ₂	10% CO ₂ , 5% O ₂ , 85% N ₂	<i>I</i> microaerobic	10% CO ₂ , 5% O ₂ , 85% N ₂	<i>I</i> microaerobic

I gas concentrations not provided

Table 2

Primer sequences used-used for qPCR

Gene name	NCBI Reference	HP G27 ID	Sense (5'-3')	Anti-sense (5'-3')
<i>ureA</i>	HP0073	HPG27_RS00385	AACCGGATGATGTGATGGAT	GGTCTGTGCGCCAAACATTTT
<i>ureB</i>	HP0072	HPG27_RS00380	CTTGGCAAAACAGCTGACAAA	GTTGGGTTTTTACGCCCAAAGA
<i>ureI</i>	HP0071	HPG27_RS00375	TTCCCTGCTGCGGATTTTATCC	CATCTCACACCCAGTGTGG
<i>ureE</i>	HP0070	HPG27_RS00370	CAAGTTGGGGGCTCTCTCAAG	GTGGGCTTTTCAAATGGTGT
<i>ureF</i>	HP0069	HPG27_RS00365	TGCGCAAAACTTCTAAACATGG	GCATCGCCTTAATGTCGTTT
<i>ureG</i>	HP0068	HPG27_RS00360	GGTAAACCGCCCTTGATTGA	CGGACAGCC'TCCTGTTTCTA
<i>ureH</i>	HP0067	HPG27_RS00355	TGCAATTAAACATCGGTCCA	CGTGGTGTGGCCCTTAAAT
<i>gyrB</i>	HP0501	HPG27_RS02415	GAAAGCGTGATGGAAATCGT	GAAATAATGGGGGTGAGCAA

Table 3
Acid regulated genes from *Helicobacter pylori* strain G27 determined by Differential Gene Expression RNAseq

Gene name	NCBI Reference	Fold Change pH 3.0/7.4	sem	p value	Fold Change pH 4.5/7.4	sem	p value	Fold Change pH 6.0/7.4	sem	p value	a.	b.	c.	d.	Annotation
NH₃ and H₂CO₃ generation															
HPG27_RS00355	HP0067	1.57	0.20	0.11	2.01	0.20	0.03	2.67	0.40	0.05	↑				urease accessory protein (<i>ureH</i>)
HPG27_RS00360	HP0068	1.93	0.22	0.05	2.27	0.23	0.02	3.45	0.52	0.04	↑				urease accessory protein (<i>ureG</i>)
HPG27_RS00375	HP0071	1.59	0.25	0.16	2.41	0.34	0.04	3.14	0.61	0.06	↑				proton-gated urea channel (<i>ureI</i>)
HPG27_RS00380	HP0072	1.08	0.10	0.49	1.69	0.17	0.05	4.12	1.02	0.09	↑				urease beta-subunit (<i>ureB</i>)
HPG27_RS00385	HP0073	1.34	0.07	0.04	1.95	0.17	0.02	2.76	0.37	0.03	↑			↑	urease alpha-subunit (<i>ureA</i>)
HPG27_RS03165	HP0649	-1.22	0.03	0.03	-1.04	0.04	0.30	1.55	0.11	0.03	↑				aspartate ammonia-lyase (<i>aspA</i>)
HPG27_RS05900	HP1186	4.86	0.67	0.02	4.22	0.60	0.03	6.28	1.15	0.04	↑			↓	α-carbonic anhydrase (<i>aca</i>)
HPG27_RS07635	HP1399	4.82	0.29	0.00	5.83	0.28	0.00	6.73	0.40	0.00	↑				arginase (<i>rocF</i>)
HPG27_RS01475	HP0294	-1.91	0.10	0.04	-1.25	0.16	0.28	-1.02	0.16	0.47	↑			↑	aliphatic amidase (<i>amiE</i>)
HPG27_RS06200	HP1238	-1.92	0.03	0.00	-1.76	0.04	0.01	-1.46	0.03	0.01	↑			↑	formamidase (<i>amiF</i>)
F₀F₁-ATPase															
HPG27_RS04065	HP0828	1.99	0.06	0.00	1.80	0.04	0.00	2.39	0.26	0.03	↓				ATP-synthase Fo A subunit (<i>atpB</i>)
HPG27_RS05645	HP1136	1.98	0.08	0.00	1.24	0.05	0.04	-1.21	0.03	0.02					ATP synthase Fo B subunit (<i>atpF</i>)
HPG27_RS06075	HP1212	1.57	0.13	0.04	2.10	0.14	0.00	2.21	0.14	0.00					ATP synthase Fo C subunit (<i>atpE</i>)
HPG27_RS05635	HP1134	1.23	0.10	0.17	1.64	0.13	0.03	3.39	0.74	0.09	↓			↑	ATP synthase F1 α-subunit (<i>atpA</i>)
HPG27_RS05630	HP1133	1.74	0.10	0.01	1.93	0.15	0.02	1.39	0.11	0.07					ATP synthase F1 γ-subunit (<i>atpG</i>)
HPG27_RS05640	HP1135	2.09	0.08	0.00	1.59	0.04	0.00	1.44	0.06	0.01					ATP synthase F1 δ-subunit (<i>atpH</i>)
HPG27_RS05620	HP1131	1.19	0.12	0.25	1.75	0.18	0.05	1.62	0.13	0.04				↑	ATP synthase F1 ε-subunit (<i>atpC</i>)
Metabolism															
TCA Pathway															
HPG27_RS00145	HP0026	1.31	0.05	0.01	1.71	0.12	0.02	2.82	0.48	0.06					citrate synthase (<i>gltA</i>)
HPG27_RS03800	HP0779	-2.15	0.03	0.00	-1.36	0.03	0.00	1.16	0.09	0.20					aconitase B (<i>acnB</i>)
HPG27_RS00150	HP0027	1.64	0.12	0.03	1.98	0.09	0.00	2.82	0.20	0.00					isocitrate dehydrogenase (<i>icd</i>)
HPG27_RS02860	HP0588	1.63	0.15	0.05	1.14	0.10	0.26	-1.19	0.03	0.02					OorD subunit of the 2-oxoglutarate oxidoreductase (<i>oorD</i>)

Gene name	NCBI Reference	Fold Change pH 3.0/7.4	sem	p value	Fold Change pH 4.5/7.4	sem	p value	Fold Change pH 6.0/7.4	sem	p value	a.	b.	c.	d.	Annotation
HPG27_RS02865	HP0589	-1.03	0.04	0.36	1.15	0.04	0.04	1.92	0.21	0.05					OorA subunit of the 2-oxoglutarate oxidoreductase (<i>oorA</i>)
HPG27_RS02870	HP0590	-1.03	0.07	0.40	1.14	0.06	0.14	1.64	0.13	0.03					OorB subunit of the 2-oxoglutarate oxidoreductase (<i>oorB</i>)
HPG27_RS00965	HP0191	-1.52	0.03	0.00	-1.22	0.02	0.01	1.13	0.04	0.09					fumarate reductase, iron-sulfur subunit (<i>frdB</i>)
HPG27_RS00970	HP0192	-2.56	0.04	0.00	-1.88	0.05	0.00	-1.19	0.02	0.01					fumarate reductase, flavoprotein subunit (<i>frdA</i>)
HPG27_RS06665	HP1325	-1.55	0.04	0.02	0.94	0.06	0.27	2.26	0.45	0.11					fumarase (<i>fumC</i>)
HPG27_RS00450	HP0086	1.35	0.16	0.17	1.63	0.12	0.02	2.88	0.52	0.07					Malate:quinone oxidoreductase (<i>mqr</i>)
HPG27_RS05490	HP1108	-1.29	0.06	0.06	-1.20	0.03	0.02	1.04	0.05	0.34					pyruvate flavodoxin oxidoreductase g-subunit gamma (<i>porG</i> , <i>porC</i>)
HPG27_RS05495	HP1109	-1.19	0.04	0.05	1.00	0.02	0.46	1.40	0.10	0.06					pyruvate flavodoxin oxidoreductase d-subunit (<i>porD</i>)
HPG27_RS05500	HP1110	-1.54	0.05	0.01	-1.15	0.04	0.10	1.54	0.19	0.11					pyruvate flavodoxin oxidoreductase a-subunit alpha (<i>porA</i>)
HPG27_RS05505	HP1111	1.06	0.08	0.37	1.36	0.05	0.01	2.08	0.18	0.02					pyruvate ferredoxin oxidoreductase, b-subunit (<i>porB</i>)
HPG27_RS00620	HP0121	1.42	0.03	0.00	1.68	0.04	0.00	2.20	0.21	0.02					phosphoenolpyruvate synthase (<i>ppsA</i>)
HPG27_RS01390	HP0277	-1.26	0.11	0.20	-1.79	0.09	0.03	-2.11	0.07	0.01	↑				ferredoxin (<i>fdxB</i>)
Entner-Doudoroff Pathway															
HPG27_RS05455	HP1101	1.77	0.18	0.05	1.47	0.15	0.10	1.90	0.09	0.00					glucose-6-phosphate 1-dehydrogenase (<i>zwf</i> , <i>g6pD</i>)
HPG27_RS05450	HP1100	1.44	0.04	0.00	1.66	0.04	0.00	2.04	0.08	0.00	↑				phosphoglucuronate dehydratase (<i>edd</i>)
HPG27_RS05445	HP1099	1.98	0.11	0.00	2.44	0.10	0.00	2.64	0.12	0.00	↑				2-keto-3-deoxy-6-phosphoglucuronate aldolase (<i>eda</i>)
Glycolysis															
HPG27_RS05840	HP1174	3.97	0.59	0.03	5.01	0.83	0.03	6.64	1.37	0.05			↑		glucose/galactose transporter (<i>galP</i>)
HPG27_RS05465	HP1103	0.67	0.05	0.02	0.73	0.06	0.04	0.99	0.03	0.43					glucokinase (<i>glk</i>)
HPG27_RS05800	HP1166	-1.22	0.07	0.13	-1.02	0.08	0.44	1.54	0.09	0.02	↑				glucose-6-phosphate isomerase (<i>pgi</i>)
HPG27_RS00885	HP0176	2.08	0.06	0.00	2.49	0.10	0.00	2.77	0.21	0.01					fructose-bisphosphate aldolase (<i>fba</i> , <i>tsr</i>)
HPG27_RS00980	HP0194	1.38	0.10	0.06	1.31	0.08	0.06	1.17	0.03	0.02					triosephosphate isomerase (<i>tpi</i>)
HPG27_RS04500	HP0921	-1.49	0.08	0.05	-1.85	0.07	0.02	-1.58	0.04	0.01					glyceraldehyde-3-phosphate dehydrogenase (<i>gap1</i> , <i>gap</i>)
HPG27_RS06770	HP1346	-1.46	0.03	0.01	-1.51	0.02	0.00	-1.13	0.04	0.12					glyceraldehyde-3-phosphate dehydrogenase (<i>gap2</i> , <i>gap</i>)

Gene name	NCBI Reference	Fold Change pH 3.0/7.4	sem	p value	Fold Change pH 4.5/7.4	sem	p value	Fold Change pH 6.0/7.4	sem	p value	a.	b.	c.	d.	Annotation
HPG27_RS06765	HP1345	1.62	0.06	0.00	1.53	0.07	0.01	1.86	0.18	0.04					phosphoglycerate kinase (<i>pgk</i>)
HPG27_RS04785	HP0974	-1.40	0.01	0.00	-1.53	0.03	0.00	-1.52	0.03	0.00					2,3-bisphosphoglycerate-independent phosphoglycerate mutase (<i>pgm</i>)
HPG27_RS00780	HP0154	-2.40	0.05	0.00	-1.71	0.06	0.01	-1.24	0.04	0.03					enolase (<i>eno</i>)
HPG27_RS00620	HP0121	1.42	0.03	0.00	1.68	0.04	0.00	2.20	0.21	0.02					phosphoenolpyruvate synthase (<i>ppsA</i>)
HPG27_RS07690	HP1385	2.13	0.04	0.00	2.30	0.07	0.00	3.23	0.29	0.01					fructose 1,6-bisphosphatase (<i>fbp</i>)
<i>pentose phosphate pathway</i>															
HPG27_RS05460	HP1102	0.95	0.07	0.38	0.77	0.06	0.07	0.81	0.03	0.02					6-phosphogluconolactonase (<i>pgl</i> , <i>devB</i>)
HPG27_RS07685	HP1386	-1.50	0.07	0.04	-1.92	0.05	0.01	-2.21	0.07	0.01	↑				ribulose-phosphate 3-epimerase (<i>rpe</i>)
HPG27_RS07415	HP1495	1.89	0.16	0.03	1.75	0.22	0.08	2.44	0.48	0.11					transaldolase (<i>tal</i>)
HPG27_RS01820	HP1088	0.65	0.06	0.03	0.62	0.07	0.02	0.74	0.04	0.01					transketolase (<i>tktA</i>)
<i>Antioxidant genes</i>															
HPG27_RS00690	HP0136	1.16	0.05	0.09	1.32	0.05	0.01	1.64	0.16	0.05					peroxiredoxin, bacterioferritin comigratory protein (<i>bcp</i>)
HPG27_RS01125	HP0224	1.49	0.09	0.03	2.00	0.05	0.00	2.67	0.25	0.01					methionine sulfoxide reductase (<i>msrA</i>)
HPG27_RS01220	HP0243	1.17	0.08	0.18	1.53	0.11	0.03	2.57	0.28	0.02					neutrophil activating protein (bacterioferritin) (<i>napA</i>)
HPG27_RS05265	HP0389	2.84	0.15	0.00	3.22	0.17	0.00	4.89	0.71	0.02					superoxide dismutase (<i>sodB</i> , <i>sodF</i>)
HPG27_RS04050	HP0825	1.94	0.21	0.04	2.33	0.22	0.02	2.97	0.41	0.04			↑		thioredoxin reductase (<i>trxB</i> , <i>trxB</i>)
HPG27_RS04280	HP0875	2.23	0.14	0.00	2.60	0.16	0.00	4.79	0.75	0.03					catalase (<i>katA</i>)
HPG27_RS05790	HP1164	1.73	0.12	0.02	1.71	0.18	0.06	1.58	0.10	0.02					thioredoxin reductase (<i>trxB</i> , <i>trxB</i>)
HPG27_RS07230	HP1458	1.14	0.20	0.40	1.15	0.14	0.35	1.66	0.12	0.02			↓		thioredoxin (<i>trxB</i>)
<i>Motility</i>															
<i>class 3 flagellar genes</i>															
HPG27_RS01995	HP1051	2.73	0.32	0.03	2.66	0.47	0.07	1.37	0.07	0.02					hypothetical protein
HPG27_RS01990	HP1052	1.88	0.13	0.01	1.81	0.07	0.00	1.95	0.08	0.00					UDP-3-O-acetyl N-acetylglucosamine deacetylase (<i>envA</i>)
<i>intermediate class flagellar genes</i>															
HPG27_RS03665	HP0753	2.39	0.11	0.00	1.98	0.14	0.01	1.41	0.10	0.05					flagellar biosynthesis protein (<i>flhS</i>)
HPG27_RS03675	HP0754	-1.07	0.04	0.21	-1.16	0.03	0.05	-1.67	0.06	0.02					flagellar chaperone (<i>flhT</i>)
HPG27_RS05575	HP1122	1.97	0.15	0.01	1.02	0.07	0.48	-1.42	0.06	0.04			↑		anti-sigma factor (<i>flgM</i>)

Gene name	NCBI Reference	Fold Change pH 3.0/7.4	sem	p value	Fold Change pH 4.5/7.4	sem	p value	Fold Change pH 6.0/7.4	sem	p value	a.	b.	c.	d.	Annotation
HPG27_RS07805	HP1557	2.43	0.19	0.01	1.30	0.13	0.15	-1.23	0.05	0.08	↑				flagellar hook-basal body complex protein (<i>flhE</i>)
HPG27_RS07815	HP1559	-1.41	0.04	0.01	-1.47	0.06	0.03	-1.96	0.09	0.02	↑	↑			flagellar biosynthesis protein (<i>flgB</i>)
HPG27_RS00830	HP0165	1.11	0.03	0.07	-1.34	0.03	0.01	-1.62	0.05	0.01					histidine kinase (<i>arsS</i>)
HPG27_RS00835	HP0166	-1.42	0.10	0.10	-1.73	0.08	0.03	-1.78	0.07	0.02					response regulator (<i>arsR</i>)
HPG27_RS05535	HP0488	-1.20	0.09	0.17	-1.21	0.07	0.11	-1.81	0.07	0.02					hypothetical protein
HPG27_RS07135	HP1440	-1.91	0.04	0.00	-2.33	0.05	0.00	-2.17	0.06	0.01	↑	↑	↑		hypothetical protein
<i>class 2 flagellar genes</i>															
HPG27_RS04430	HP0906	1.42	0.26	0.24	1.89	0.24	0.06	1.81	0.20	0.05					hypothetical protein
HPG27_RS00600	HP0115	1.56	0.27	0.18	2.56	0.35	0.04	3.23	0.51	0.04	↑	↑			flagellin B (<i>flaB</i>)
HPG27_RS01480	HP0295	1.31	0.22	0.27	1.43	0.13	0.09	1.76	0.18	0.04			↑		flagellar hook-associated protein (<i>flgL</i>)
HPG27_RS01870	HP1076	3.48	0.88	0.11	2.84	0.57	0.09	2.16	0.20	0.02			↑		hypothetical protein
HPG27_RS05740	HP1154	2.63	0.71	0.16	2.17	0.40	0.11	2.12	0.26	0.05			↑		flagellar assembly protein (<i>flaW</i>)
<i>class 1 flagellar genes</i>															
HPG27_RS00520	HP0099	1.37	0.09	0.05	1.46	0.08	0.02	2.25	0.17	0.01					methyl-accepting chemotaxis transmembrane sensory protein (<i>tlpA</i>)
HPG27_RS00540	HP0103	-1.26	0.04	0.04	1.25	0.05	0.03	1.66	0.14	0.03					methyl-accepting chemotaxis transmembrane sensory protein (<i>tlpB</i>)
HPG27_RS00870	HP0173	-1.32	0.13	0.19	-1.87	0.08	0.02	-2.08	0.06	0.01					flagellar biosynthesis protein (<i>flhR</i>)
HPG27_RS01225	HP0244	1.79	0.05	0.00	1.35	0.08	0.05	-1.03	0.06	0.41					histidine kinase (<i>flgS</i>)
HPG27_RS01235	HP0246	-1.22	0.06	0.09	-1.72	0.07	0.02	-1.93	0.07	0.02					flagellar P-ring protein (<i>flgH</i>)
HPG27_RS01635	HP0325	-1.73	0.07	0.02	-1.83	0.07	0.01	-1.54	0.03	0.00	↑				flagellar L-ring protein (<i>flgH</i>)
HPG27_RS05250	HP0392	-1.07	0.04	0.19	1.05	0.05	0.33	1.50	0.10	0.03					chemotaxis protein A (<i>cheA</i>)
HPG27_RS05245	HP0393	-1.57	0.02	0.00	-1.78	0.03	0.00	-1.76	0.06	0.01	↑				chemotaxis protein (<i>cheY</i>)
HPG27_RS02835	HP0584	-1.24	0.02	0.01	-1.60	0.01	0.00	-2.05	0.06	0.01					flagellar motor switch protein (<i>flhN</i>)
HPG27_RS02915	HP0599	1.14	0.02	0.01	1.28	0.02	0.00	1.94	0.20	0.04					chemotaxis receptor (<i>tlpB</i>)
HPG27_RS02995	HP0616	-1.68	0.04	0.01	-1.72	0.07	0.02	-1.78	0.07	0.02					chemotaxis protein (<i>cheY</i>)
HPG27_RS03320	HP0685	1.12	0.08	0.26	-1.43	0.04	0.02	-2.01	0.04	0.00					flagellar biosynthesis protein (<i>flhP</i>)
HPG27_RS03460	HP0714	-1.21	0.18	0.30	-1.70	0.09	0.04	-1.66	0.06	0.03					RNA polymerase sigma54 factor (<i>spoN</i>)
HPG27_RS03980	HP0815	-1.68	0.09	0.05	-1.69	0.08	0.03	-1.60	0.07	0.03					motility protein A (<i>motA</i>)
HPG27_RS03985	HP0816	-1.46	0.09	0.08	-1.64	0.05	0.01	-1.72	0.04	0.01			↑		flagellar motor proteinB (<i>motB</i>)

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HPG27_RS04125	HP0840	-1.32	0.10	0.15	-1.30	0.08	0.11	-1.61	0.04	0.01					flagella associated protein (<i>flaA</i>)
HPG27_RS02045	HP1041	-1.16	0.11	0.27	-1.46	0.09	0.08	-1.70	0.08	0.03					flagellar biosynthesis protein (<i>flhA</i>)
HPG27_RS01910	HP1067	1.74	0.09	0.01	-1.11	0.04	0.14	-1.42	0.09	0.08	↑				chemotaxis protein (<i>cheY</i>)
HPG27_RS06480	HP1286	1.02	0.07	0.46	1.60	0.08	0.01	2.28	0.35	0.07					FliZ protein (<i>fliz</i>)
HPG27_RS07030	HP1420	-1.71	0.03	0.00	-1.44	0.04	0.02	-1.70	0.02	0.00					flagellum-specific ATP synthase (<i>flh</i>)
HPG27_RS07135	HP1440	-1.91	0.04	0.00	-2.33	0.05	0.00	-2.17	0.06	0.01		↑		↑	hypothetical protein
HPG27_RS07250	HP1462	2.69	0.52	0.08	2.50	0.40	0.06	1.81	0.18	0.03					flagellar motility secretory protein
HPG27_RS07895	HP1575	-1.51	0.08	0.05	-1.66	0.09	0.04	-1.96	0.08	0.02					flagellar biosynthesis protein (<i>flhB/yqH</i>)
HPG27_RS07945	HP1585	-2.16	0.07	0.01	-2.31	0.06	0.00	-2.37	0.06	0.01					flagellar basal body rod protein (<i>flgG</i>)
<i>other</i>															
HPG27_RS05160	HP0410	1.45	0.03	0.00	1.53	0.04	0.00	2.07	0.19	0.02					neuraminylactose-binding hemagglutinin (<i>hpaA2</i>)
HPG27_RS02370	HP0492	1.55	0.11	0.03	1.29	0.07	0.05	1.40	0.06	0.02					flagellar sheath associated protein paralog (<i>hpa3</i>)
HPG27_RS03890	HP0797	2.34	0.11	0.00	3.06	0.20	0.00	4.62	0.68	0.03					neuraminylactose-binding hemagglutinin (<i>hpaA</i>)
Outer membrane proteins															
<i>hop genes</i>															
HPG27_RS00060	HP0009	1.33	0.07	0.03	1.81	0.05	0.00	2.62	0.30	0.03		→			outer membrane protein HopZ (<i>hopZ</i> , <i>ompI</i>)
HPG27_RS04380	HP0317	-1.93	0.05	0.02	-1.56	0.06	0.04	-1.94	0.06	0.02		→			outer membrane protein HopU (<i>hopU</i> , <i>omp9</i> , <i>babC</i>)
HPG27_RS03495	HP0722	3.19	0.45	0.04	4.41	0.79	0.05	6.19	1.53	0.08		→		→	outer membrane protein HopO (<i>hopO</i> , <i>omp16</i> , <i>stabB</i>)
HPG27_RS03510	HP0725	9.14	1.83	0.04	11.22	3.42	0.10	13.00	4.12	0.11		→		→	outer membrane protein HopP (<i>hopP</i> , <i>omp17</i> , <i>stabA</i>)
HPG27_RS04465	HP0912	1.42	0.10	0.04	2.05	0.11	0.00	4.30	0.91	0.07		→			outer membrane protein HopC (<i>hopC</i> , <i>alpA</i> , <i>omp20</i>)
HPG27_RS04470	HP0913	1.20	0.06	0.08	2.34	0.22	0.02	4.23	0.94	0.08		→			outer membrane protein HopB (<i>hopB</i> , <i>alpB</i> , <i>omp21</i>)
HPG27_RS05850	HP1177	1.73	0.13	0.02	1.97	0.19	0.03	2.42	0.37	0.06		→		→	outer membrane protein HopQ (<i>hopQ</i> , <i>omp27</i>)
HPG27_RS06750	HP1342	-6.21	0.01	0.00	-3.09	0.07	0.00	-2.70	0.09	0.01					outer membrane protein HopN2 (<i>hopN</i> , <i>omp29</i>)
<i>hor genes</i>															
HPG27_RS01630	HP0324	1.52	0.08	0.01	1.25	0.03	0.00	1.18	0.04	0.04					outer membrane protein HorC (<i>horC</i> , <i>omp10</i>)
HPG27_RS03885	HP0796	3.01	0.45	0.04	2.88	0.58	0.08	1.04	0.07	0.45		→			outer membrane protein HorG (<i>horG</i> , <i>omp18</i>)

Gene name	NCBI Reference	Fold Change pH 3.0/7.4	p value	sem	Fold Change pH 4.5/7.4	p value	sem	Fold Change pH 6.0/7.4	p value	sem	a.	b.	c.	d.	Annotation
HPG27_RS01915	HP1066	-1.69	0.04	0.01	-1.50	0.05	0.02	-1.78	0.09	0.04					outer membrane protein Hord (<i>hordD</i>)
HPG27_RS05515	HP1113	-1.86	0.02	0.00	-1.26	0.02	0.01	-1.05	0.06	0.34					outer membrane protein Hori (<i>hori_omp24</i>)
HPG27_RS07650	HP1395	2.55	0.18	0.00	3.08	0.22	0.00	3.75	0.41	0.01				→	outer membrane protein HoriL (<i>horiL</i>)
<i>hof genes</i>															
HPG27_RS01055	HP0209	-1.64	0.03	0.00	-1.59	0.03	0.00	-1.69	0.05	0.01					outer membrane protein HofA (<i>hofA</i>)
HPG27_RS01845	HP1083	1.87	0.23	0.07	2.07	0.26	0.05	1.88	0.16	0.03					outer membrane protein HofB (<i>hofB</i>)
HPG27_RS03815	HP0782	-1.50	0.08	0.05	-1.71	0.07	0.02	-1.97	0.07	0.01					outer membrane protein HofE (<i>hofE</i>)
<i>hom genes</i>															
HPG27_RS04570	HP0710	2.58	0.22	0.01	2.47	0.25	0.02	2.59	0.33	0.04				→	outer membrane protein HomaA (<i>homaA</i>)
<i>FecA-like genes</i>															
HPG27_RS03325	HP0686	-2.79	0.06	0.00	-2.37	0.07	0.01	-2.01	0.08	0.02					outer membrane protein (<i>fecA-1</i>)
HPG27_RS03940	HP0807	-1.81	0.05	0.01	-1.64	0.05	0.01	-1.12	0.04	0.13					outer membrane protein (<i>fecA-2</i>)
HPG27_RS07505	HP1512	0.98	0.04	0.39	1.15	0.04	0.08	1.50	0.07	0.01				→	outer membrane protein (<i>tirB-3</i>)
<i>efflux pump genes</i>															
HPG27_RS02945	HP0605	1.61	0.13	0.04	1.73	0.16	0.04	1.41	0.11	0.06					outer-membrane protein of the hefABC efflux system (<i>hefA</i>)
HPG27_RS04770	HP0971	-1.12	0.06	0.21	-1.45	0.03	0.00	-1.83	0.04	0.00					outer membrane protein HefD
HPG27_RS06675	HP1327	1.49	0.15	0.08	-1.17	0.03	0.05	-1.57	0.07	0.03					outer membrane protein HefG
<i>other omp genes</i>															
HPG27_RS03360	HP0694	-1.53	0.07	0.04	-1.67	0.06	0.02	-1.98	0.05	0.00					outer membrane protein
HPG27_RS03515	HP0726	-1.54	0.03	0.00	-1.51	0.03	0.00	-1.48	0.06	0.03					outer membrane protein
HPG27_RS03830	HP0785	1.70	0.16	0.04	1.58	0.07	0.00	1.71	0.09	0.00					outer-membrane lipoprotein carrier protein (<i>tolA</i>)
HPG27_RS07275	HP1467	-1.65	0.07	0.03	-1.62	0.06	0.03	-1.67	0.06	0.02				↑	outer membrane protein
HPG27_RS07835	HP1564	2.71	0.55	0.09	3.29	0.54	0.04	3.80	0.69	0.05					outer membrane lipoprotein (<i>plpA</i>)
HPG27_RS01635	HP0325	-1.73	0.07	0.02	-1.83	0.07	0.01	-1.54	0.03	0.00					outer membrane protein (<i>flgH</i>)
HPG27_RS05595	HP1125	1.62	0.03	0.00	1.44	0.03	0.00	1.55	0.05	0.00					outer membrane protein (<i>palA</i>)
Type 4 Secretion System (T4SS)/Cag pathogenicity island (PAI)															
HPG27_RS02505	HP0521	1.54	0.12	0.04	1.29	0.08	0.06	-1.44	0.10	0.09					cag pathogenicity island protein 2 (<i>cag2</i>)
HPG27_RS02510	HP0522	1.83	0.07	0.00	1.57	0.03	0.00	1.42	0.04	0.00					cag pathogenicity island protein 3 (<i>cag3</i>)
HPG27_RS02520	HP0524	1.50	0.10	0.03	1.09	0.05	0.18	1.19	0.08	0.14					cag pathogenicity island protein 5 (<i>cag5</i>)

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HPG27_RS02525	HP0525	1.63	0.05	0.00	1.25	0.07	0.07	1.33	0.07	0.04					cag pathogenicity island encoded (<i>virB11</i>)
HPG27_RS02530	HP0526	3.74	0.20	0.00	3.22	0.25	0.00	3.48	0.39	0.01					cag pathogenicity island protein Z (<i>cag6</i>)
HPG27_RS02545	HP0529	1.53	0.13	0.05	-1.09	0.06	0.26	-1.46	0.07	0.04					cag pathogenicity island protein W (<i>cag9</i>)
HPG27_RS02555	HP0531	1.07	0.04	0.20	-1.45	0.03	0.00	-1.50	0.06	0.02					cag pathogenicity island protein U (<i>cag11</i>)
HPG27_RS02560	HP0532	2.70	0.12	0.00	2.34	0.13	0.00	2.44	0.16	0.00	↑				cag pathogenicity island protein T (<i>cag12</i>)
HPG27_RS02565	HP0534	2.47	0.18	0.00	1.77	0.11	0.01	2.08	0.16	0.01					cag pathogenicity island protein S (<i>cag13</i>)
HPG27_RS02580	HP0535	2.43	0.10	0.00	1.99	0.05	0.00	1.91	0.07	0.00					cag pathogenicity island protein Q (<i>cag14</i>)
HPG27_RS02595	HP0537	-1.41	0.06	0.03	-1.91	0.06	0.01	-2.04	0.04	0.00	↑			↑	cag pathogenicity island protein M (<i>cag16</i>)
HPG27_RS02615	HP0541	3.09	0.09	0.00	2.42	0.10	0.00	2.00	0.05	0.00	↑				cag pathogenicity island protein H (<i>cag20</i>)
HPG27_RS02625	HP0542	5.88	0.40	0.00	4.51	0.51	0.01	4.29	0.32	0.00	↑				cag pathogenicity island protein G (<i>cag21</i>)
HPG 27_RS02630	HP0543	6.50	0.90	0.01	5.34	0.71	0.01	7.09	1.05	0.02	↑				cag pathogenicity island protein F (<i>cag22</i>)
HPG27_RS02640	HP0545	2.28	0.15	0.00	2.23	0.22	0.02	2.24	0.18	0.01					cag pathogenicity island protein D (<i>cag24</i>)
HPG27_RS02650	HP0546	2.15	0.24	0.04	1.15	0.09	0.22	-1.31	0.12	0.18					cag pathogenicity island protein C (<i>cag25</i>)
HPG27_RS02655	HP0547	-1.61	0.11	0.08	-1.30	0.12	0.19	-1.04	0.07	0.41					cag pathogenicity island protein A (<i>cag26</i>)
Cell division															
HPG27_RS01435	HP0286	-1.54	0.06	0.02	-1.56	0.06	0.02	-1.59	0.06	0.02			↑		cell division protein (<i>fstH</i>)
HPG27_RS01985	HP1053	1.87	0.30	0.11	1.94	0.22	0.05	1.91	0.19	0.03					septum site-determining protein (<i>minC</i>)
HPG27_RS01675	HP0331	2.11	0.22	0.03	1.69	0.13	0.02	1.25	0.06	0.05	→			↑	septum site-determining protein (<i>minD</i>)
HPG27_RS01680	HP0332	1.99	0.16	0.01	1.45	0.16	0.12	-1.24	0.08	0.15					cell division topological specificity factor (<i>minE</i>)
HPG27_RS03280	HP0675	-1.75	0.05	0.01	-1.82	0.06	0.01	-1.92	0.07	0.02					tyrosine recombinase (<i>xerC</i>)
HPG27_RS03645	HP0749	-1.20	0.07	0.15	-1.50	0.06	0.02	-1.51	0.05	0.02					cell division protein (<i>fisX</i>)
HPG27_RS04805	HP0978	1.49	0.10	0.03	1.54	0.10	0.02	2.01	0.21	0.03					cell division protein (<i>fisA</i>)
HPG27_RS04810	HP0979	-2.01	0.06	0.01	-1.91	0.05	0.01	-1.51	0.03	0.01					cell division protein (<i>fisZ</i>)
HPG27_RS01810	HP1090	-1.21	0.12	0.24	-1.58	0.06	0.02	-1.56	0.06	0.03					cell division protein (<i>fisK</i>)
HPG27_RS01900	HP1069	1.28	0.06	0.04	1.47	0.03	0.00	1.84	0.14	0.02					cell division protein (<i>fisH</i>)

Induced genes > 1.5 fold font, repressed genes <1.5 fold bold and italic font

^aWen 2003

^bMerrell 2003

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Bury-mone 2004
Ang 2001

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