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The Characterization of the Type I DNA Methyltransferase *hsdM1* (HP0463) in *Helicobacter pylori*

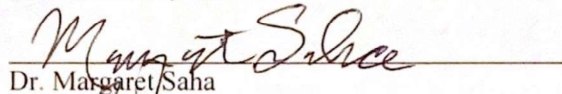
A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Biology from William & Mary

by

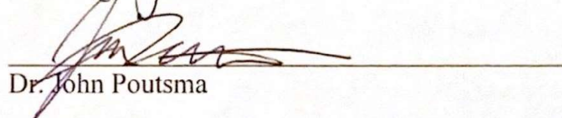
Elise Hannah Zimmerman

Accepted for __________Honors__________

Dr. Mark Forsyth, Director



Dr. Margaret Saha



Dr. John Poutsma

Williamsburg, VA
May 4, 2022

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Abstract

Helicobacter pylori is a gram-negative spiral-shaped bacterium that colonizes the gastric epithelium and is the leading cause of gastric adenocarcinoma globally. For both *H. pylori* and many other bacterial species, there is an increasing body of evidence that methylation by restriction-modification systems regulates gene expression in addition to its traditional role in genome protection. The study aimed to further elucidate the mechanisms through which *H. pylori* achieves methylome plasticity. We demonstrated that the Type I DNA methyltransferase *hsdM1* (HP0463) is regulated by the main acid sensing mechanism of *H. pylori*, the two-component system (TCS) ArsRS. ArsRS induces *hsdM*1 expression under acidic conditions. The acid response of *hsdM1* is unique among the Type I DNA methyltransferases (MTases) and the two Type II MTases in *H. pylori* 26695 selected for the current study. In addition, transcription of *hsdM1* and *hsdM2* (HP0850), another Type I MTase, appear to be under the regulation of the protein LuxS. While there is debate surrounding the role of LuxS in *H. pylori,* we propose that LuxS is acting in a quorum-sensing role within this system. The selected Type II MTases, *M.HpyAI* (HP1208) and *M.HpyAII* (HP1368), do not follow the observed patterns or exhibit any significant changes in gene expression upon acid exposure or resuspension in new media. This indicates that our findings are *hsdM1* and Type I methyltransferase-specific. We also conducted a prolonged acid exposure experiment with the *H. pylori* 26695 control mutant, *ΔarsS* mutant, *ΔhsdM1* mutant, and *hsdM1*-repair mutant to determine the impacts of differential acid conditions and MTase expression on the methylome. The DNA methyltransferase *hsdM1* in *H. pylori* strain 26695 is acid and quorum-sensitive, and thus may contribute to methylome plasticity to aid the bacterium in colonizing the harsh and volatile environment of the human gastric epithelium.

Background

Helicobacter pylori is a Gram-negative spiral-shaped bacterium that colonizes the gastric epithelium and can cause gastritis, gastric and duodenal ulcers, and gastric adenocarcinoma.[1,3] *H. pylori* is one of the most common bacterial infections in the world, infecting about half of the world's population.[3] Infection generally occurs in childhood and persists for the host's lifetime if left untreated.[2] Infection can occur via oral-oral transmission, fecal-oral transmission, or can be food-borne or water-borne.[4] While most cases are asymptomatic, 10-15% of infections lead to peptic ulcer disease or gastric cancer.[5] *H. pylori* is the leading cause of gastric adenocarcinoma which is the second leading cause of cancer-related deaths worldwide.[2,5] Infection is more prevalent in developing countries than in developed countries, though global infection rates are declining due to the widespread use of antibiotics.[2]

Restriction-Modification Systems

There are four main classes of restriction-modification (R-M) systems in bacteria: Types I, II, III, and IV.[12] They differ in subunit composition of the enzymes, motif recognition, and recombination ability.[12] This study was conducted using *H. pylori* strain 26695 and focuses primarily on its three Type I R-M systems with two selected Type II systems.

Type I restriction-modification enzyme systems are complexes composed of three subunits: a DNA methyltransferase (MTase), a restriction endonuclease, and a specificity subunit.[34] *H. pylori* strain 26695 has three complete Type I R-M systems with two orphan specificity subunits which are outlined in **Figure S2.** Type II restriction-modification systems are composed of separate restriction endonuclease and DNA MTase subunits which exhibit identical DNA binding specificity.[33] These systems are best known for their role as primitive bacterial immune systems in which DNA methylation is a mechanism to label the genome as self.[12] Both

the DNA methyltransferase and the restriction endonuclease can only act on nucleotide sequences determined by the specificity subunit. [12] Thus, restriction endonucleases selectively cleave foreign DNA, as the host genome is protected by methyl groups at the target sites.[12] Type I restriction modification systems control sequence recognition via two ***t***arget ***r***ecognition ***d***omains, TRD1 and TRD2, within the specificity (S) subunit which together allow the protein to recognize a bipartite target sequence.[17] The TRDs are flanked by repeat sequences, the length of which determines the distance between the target sequences.[17] Type II systems bind short, generally palindromic, motifs.[12] There are three types of DNA methylation: N^6-methyladenosine (m6A), N^4-methylcytosine (m4C), and N^5-methylcytosine (m5C).[11] Adenine methyltransferases are the most prevalent because m5C methylation is highly susceptible to C to T mutations via deamination.[35] Deamination leads increased mutation rates and decreased DNA stability.[13,35] Thus, bacteria have evolved the utilize m6A and m4C more frequently to avoid these impacts.[36]

There is a growing body of research demonstrating that R-M systems have functions beyond genome protection. In a study of DNA methylation across 230 bacterial and archaeal species, over 800 methylation motifs by 620 different DNA MTases were found.[12] In addition, over 100 Type II MTases without a cognate restriction enzyme were found. [12] The traditional function of degrading foreign DNA does not provide sufficient explanation for the high level of specificity in sequence recognition, the diversity in kind and number of R-M systems in the bacterial kingdom, or the independent evolution of restriction endonucleases and methyltransferases in relation to each other.[13] Gene comparison has shown that Type I systems can alter sequence specificity via homologous recombination of target recognition domains and point deletion/duplication mutations within the central repeat region of the specificity subunit.[19, 38]

It is possible that methylation changes the secondary structure of DNA and this could disrupt simultaneous transcription and translation.[15] In *H. pylori,* NusG mediates the coupling of RNA polymerase and ribosomes to prevent RNA polymerase backtracking and mediate synchronized mRNA production and protein synthesis.[15, 37] This complex functions close to the template DNA, thus may be impacted by methyl groups and proteins bound to methylated sequences.[15, 37] Specifically, methylation on long palindromic sequences by Type I systems has been shown to decrease transcription of operons.[19] In addition, start codon methylation has been shown to increase transcription.[15] There is also evidence suggesting that methylation at translational stop codons impacts transcription.[15] It is becoming clear that restriction-modification systems play a larger role in genome regulation and genetic diversity than previously recognized.

The *H. pylori* Methylome

Within *H. pylori's* relatively small genome of 1.64–1.67 Mb, there are over 20 R-M systems.[14, 48] This makes *H. pylori* an organism of interest for R-M system studies as the average number of methyltransferase (MTase) genes in a prokaryote is five.[15] *H. pylori* has three Type I MTases, all m6A-specific, and 16 Type II MTases of all three methylation types.[14] The five methyltransferase genes of interest in this study can be found in **Table 1.** Lin et al. 2001 conducted a comparative genomics study in *H. pylori* strains 26695 and J99, the first two sequenced strains of *H. pylori*.[14] They found these strains share 90% of their R-M genes, however most of the shared genes are inactive while all the strain-specific genes are active.[14] In fact, R-M genes account for 15-20% of strain-specific genes between 26695 and J99.[14] The large diversity of methylomes even between closely related strains indicate that adaptive evolution may be occurring via epigenetics.[15]

Locus Tag	Gene Name	Methyltransferase Class
HP0463	*hsdM*1	Type I, m6A
HP0850	*hsdM*2	Type I, m6A
HP1403	*hsdM*3	Type I, m6A
HP1208	*M.HpyAI*	Type II, m6A
HP1368	*M.HpyAII*	Type IIS M2 mod protein, m4C

Table 1: Genes of Interest. *The five main genes of interest of this study.*

Type I R-M system complexes exist in two configurations. They primarily exist in a pentamer with two methyltransferase subunits, one specificity subunit, and two restriction endonuclease subunits.[21] This pentameric complex is able to both methylate and cleave DNA.[21] The system can also exist as a trimer of two MTase subunits and one specificity subunit, with no restriction endonuclease subunit, that can only methylate DNA.[21] This may indicate that the system is functioning in its methylase role significantly more than in its traditional role of cleavage of foreign DNA role. This may also be a mechanism through which orphan MTases are able to maintain functionality without a cognate restriction endonuclease, and thus beyond the role of genome protection.

H. pylori strain 26695 only has one m4C methyltransferase, M2.HpyAII (locus tag; HP1368), which is a Type II MTase.[40] A study by Kumar et al. in 2018 created a ΔHP1368 mutant which exhibited differential expression of 102 genes associated with ribosome assembly, virulence, and cellular components.[20] This mutant also had lower adherence to host cells and reduced ability to induce inflammation and apoptosis.[20] This suggests that M2.HpyAII acts as a global epigenetic regulator, possibly both through direct transcriptional regulation and through regulation of other methyltransferases.[20] Before the Kumar et al. 2018 study, m4C methylation

was not known to function beyond traditional protection against restriction endonucleases.[41] Type I R-M systems were thought to only use m6A modification of bacterial DNA, but they appear to use m4C as well.[42]

DNA methyltransferases interact in a hierarchical manner, possessing the ability to both positively and negatively regulate one another.[15] R-M systems may also be transcriptionally regulated by modification enzymes, anti-sense promoters, or methylation of its own target sequence within the promoter region.[18,39] With so many avenues of regulation, it's logical that the previously held hypothesis that methylation is turned completely on or off has been largely disproven.[19] Intermediate methylation activity, in which only 50-60% of target sites are methylated, has been demonstrated.[19] Thus, R-M systems may be a mechanism through which the bacterium fine-tune gene expression levels. As these systems are over-represented in the *H. pylori* genome, the possibilities for such regulation are significant.

Operon *hsdR*1-*hsdM*1 (HP0464-0463)

The *H. pylori* strain 26695 operon *hsdR*1-*hsdM*1 (annotated locus tags; HP0464-0463), the principal operon under investigation in my honors studies, consists of genes for the restriction endonuclease and DNA methyltransferase (MTase) subunits of a Type I restriction-modification system in *H. pylori* strain 26695.[16] This operon is also phase-variable.[16] Across all *H. pylori* strains, restriction-modification genes are phase-variable at a disproportionately high rate.[14] Phase variation occurs via slipped-strand mispairing during replication at short tandem repeat sequences at the beginning of the operon.[14] This frameshift mutation allows these genes to be turned on and off between generations.[14] Phase-locking *hsdR*1 (HP0464), the restriction endonuclease gene, on and off has significant impacts on the transcription of the downstream operon gene, *hsdM*1 (Villadelgado and Forsyth, unpublished data). A study by Gauntlett et al. in

2014 found that phase-locking *hsdR*1 on had a detrimental effect on colonization of mice.[16] This suggests that this R-M system regulates the transcription of genes involved in colonization.[16] This supports the hypothesis that plasticity of the epigenome is essential for host adaptation and colonization.

Multiple conditions have been shown to impact the expression of *hsdM*1 (HP0463). This gene has been found to be upregulated in acidic conditions.[9] In addition, a study in 2020 by Yano et al. found that *hsdM*1 is directly activated by Type III MTase gene HP0593 and a Type I specificity gene HP0848 (*hsdS*2). [15] It is further indirectly activated by Type II MTase gene HP0260.[15] However, the mechanisms through which *hsdM*1 is acid responsive and the impacts of the changes in *hsdM*1 expression on the methylome have yet to be elucidated.

Two-Component Systems and Acid Response in *Helicobacter pylori*

H. pylori grows optimally in vitro at or close to neutral pH, however the human stomach fluctuates between pH < 1.0 and pH 6.0 throughout the day, with a median pH of 1.4.[6] Therefore, acid acclimation strategies are vital for *H. pylori* long-term survival and colonization.[6] The bacteria are able to maintain a cytoplasmic pH greater than 4 even at external pH < 2.[6] Maintenance of the cytoplasmic pH is done primarily through the production of high amounts of the enzyme urease.[43] The urease gene cluster is regulated by the two-component system (TCS) ArsRS which senses periplasmic pH via the sensory histidine kinase ArsS.[6] Once activated by acidic pH, ArsS autophosphorylates a cytoplasmic histidine residue then transfers the phosphoryl group to an aspartic acid residue on its cognate response regulator ArsR.[11] ArsR~P then acts as a transcription factor[11] (**Fig. 1**). *H. pylori* possesses only three complete two-component systems as well as two orphan response regulators, in comparison to the average 52 two-component systems in bacteria, suggesting that each histidine kinase has a broad impact on cellular

function.[10] Interestingly, Δ*arsS* strains are viable in the laboratory while Δ*arsR* strains are not, indicating that ArsR has essential function even in its non-phosphorylated state.[11] ArsR and ArsR~P have been found to bind to the same DNA site, with ArsR~P binding with much higher affinity.[11] Thus, ArsR may be acting as a transcriptional regulator in both neutral and acidic pH.[10] This contradicts the traditional two-component system model in which the response regulator must be phosphorylated to be active.

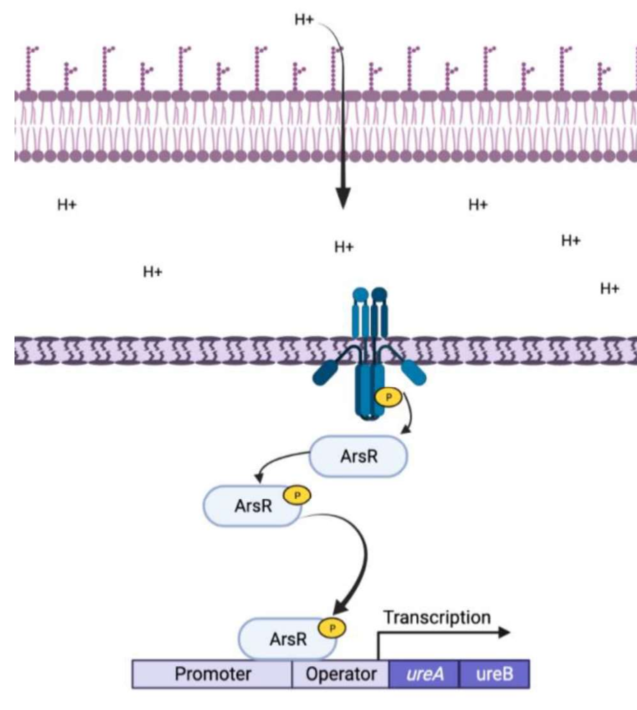

Figure 1: Traditional model of the two-component system ArsRS. *Upon detection of acidic pH in the periplasm, the histidine kinase ArsS autophosphorylates. ArsS then phosphorylates its cognate response regulator ArsR. ArsR~P then binds to the genome to regulate transcription. In this figure, ArsR~P is inducing the transcription of urease subunits ureA and ureB. This figure was created in BioRender.*

In addition to the regulation of urease, shown in **Fig. 1**, there are many genes that are differentially regulated in response to changes in acidity. A study by Wen et al. in 2003 found ~300 differentially regulated genes in *H. pylori* strain 26695, with more genes being upregulated

at pH 4.5 than downregulated.[8] They also found that the number of genes altering expression increased with decreasing pH, including several restriction-modification system subunit genes.[8] ArsS adopts different conformations based on the level of acidity via varying degrees of protonation, thus produces varying concentrations of ArsR~P.[45] As different layers of the gastric mucosa have different pH levels, it's important for *H. pylori* to be able to sense where they are in relation to lethal pH values found in stomach and respond accordingly.[9] A different study from Bury-Moné et al. in *H. pylori* 26695 found 56 genes upregulated and 45 genes downregulated in acid conditions, including multiple restriction-modification system subunit genes.[9] Our main gene of interest in the study described in this thesis, *hsdM*1 (HP0463), was demonstrated by Bury-Moné and colleagues to be upregulated in low pH.[9] There is significant discrepancy in the full regulon of ArsS between individual studies, likely due to differences in experimental setup.[11] However, all studies agree that the two-component system ArsRS is the main acid sensing and response mechanism in *H. pylori* with a regulon of at least 100 genes.[11]

LuxS and AI-2

A controversy has developed surrounding the role of LuxS and autoinducer-2 (AI-2) in *H. pylori*. *H. pylori* contains an orthologue of *luxS* which is essential for the production of extracellular signaling molecule AI-2.[22] Many organisms, including *H. pylori*, produce LuxS and AI-2, but do not produce the known AI-2 receptor complex LuxPQ.[23] Through phylogenetic analysis, it has been suggested that *H. pylori* acquired *luxS* and *AI-2* via horizontal gene transfer, but did not acquire its cognate receptors.[23, 24] However, it's possible that *H. pylori* has unknown or pre-existing adapted receptors for AI-2.[23] Rezzonico and Duffy 2008 use the lack of AI-2 receptors as an argument for LuxS providing a primarily metabolic role in the cell.[23] However,

Doherty et al. 2010 use this same phylogenetic information to come to the conclusion the LuxS has a primary role in quorum sensing.[28]

In support of the argument that LuxS has an essential role in quorum sensing, *H. pylori* has been demonstrated to have quorum sensing ability.[22] A study by Forsyth and Cover in 2000 found that *H. pylori* conditioned media possess the known quorum-sensing molecule autoinducer-2 (AI-2) as evidenced by its ability to induce bioluminescence, an AI-2 controlled event, in *Vibrio harveyi*.[22] AI-2 accumulates in the environment as the bacterial population grows and signals cell density to the bacterium which may induce changes in gene expression.[22] This study showed that deleting *luxS* prevents quorum sensing, and that quorum sensing ability was restored in the *luxS* complementation mutant.[22] However, this study did not identify *H. pylori* gene targets of AI-2 regulation.

In another function of this quorum-sensing system, *H. pylori* has also been shown to perceive AI-2 as a chemorepellent via the chemoreceptor TlpB.[29] AI-2 has also been shown to regulate flagellar gene transcription.[30] A study by Loh, Forsyth, and Cover in 2004 found that expression of the flagellar gene *flaA* is induced as culture density increased and that this induction is *luxS*-dependent.[44] Thus, it is hypothesized that *H. pylori* utilizes AI-2 to regulate motility and subsequently its distribution within the stomach.[29] It may help the bacterium distribute evenly across the gastric epithelium and avoid competition for resources within densely populated areas.[29] In addition, this may impact where *H. pylori* colonizes within the stomach.[29] This is important as the various disease outcomes associated with *H. pylori* infections are related to the specific site of infection.[31]

In addition to a quorum sensing role, LuxS in most cells also plays a significant metabolic role in the activated methyl cycle (AMC). Thus, it is maintained in bacteria that lack

the LuxS receptor, such as *H. pylori*. Within the AMC, LuxS is involved in the generation of the cell's major methyl donor, S-adenosyl-L-methionine (SAM), and the recycling of methionine by detoxification of S-adenosyl-L-homocysteine (SAH).[23] LuxS produces the direct AI-2 precursor, (S)- 4,5-dihydroxy-2,3-pentanedione (DPD), and the methionine precursor, homocysteine.[28, 46] Methionine is then converted back to SAM via the enzyme MetK.[28] DNA methyltransferases are SAM-dependent enzymes and catalyze the transfer of methyl groups from SAM to themselves to become active.[24] Thus, it is possible that a deletion of *luxS* would indirectly impact the functionality of DNA MTases.

However, *H. pylori* is missing genes encoding the enzymes MetE/MetH which catalyze the production of methionine from homocysteine within a traditional activated methyl cycle.[28] Therefore, *H. pylori* does not have a traditional AMC.[28] This is consistent with the genome-based prediction that *H. pylori* is auxotrophic for methionine.[47] Instead, LuxS is involved in the conversion of homocysteine to cysteine via a reverse transsulfuration pathway.[28] However, it is still possible that LuxS is linked to SAM via its role in the breakdown of S-adenosyl-L-homocysteine (SAH) which is the toxic byproduct of the removal of methyl from SAM by DNA methyltransferases.[23, 28]

The function of LuxS in *H. pylori* is not widely agreed upon, thus additional research is crucial to determine its role in *H. pylori* physiology and gene regulation. Its potential links to the regulation of gene expression and methyltransferase production warranted experimentation within the studies described in this thesis.

Aim and Hypotheses

The principle aim of this study was to elucidate the mechanisms of regulation of the Type I DNA methyltransferase *hsdM*1 (HP0463) in *H. pylori* strain 26695. This study began as a further investigation into a finding in our lab that *hsdM*1 is significantly upregulated when the gene encoding the sensory histidine kinase ArsS is experimentally deleted. Based on this finding, we hypothesized that *hsdM*1 is acid-responsive and regulated by the two-component system ArsRS. We also wanted to investigate other systems, such as quorum-sensing or methyl cycle-dependent factors, that may be regulate expression of *hsdM*1.

In addition to determining the regulation of the DNA methyltransferase itself, we sought to determine the impacts of acid conditions and differential *hsdM*1 expression on the methylome of *H. pylori* 26695. Several studies[8,9,11] have linked acid conditions to differential expression of restriction-modification genes via transcriptome analysis, but the consequences of differential expression and the mechanisms through which this occurs are not well understood. An important piece missing in this logical progression linking differential methyltransferase expression to the altered transcriptome may lie in the resulting alterations in the *H. pylori* methylome.

Our studies to characterize changes to DNA methyltransferase expression mediated by acid, ArsRS, and LuxS may lead to a much clearer understanding of *H. pylori* genome plasticity and its ability to colonize the human stomach through conditions that vary greatly both daily and over the lifetime of an infected host. This may aid in creating more targeted, effective treatments as *H. pylori* is becoming increasingly antibiotic resistant.[27] We hypothesize that prolonged acid exposure, as would occur in proximity to the gastric epithelium, results in a shift in genome-wide expression patterns partially due to methyltransferases altering the epigenome.

Materials and Methods

Culture Conditions and Bacterial Mutants

H. pylori strains were grown on Trypticase Soy Agar II plates with 5% sheep blood at 37°C in 5% CO_2/ 95% ambient air. *H. pylori* strain 26695 Δ*rdxA*, a metronidazole resistant mutant, served as the control and is referred to hereafter as the control mutant. The Δ*rdxA* mutant was used as part of a counter-selection procedure to create the Δ*arsS,* Δ*hsdM*1, and *hsdM*1-repair strains described below. The counter-selection procedure is detailed in Loh et a., 2011.[25] This controls for any potential impacts the Δ*rdxA* locus. This locus is unrelated to any studied functions as it encodes an oxygen-insensitive NADPH nitro-reductase that confers metronidazole resistance. This strain was used as the control in all experiments.

H. pylori 26695 Δ*rdxA*-Δ*arsS* mutant referred to hereafter as Δ*arsS* contains a deletion of the sensory histidine kinase ArsS of the ArsRS two-component system.[26] Deleting this histidine kinase removes *H. pylori*'s ability to detect changes in acidity and thus nullifies the major acid-response pathway. This allows us to identify acid-sensitive genes and pathways within the bacterium.

We made two separate mutants of *H. pylori* involving the gene *hsdM*1 (HP0463), encoding a Type I DNA Methyltransferase HsdM1, the focus of this study. The first mutant, *H. pylori* 26695 Δ*rdxA*-Δ*hsdM*1, referred to hereafter as Δ*hsdM*1, which is the deletion of HP0463 to identify methylation function of *hsdM*1 (K.E. Hunter & M.H. Forsyth. Unpublished data). The second was *H. pylori* 26695 Δ*rdxA*-*hsdM*1-repaired referred to hereafter as *hsdM*1-repaired. The protein HsdM1 in *H. pylori* 26695 is predicted to be missing the last ~56 amino acids (of ~543) due to a frameshift mutation in HP0463. We re-inserted the adenine present in full length strains to reverse the frameshift mutation.

This study also utilized *H. pylori luxS* mutants to determine if there are non-acid related mechanisms, such as quorum sensing or methyl cycle-dependent factors impact *hsdM*1 expression. We used the *luxS* deletion mutant, *luxS*::CAT, referred to hereafter as L26-1 and a *luxS* complementation mutation, referred to hereafter as L26-2.[22] The *luxS* mutants were made at Vanderbilt University and donated by Mark H. Forsyth. Mutant construction is detailed in *Forsyth & Cover 2000*.[22]

RNA Extraction

Samples preserved in RNAzol were transferred to separate 2 ml screw-cap tubes with 1 mm glass beads. To lyse the *H. pylori* cells, the samples were shaken in the beadrupter for 45 seconds on speed setting 5. Two µL of polyacryl carrier (Molecular Diagnostics Inc.) was added to each sample to assist in the precipitation of RNA and prevent its loss when samples were transferred to clean tubes containing 400 µL of molecular grade water. Samples were added one at a time, immediately capped, and shaken vigorously for 10 seconds. The samples incubated for 15 minutes at room temperature. The samples were then centrifuged for 15 minutes at 12,000 x g at 22° C to pellet all cell debris. One mL of supernatant was taken from each sample and added to new clean tubes containing 400 µL of 75% ethanol and immediately mixed. The samples incubated for 10 minutes at room temperature then centrifuged at 12,000 x g for 8 minutes. All supernatant was gently decanted leaving the RNA pellet in the tube. 1 mL of 75% ethanol was added to each tube and shaken to dislodge and wash the pellet. The samples were centrifuged again for 2 minutes at 12,000 x g. The samples then underwent a second ethanol wash, and the supernatants were decanted again. The tubes were placed upside down on a Kimwipe under a lamp to dry all remaining ethanol. After about 20 minutes and once all the ethanol dried, the RNA pellets were resuspended in 50 µL of molecular grade water. The RNA was quantified for

concentration and purity using a Nanodrop (ThermoFisher), and the purified RNA samples were stored at -80°C.

cDNA Synthesis

All samples that underwent RNA extraction were used as templates in cDNA synthesis for subsequent use as templates in qRT-PCR analysis. One µg of RNA, 4 µL of iScript (Bio-Rad), and molecular grade water were combined to make a 20 µL total reaction. Each reaction was cycled through the thermo-cycler as specified by the manufacturer (Bio-Rad).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was conducted to quantify mRNA levels of *H. pylori* genes of interest. We conducted qRT-PCR for all three Type I DNA methyltransferases (MTases) in *H. pylori* 26695 and two selected Type II DNA methyltransferases. TaqMan probes were ordered from ThermoFisher for the Type I MTases *hsdM*1 (HP0463), *hsdM*2 (HP0850), and *hsdM*3 (HP1403), and the Type II MTases *M.HpyAI* (HP1208) and *M.HpyAII* (HP1368). The sequences of all probes and reporters used can be found in **Figure S1**. All reporters have FAM as the fluorophore at the 5' end and NQR as the quencher at the 3' end. For each qRT-PCR reaction, 10 µL of qPCR TaqMan Universal Master Mix II, with UNG (Thermo Fisher) was mixed with 8 µL of molecular grade water, 1 µL of gene-specific TaqMan Custom Gene Expression Assay with Fam (ThermoFisher), and 1 µL of cDNA diluted 1:8 with molecular grade water in a 96-well plate (StepOne Plus System by Thermo Fisher). Target and normalizing genes in each sample were analyzed in triplicate. The DNA Gyrase B subunit gene (*gyrB*) was used as the normalizing gene. GyrB is the beta subunit of DNA gyrase, responsible for uncoiling DNA for DNA synthesis. *gyrB* is not known from the literature to be acid regulated and has not shown differential regulation is any experiments

performed by our lab, therefore it offers a good reference point for natural variance in gene expression. qRT-PCR data was analyzed by calculating Relative Quantity (RQ) values using the $2^{-\Delta\Delta CT}$ method.[7]

Acid Shock Experiments

H. pylori 26695 control mutant and the isogenic Δ*arsS* mutant were subjected to acid shock experiments at pH 5 for 60 minutes. Both strains were grown on Trypticase Soy Agar/ 5% sheep's blood plates, being passed to new plates every 24-48 hours. Using a sterile cotton swab the *H. pylori* cells were harvested from plates and resuspended in 2.5 ml of Sulfite-Free Brucella Broth containing cholesterol (Gibco-BRL) and 10 μg vancomycin/ml (SFBB-chol-vanc) at pH 7. One mL of the sterile SFBB-cholesterol-vancomycin was used to blank the spectrophotometer, and 500 μL of the media was used to dilute 500 μL of the culture to determine the OD_{600} (Optical Density at 600 nm) using spectrophotometry. The OD_{600} is used to determine the progression of microbial growth in broth cultures by measuring the optical density of a culture compared to the sterile media that was used to make the cultures (blank reading). The spectrophotometer reading was multiplied by two to determine the inoculated broth concentration. To achieve an OD_{600} of 0.2 in new 3 mL cultures, the concentrations were used to calculate the appropriate volumes of each culture to transfer.

$$0.2(\text{final } OD_{600}) / \text{current } OD_{600} = X * 3 \text{ ml (final volume)} = n$$

Sterile 3 mL cultures of SFBB-chol-vanc were started in a six-well plate, removing n mL of sterile media from the 3 mL, and adding n ml of the inoculated media. These mutants were cultured in an incubator at 37° C in a 5% CO_2/ 95% ambient air environment with shaking at 150

rpm. The growth of the two overnight cultures was quantified via OD_{600}. New broth cultures were begun by subculturing each overnight culture to achieve a final OD_{600} of 0.4 in new 6 mL cultures. These cultures were incubated for 7 hours shaking in a 5% CO_2 incubator at 37° C under the conditions described before.

After 7 hours, the OD_{600} of the two cultures were taken and 1 OD_{600} unit (~10^9 cells) from the control and Δ*arsS* mutant cultures were collected as the time zero samples. The control and Δ*arsS* mutant cultures were each split into two 2.5 mL aliquots in 15 mL falcon tubes. *H. pylori* cells were harvested by centrifugation at 5,000 x g, 20°C, for 10 min. The supernatant media was decanted, and one of the two pellets of each mutant *H. pylori* was resuspended in 3 mL of SFBB-chol-vanc at pH 7, while the other pellet was resuspended in the same volume of medium, but at pH 5 and then transferred to a six-well plate (Fig. 2). The plate was incubated for 60 minutes under the same conditions described for broth cultures above with samples taken at 30 minutes and 60 minutes. To collect the *H. pylori* cells, 1 OD_{600} unit of the cultures was transferred to microcentrifuge tubes which were centrifuged at 6,000 x g for 7 minutes. The supernatant media was decanted, cells were resuspended in 1mL RNAzol, and samples were stored at -80°C. All samples were subsequently subjected to RNA extraction and cDNA synthesis in preparation for quantitative Real-Time PCR (qRT-PCR) to determine the concentrations of mRNA of interest.

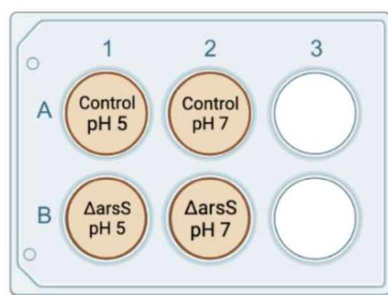

Figure 2. **Acid Shock plate setup.**

LuxS Experiments

Bacterial strains *H. pylori* 26695 control mutant, *luxS* deletion mutant L26-1, and *luxS* complementation mutant L26-2 were used. LuxS is an enzyme essential to the production of quorum sensing molecule auto-inducer 2 (AI-2) and may play a role in the production and recycling of the major methyl donor S-adenosyl-L-methionine (SAM).[22, 23] Overnight broth cultures and subcultures were performed as described in the Acid Shock experiments above. Seven hours after sub-culture of an overnight culture of each of the *H. pylori* mutants, the OD_{600} of the cultures were taken, and 1 OD_{600} volume of culture was collected from each sample. The *H. pylori* cells were harvested by centrifugation at 6,000 x g for 7 minutes. The supernatant media was decanted, cells were resuspended in 1mL RNAzol, and samples were stored at -80°C. All samples then underwent RNA extraction and cDNA synthesis in preparation for quantitative Real-Time PCR (qRT-PCR) to determine the concentrations of mRNA of interest.

Methylome Experiment

To determine the role of acid conditions, the two-component system ArsRS, and the Type I DNA Methyltransferase *hsdM*1 on the *H. pylori* methylome, we conducted a long-term acid exposure experiment on the *H. pylori* 26695 control mutant, Δ*arsS,* Δ*hsdM*1, and Δ*hsdM*1 - repair mutants. All strains were grown on Trypticase Soy Agar/5% sheep's blood plates, passed to new plates every 24-48 hours. Overnight broth cultures and subcultures were performed as in the Acid Shock experiments. After 7 hours of subculture, each strain was split into two 2.5 mL aliquots and *H. pylori* cells were isolated by centrifugation at 5,000 x g, 20° C, for 10 min. The supernatant media was decanted, and one of the two pellets of each mutant *H. pylori* was resuspended in 6 mL of SFBB-cholesterol-vancomycin at pH 7, while the other pellet was resuspended in the same media at pH 5 and transferred to a six-well plate (**Fig. 3**). These cultures

were grown overnight under standard broth culture conditions. The next morning, each culture was subcultured by taking transferring 0.2 OD_{600} units of each culture into 6 mL of new media to make a new overnight culture. This subculture procedure was repeated the next morning, and samples were collected the morning after that so that each mutant was growing in pH 5 or pH 7 broth for 72 hours total. We hypothesized this long-term acid exposure across multiple generations allowed sufficient time and environmental pressure for the *H. pylori* cells to alter their methylome in response to pH 5. To collect samples, 4 mL of each culture was transferred to Falcon tubes and centrifuged at 4,500 rpm, 20°C, for 10 min. The supernatant was decanted, and the pellets were stored at -20°C. The samples were then prepped for methylome sequencing, described below.

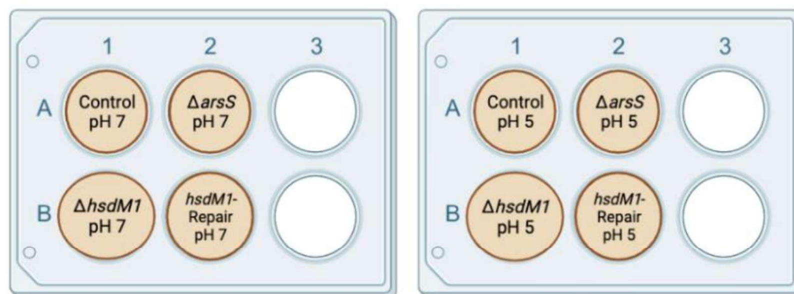

Figure 3. **Methylome Experiment plate setup.**

Methylome Sequencing

To prepare our 8 samples (*H. pylori* 26695 control mutant, Δ*arsS,* Δ*hsdM*1, and *hsdM*1-repair strains, each cultured at pH 5 and at pH 7), for methylome sequencing, they first underwent genomic DNA extraction using the Bio-Rad genomic DNA extraction kit via the manufacturer's suggested protocol including the optional RNA elimination step. The extracted DNA was quantified for concentration and purity using a Nanodrop (Thermo Fisher). As an additional quality check, we performed agarose gel electrophoresis to ensure all extracted DNA was above 5kB and had not been fragmented.

The samples were sequenced by Azenta Life Sciences using the PacBio Sequel machine. This machine can determine N^6-methyladenosine and N^4-methylcytosine methylation status genome-wide and identify methylation motifs using Single Molecule, Real-Time (SMRT) sequencing.

Results

Expression patterns of Type I Restriction-Modification System *hsdR1-hsdM1-hsdS1*

A previous student in our lab, Ryan Shipman, conducted experiments on the expression patterns of the genes HP0465-HP0462 (**Fig. 4**) in the absence of all three of the two-component systems in *H. pylori.* It was determined that there is an increase in transcription of *hsdM*1 (HP0463) by 2.5-fold in this triple deletion mutant (**Fig. 5**). This led us to investigate which two-component system regulates *hsdM*1. Interestingly, *hsdR*1 (HP0464), the other gene in the operon, and *hsdS*1b (HP0462), the cognate specificity subunit of this Type I restriction modification system, are unaffected by the total absence of sensory histidine kinases **(Fig. 5).** In addition, our lab has preliminary data suggesting that when *hsdM*1 is differentially regulated in acid, *hsdR*1 expression remains constant (data not shown). This finding suggests the intriguing possibility that *hsdM*1 may be independently regulated from its cognate subunits. While *hsdM*1 *and hsdR*1 are in the same operon, we hypothesize that *hsdM*1 may have its own uncharacterized promoter.

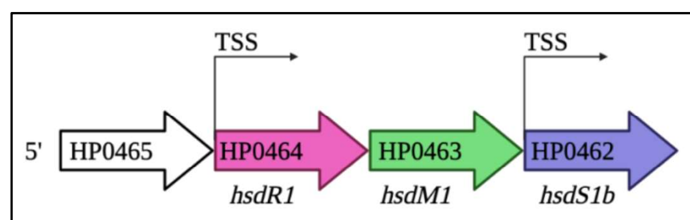

Figure 4. Type I R-M System *hsdR1-hsdM1-hsdS1b* (HP0464-0462) and HP0465 in the *H. pylori* 26695 genome. *The organization of the restriction-modification system operon HP0464-0463 (hsdM1-*

hsdR1) and its cognate specificity unit HP0462 (hsdS1b) in the H. pylori 26695 genome. HP0465 is an unrelated gene used as a control. Bent arrow indicates transcription start site (TSS). Figure made in BioRender.

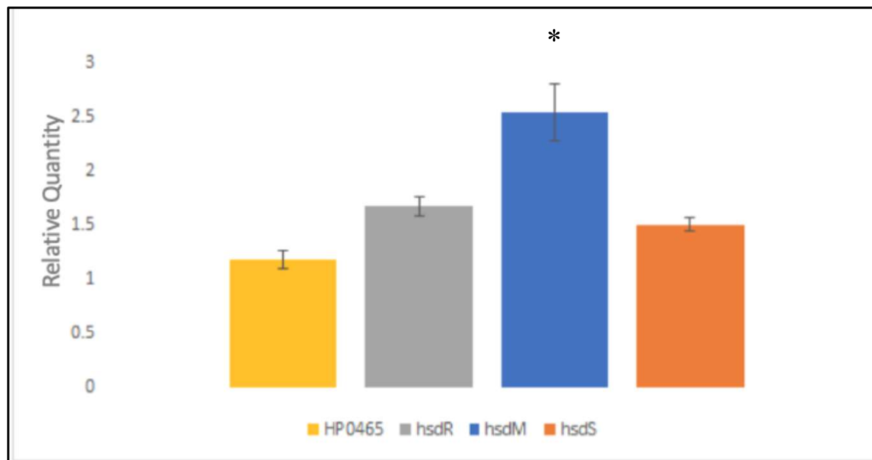

Figure 5. Expression of *hsdR1-hsdM1-hsdS1* (HP0464-0462) and HP0465 in *H. pylori* 26695 containing deletions of all three two-component systems. *Expression of HP0465, HP0464 (hsdR1), HP0463 (hsdM1), and HP0462 (hsdS1b) was measured using qRT-PCR in a triple deletion mutant of H. pylori in which all three sensory histidine kinases (arsS, crdS, and flgS) are experimentally ablate. Genes HP0464-0462 encode the Type I restriction modification system hsdM1/hsdR1/hsdS1b. HP0465 is an unrelated gene directly upstream of the operon. mRNA levels are expressed as relative quantities in relation to their expression in an isogenic control strain possessing all three of the sensory histidine kinases. Error bars; standard deviation. Statistical analysis is performed via unpaired one-tailed t-test. *;P≤0.05. This is a representative of three biological replicates.*

Acid Response and LuxS regulation in *hsdM*1

*hsdM*1 transcription is significantly upregulated in the absence of the major acid sensing protein ArsS **(Fig. 6)** thus demonstrating that *hsdM*1 is under the regulation of the two-component system (TCS) ArsRS. Under pH neutral conditions, ArsRS apparently represses the expression of *hsdM*1 **(Fig. 6)**. Given that ArsRS is the major acid sensing and response

mechanism of *H. pylori,* and *hsdM*1 is repressed by this system in the absence of acid conditions, we hypothesized that *hsdM*1 is acid responsive.

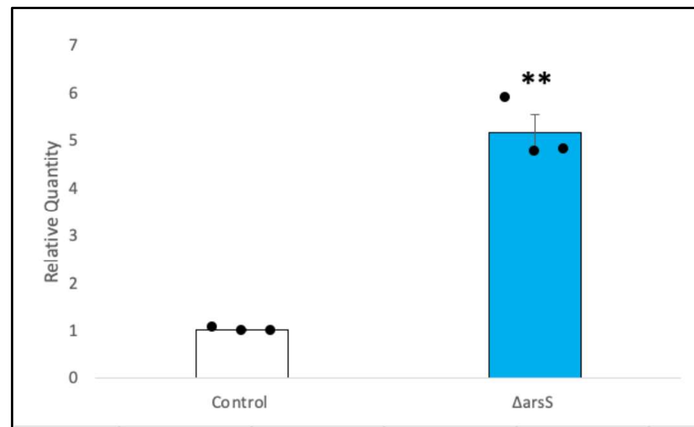

Figure 6: Expression of *hsdM*1 (HP0463) in *H. pylori* is controlled by ArsS. *mRNA levels are expressed as relative quantities in relation to the control strain. Each dot represents a biological replicate, each done in a triplicate. Error bars; standard error of the mean. Statistical analysis is performed via unpaired one-tailed t-test. **; P≤0.01.*

As predicted, *hsdM*1 is acid responsive as it is upregulated at pH 5 in the control strain of *H. pylori* 26695 and not in the isogenic *ΔarsS* mutant **(Fig 7)**. The upregulation peaks at 30 minutes and returns to base levels at 60 minutes. This may indicate that *hsdM*1 is implicated in immediate acid response. However, the decrease in *hsdM*1 transcription at pH 5 at 60 minutes may be due to the induction of urease expression by pH 5 which raises the pH and thus may turn off the acid induction of *hsdM*1. There is also repression occurring. *hsdM*1 is significantly downregulated at pH 5 in the *ΔarsS* mutant **(Fig. 7)**. We speculate that this may be due to the inability of this mutant to mount an acid acclimation response and thus its physiology is impaired by the continued low pH exposure.

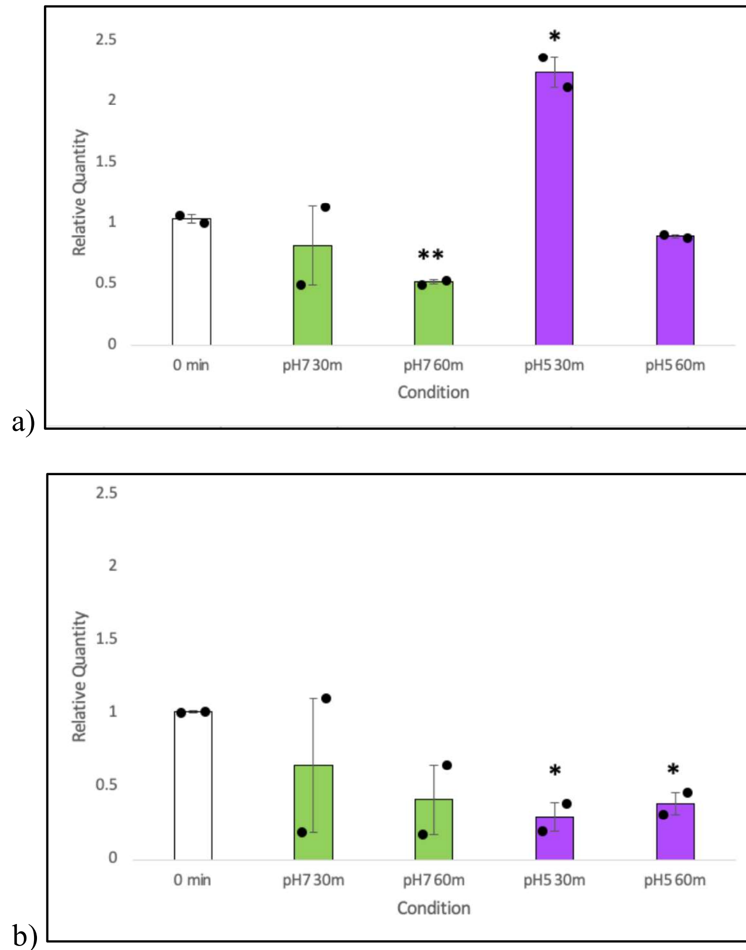

Figure 7: The acid-responsive nature of *hsdM*1 transcription is ArsS-dependent. *Both the control H. pylori mutant, possessing an intact arsRS locus, and an isogenic ΔarsS mutant were grown to mid-logarithmic stage of growth and equal aliquots were harvested and resuspended in pH 7 or pH 5 broth.* ***a)*** *The expression of hsdM1 in the H. pylori 26695 control mutant.* ***b)*** *The expression of hsdM1 in H. pylori ΔarsS. mRNA levels are expressed as relative quantities in relation to the time zero sample. Each dot represents a biological replicate, each done in a triplicate. Error bars; standard error of the mean. Statistical analysis was performed via unpaired one-tailed t-test. *; P≤0.05, **; P≤0.01.*

In addition to acid response, we investigated the *hsdM*1 response to other regulatory mechanisms within *H. pylori.* Specifically, we hypothesized that the protein LuxS may be regulating methyltransferases due to its role in quorum sensing and the possible role of LuxS in

cellular metabolism relating to methyltransferase production. Our hypothesis is based on the observation that *hsdM*1 expression in pH 7 drops upon resuspension in new media, which lacks the quorum-signaling molecule AI-2, in the acid shock experiment **(Fig. 7a)**.

To test this hypothesis, we grew the control *H. pylori* 26695, an isogenic *ΔluxS* mutant**,** L26-1, and an isogenic *luxS* complemented *H. pylori* strain, L26-2 (*luxS*::CAT-*ureA::luxS*)[22] in pH 7 broth to mid-logarithmic stage of growth and equal aliquots were harvested. We observed a decreased transcription of *hsdM*1 in the *ΔluxS* mutant **(Fig. 8)**. This indicates that LuxS is necessary for normal levels of transcription of *hsdM*1. This supports the hypothesis that *hsdM*1 expression is induced at quorum, i.e., a threshold cellular population, as there is decreased *hsdM*1 transcription when the cell is unable to determine the concentration of cells surrounding it as LuxS is responsible for the production of the quorum sensing signaling molecule, autoinducer-2 (AI-2). These data also support the argument that LuxS has a primarily quorum-sensing role within *H. pylori*. While the complementation mutant, L26-2, did bring average expression back to control levels, no single biological replicate was at control expression levels. Two biological replicates exhibited downregulation similar to L26-1, and one biological replicate exhibited upregulation.

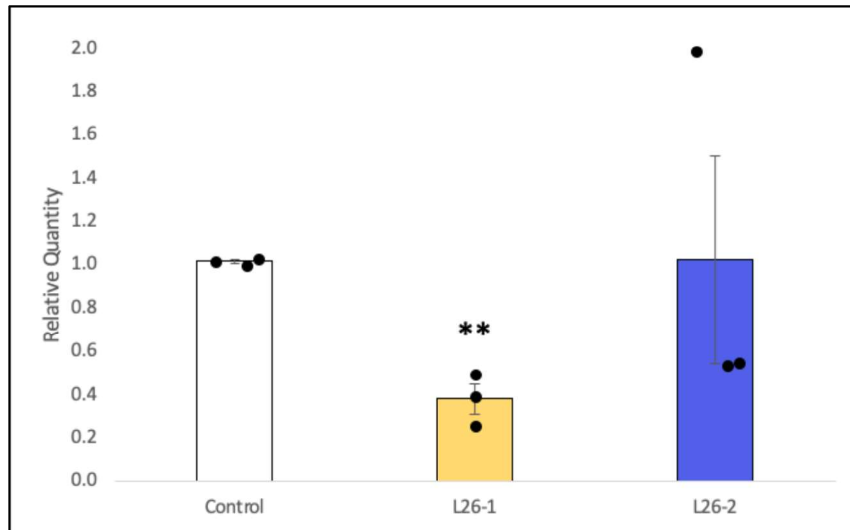

Figure 8. *hsdM1* expression is regulated by LuxS. *The control strain, H. pylori L26-1 (ΔluxS), and L26-2 (luxS-complementation) were grown in pH 7 broth to mid-logarithmic stage of growth and equal aliquots were harvested. mRNA levels are expressed as relative quantities in relation to the control strain. Each dot represents a biological replicate, each done in a triplicate. Error bars; standard error of the mean. Statistical analysis was performed via unpaired one-tailed t-test. **; $P \leq 0.01$.*

While further investigation is needed regarding the control of expression of this Type I methyltransferase, it is clear that *hsdM*1 expression is acid responsive, that responsiveness is dependent upon the two-component system ArsRS, and it may also be regulated by cell-density in a LuxS-dependent manner.

Acid Response and LuxS regulation of the other Type I DNA Methyltransferases

Given *hsdM*1 is acid responsive, and there are only three Type I DNA methyltransferases in *H. pylori,* we asked the question; could this ArsRS acid regulation be a pan-Type I methyltransferase mechanism? To determine the specificity of ArsRS regulation, we also quantified mRNA via qRT-PCR for the other Type I MTases, *hsdM*2 (HP0850) and *hsdM*3 (HP1403). Neither *hsdM*2 or *hsdM*3 are apparently regulated by the two-component system ArsRS nor are they acid sensitive (**Fig. 9** and **Fig. 10**, respectively). Therefore, acid response and

thus ArsRS control is unique to *hsdM*1 among Type I DNA methyltransferases in *H. pylori* 26695.

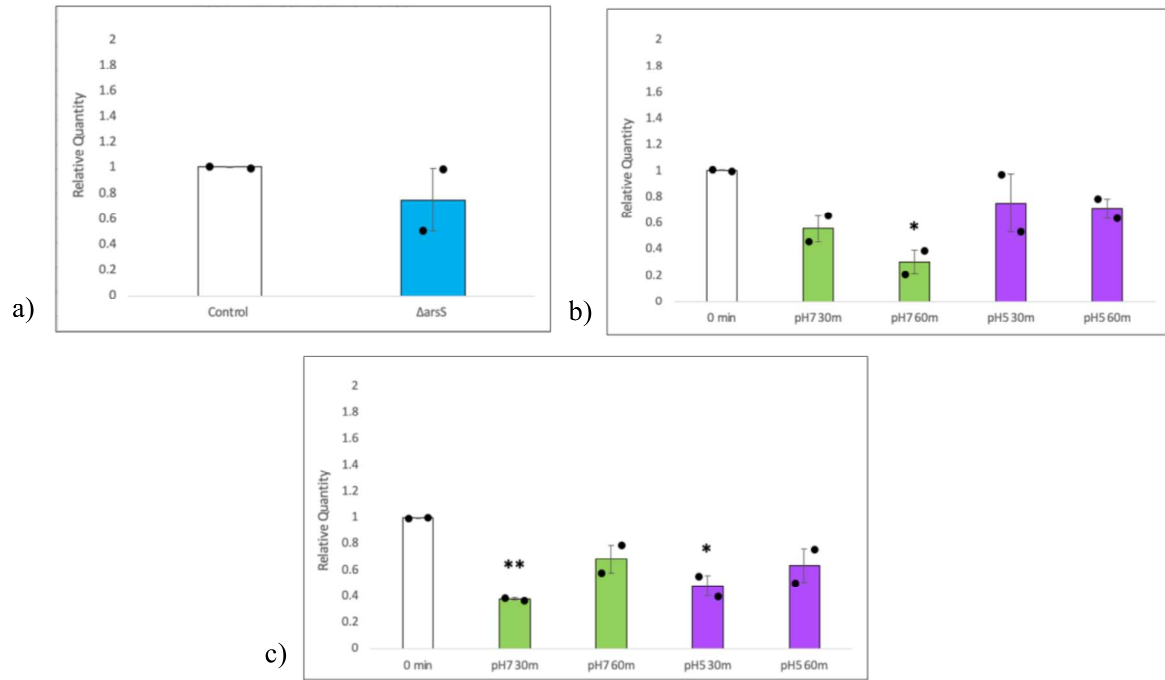

Figure 9. *hsdM*2 (HP0850) is not regulated by ArsRS. *Both the control H. pylori mutant, possessing an intact arsRS locus, and an isogenic ΔarsS mutant were grown to mid-logarithmic stage of growth and equal aliquots were harvested and resuspended in pH 7 or pH 5 broth.* ***a)*** *Expression of hsdM2 in H. pylori 26695 control mutant vs H. pylori ΔarsS. mRNA levels are expressed as relative quantities in relation to the control strain.* **b)** *The expression of hsdM2 in the H. pylori 26695 control mutant during acid shock.* ***c)*** *The expression of hsdM2 in H. pylori ΔarsS during acid shock. In b and c, mRNA levels are expressed as relative quantities in relation to the time zero sample. Each dot represents a biological replicate, each done in a triplicate. Error bars; standard error of the mean. Statistical analysis is performed via unpaired one-tailed t-test. *; P≤0.05, **; P≤0.01.*

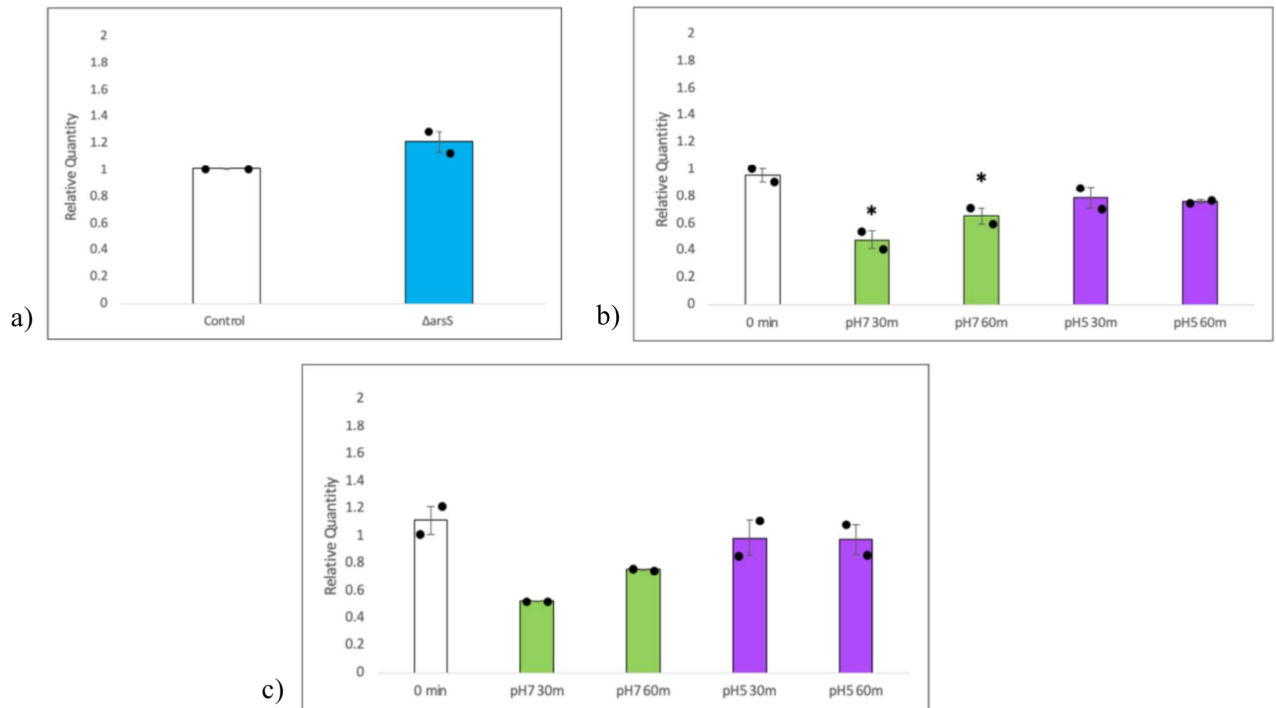

Figure 10. *hsdM*3 (HP1403) is not regulated by ArsRS. *Both the control H. pylori mutant, possessing an intact arsRS locus, and an isogenic ΔarsS mutant were grown to mid-logarithmic stage of growth and equal aliquots were harvested and resuspended in pH 7 or pH 5 broth.* ***a)*** *Expression of hsdM3 in H. pylori 26695 control mutant vs H. pylori ΔarsS. mRNA levels are expressed as relative quantities in relation to the control strain.* ***b)*** *The expression of hsdM3 in the H. pylori 26695 control mutant during acid shock.* ***c)*** *The expression of hsdM3 in the ΔarsS mutant during acid shock. In b and c, mRNA levels are expressed as relative quantities in relation to the time zero sample. Each dot represents a biological replicate, each done in a triplicate. Error bars; standard error of the mean. Statistical analysis is performed via unpaired one-tailed t-test. *; P≤0.05, **; P≤0.01.*

However, each of these methyltransferases exhibit the same pattern of decreased transcription upon resuspension in fresh medium at pH 7 (**Fig. 9b** and **Fig. 10b**) as seen in *hsdM*1 in the control strain (**Fig.7a**). Therefore, we also quantified mRNA for each of these

methyltransferases via qRT-PCR on *hsdM*2 and *hsdM*3 using the *H. pylori luxS* mutants to determine if quorum sensing may be involved in the regulation of these genes.

Transcription of *hsdM*2 (HP0850) appears to follow the same expression pattern as *hsdM*1 and is significantly downregulated in *H. pylori* L26-1. Expression of this Type I methyltransferase in the *luxS* complemented strain, L26-2, indicates *hsdM*2 expression responds to the presence of LuxS (**Fig. 11a**). Expression of *hsdM*3 (HP1403) did not yield consistent results in the three biological replicates shown in **Figure 11b**. Therefore, more biological replicates must be done before conclusions can be made regarding the impact of LuxS on *hsdM*3 expression.

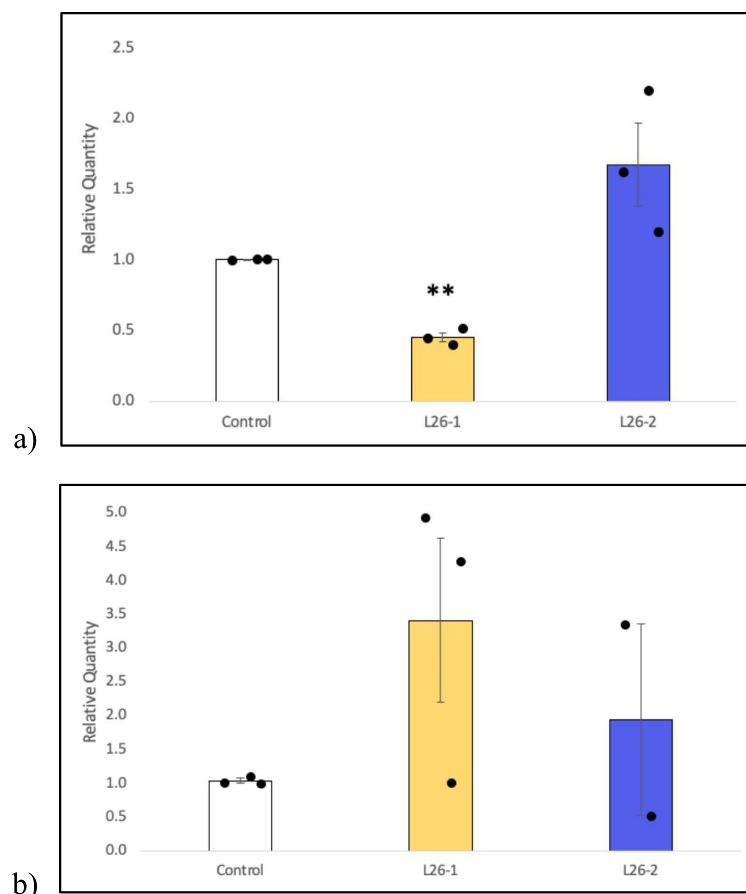

Figure 11. *hsdM2* expression is regulated by LuxS, *hsdM3* expression is not consistent. *The control strain, L26-1 (ΔluxS), and L26-2 (luxS-complementation) were grown in pH 7 broth to mid-logarithmic*

stage of growth and equal aliquots were harvested. ***a)*** *hsdM2 expression.* ***b)*** *hsdM3 expression. mRNA levels are expressed as relative quantities in relation to the control strain. Each dot represents a biological replicate, each done in a triplicate. Error bars; standard error of the mean. Statistical analysis was performed via unpaired one-tailed t-test. *; P≤0.05, **; P≤0.01.*

Acid Response in selected Type II DNA Methyltransferases

As part of our aim to examine the effects of environmental pH on the potential to methylate the *H. pylori* genome, we also conducted qRT-PCR for two selected Type II DNA methyltransferases to investigate if observed regulation patterns of *hsdM*1 were apparent in other families of MTases.

Results illustrated in **Figures 12 & 13** indicate there are no significant changes in expression among the Type II DNA methyltransferases *M.HpyAI* (HP1208) and *M.HpyAII* (HP1368) in response to pH 5 conditions and that the ablation of the sensory histidine kinase ArsS has no apparent effect on these methyltransferases. This indicates that the expression patterns revealed in the current study in *hsdM*1 and the other Type I DNA methyltransferases are unique to the Type I R-M systems. The expression patterns seen in *M.HpyAI* and *M.HpyAII* (**Fig. 12 & Fig. 13**, respectively) serve as a control to ensure that neither our in vitro acid shock itself nor the deletion of *arsS* are causing genome-wide changes in MTase expression.

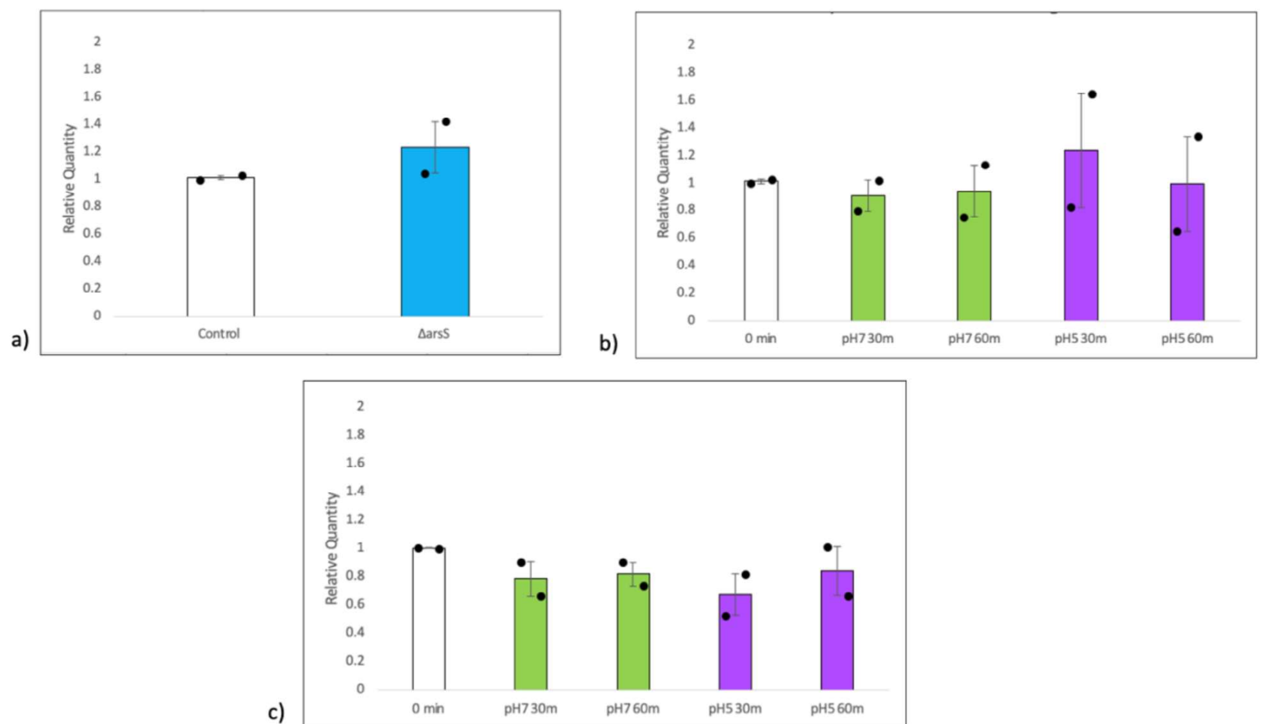

Figure 12. ***M.HpyAI*** **(HP1208) is not acid-responsive.** *Both the control H. pylori mutant, possessing an intact arsRS locus, and an isogenic ΔarsS mutant were grown to mid-logarithmic stage of growth and equal aliquots were harvested and resuspended in pH 7 or pH 5 broth.* ***a)*** *Expression of M.HpyAI in H. pylori 26695 control mutant vs H. pylori ΔarsS. mRNA levels are expressed as relative quantities in relation to the control strain.* ***b)*** *The expression of M.HpyAI in the H. pylori 26695 control mutant during acid shock.* ***c)*** *The expression of M.HpyAI in the ΔarsS mutant during acid shock. In b and c, mRNA levels are expressed as relative quantities in relation to the time zero sample. Each dot represents a biological replicate, each done in a triplicate. Error bars; standard error of the mean. Statistical analysis is performed via unpaired one-tailed t-test. *; P≤0.05, **; P≤0.01.*

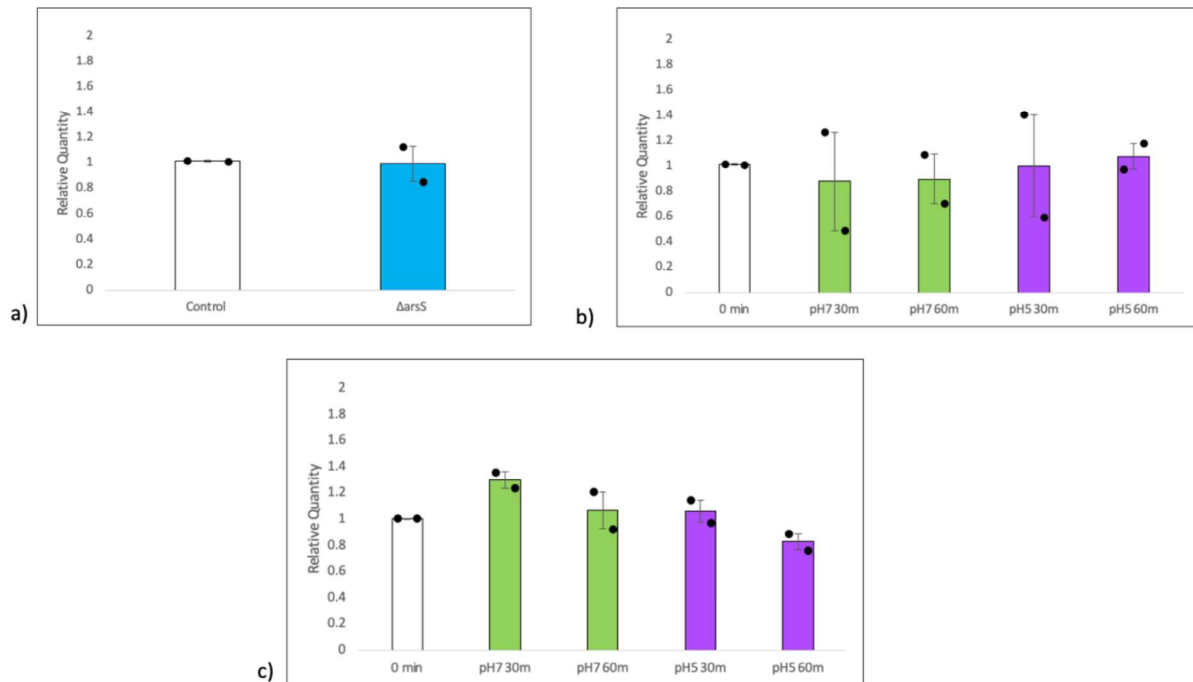

Figure 13. ***M.HpyAII* (HP1368) is not acid-responsive.** *Both the control H. pylori mutant, possessing an intact arsRS locus, and an isogenic ΔarsS mutant were grown to mid-logarithmic stage of growth and equal aliquots were harvested and resuspended in pH 7 or pH 5 broth.* ***a)*** *Expression of M.HpyAII in H. pylori 26695 control mutant vs H. pylori ΔarsS. mRNA levels are expressed as relative quantities in relation to the control strain.* ***b)*** *The expression of M.HpyAII in the H. pylori 26695 control mutant during acid shock.* ***c)*** *The expression of M.HpyAII in H. pylori ΔarsS during acid shock. In b and c, mRNA levels are expressed as relative quantities in relation to the time zero sample. Each dot represents a biological replicate, each done in a triplicate. Error bars; standard error of the mean. Statistical analysis is performed via unpaired one-tailed t-test. *; P≤0.05, **; P≤0.01.*

Discussion

This study has uncovered regulation mechanisms and patterns of the Type I DNA methyltransferase *hsdM*1 (HP0463). Preliminary data shows that, while *hsdM*1 and *hsdR*1 (HP0464) are located on the same operon, *hsdM*1 has independent expression patterns and thus may have its own uncharacterized promoter. This may allow *hsdM*1 to have function in the cell independently of the entire restriction-modification complex.

Our data demonstrate that *hsdM*1 is upregulated under acidic conditions in vitro and that this acid response is ArsS-dependent. In addition, *hsdM*1 is downregulated in neutral conditions. However, these expression patterns do not follow the tradition model of two-component system (TCS) regulation. In neutral pH conditions, the standard view of TCS functioning predicts there is a high quantity of unphosphorylated response regulator, ArsR, and a low quantity of phosphorylated ArsR (ArsR~P).[45] ArsS adopts different conformations based on the level of acidity via varying degrees of protonation, thus produces varying concentrations of ArsR~P.[45] In the traditional model, response regulators are only dimerized and active transcription factors when phosphorylated.[11] Thus, we would predict that since ArsR is repressing *hsdM*1 in neutral conditions, *hsdM*1 would be further repressed in acidic conditions as there is a substantially higher concentration of ArsR~P predicted due to protonated and thus activated ArsS. However, we observed the opposite. This leads us to believe that unphosphorylated ArsR may also be acting as a transcriptional regulator of *hsdM*1. Non-phosphorylated ArsR has been found to act as a transcriptional regulator, and Δ*arsR* strains are not viable which indicates that ArsR has an essential role within *H. pylori*.[11] It is possible that ArsR and ArsR~P are binding to the same DNA site with different affinities, or that the manner in which ArsR and ArsR~P bind the DNA results in different DNA conformations and thus different transcriptional outcomes.

In addition, all three Type I DNA methyltransferases demonstrated decreased expression at 30 minutes and/or 60 minutes than at zero minutes in pH 7 in the acid shock experiments. The repression is puzzling because the pH does not change between the time zero samples and the 30 minute and 60 minute samples. The time zero samples are taken before centrifugation and resuspension, and the remaining cells are resuspended in fresh media and incubated in the same conditions as it had been in the overnight culture and subculture. Our initial hypothesis was that this downregulation was caused by the manipulation of the cells during the resuspension procedure, but the Type II MTases do not exhibit this expression pattern despite identical experimental manipulations.

While the pH of the media does not change, the nutrient content and AI-2 concentration changes. Thus, we hypothesized that *hsdM*1 is also quorum-sensitive and is responding to the decreased cell concentration, and thus decreased AI-2 concentration, in the new media. This led us to conduct experiments with L26-1 and L26-2, the *luxS* deletion and complementation mutants, respectively[22]. LuxS is a protein involved in both quorum sensing, via the production of extracellular signaling molecule AI-2, and metabolism, possibly in the cycling of major methyl donor, S-Adenosyl methionine (SAM), in *H. pylori*.[22,23] We found that *hsdM*1 and *hsdM*2 are downregulated in the absence of *luxS*. This aligns with our hypothesis that expression of *hsdM*1 and *hsdM*2 is induced by quorum, as expression is not induced when the cell is unable to determine the concentration of cells around it. This supports the hypotheses of Doherty et al. 2010[28] and Forsyth & Cover 2000[22] that LuxS plays a quorum-sensing role in *H. pylori*. Our data aligns with their findings that LuxS is necessary for the production of AI-2[22]. However, it is unclear how this data fits within the findings that *H. pylori* does not have a traditional activated methyl cycle and LuxS instead acts in cysteine production[28].

While L26-2 was demonstrated by Forsyth & Cover 2000[22] to restore AI-2 production, and therefore LuxS production, it was not necessarily proven to restore LuxS production to equivalent levels. *luxS* was reinserted within the *ureAB* operon, and thus may not be as responsive to the complex mechanisms of regulation wild-type *luxS* is under. This complementation strain has also lost its ability to produce urease, thus its ability to change the pH of its growth medium is affected. It may also be impacted by the transcriptional regulators of *ureA*. Thus, it is perhaps not surprising that a downstream target of LuxS regulation is not returned to normal expression levels within this complementation mutant.

We also conducted this experiment with other *luxS* deletion mutants (data not shown) and got inconsistent results across the different mutants. Therefore, more experiments need to be done to verify that this pattern of downregulation is real. In addition, experiments comparing the expression of Type I MTases in the control strain vs L26-1 and L26-2 in varying degrees of conditioned media should be conducted to verify that LuxS is acting on the MTases in a quorum sensing role.

As there are 16 Type II DNA MTases in *H. pylori* 26695, we investigated two select Type II DNA methyltransferases. We chose *M.HpyAI* (HP1208) because it has previously been proven to be active[14] and has been shown to be differentially regulated in acid.[8] We also chose *M.HpyAII* (HP1368) because it has previously been demonstrated to be active and is the only N^4-methylcytosine (m4C) methyltransferase in *H. pylori* 26695, thus may exhibit unique expression patterns.[14] Neither of these two Type II MTases had any significant changes in expression. This suggests that the regulation patterns we have discovered are unique to Type I MTases.

Given that *hsdM*1 (HP0463) is acid responsive, and none of the other Type I or selected Type II DNA methyltransferases are, we aim to characterize the impact that differential acid

conditions, and thus differential *hsdM*1 expression, has on the methylome. We conducted a 3-day, continuous acid culture experiment in both pH 5 and pH 7 broth in four strains: *H. pylori* 26695 control mutant, *ΔarsS* mutant, *ΔhsdM*1 mutant, and *hsdM*1-repair mutant. Through this data, we should be able to determine the impact of prolonged acid exposure on the methylome, if the changes in the methylome in acid are ArsS-dependent and/or *hsdM*1-dependent, and if *hsdM*1-repaired has differential function than wild-type 26695 *hsdM*1.

The protein HsdM1 in *H. pylori* 26695, the first strain with a sequenced genome, is predicted to be missing the last ~56 amino acids (of ~543) due to a frameshift mutation in *hsdM*1 (HP0463). We re-inserted the adenine present in the subsequently sequenced *H. pylori* genomes to reverse the frameshift mutation. A similar frameshift mutation also exists in at least one other *H. pylori* strain, K2681, an *H. pylori* strain isolated in Angola (Montano,V., Didelot,X., Foll,M., Linz,B., Reinhardt,R. Suerbaum,S., Moodley,Y. and Jensen,J.D. Detecting global and local adaptation in a worldwide sample of *Helicobacter pylori* genomes. Unpublished. https://www.ncbi.nlm.nih.gov/nuccore/CP011486). The full protein exists in the majority of sequenced *H. pylori* strains, including commonly studied *H. pylori* strains J99 and G27 (data not shown). This led us to hypothesize that the full and truncated proteins may have different functions. This repair mutant will be compared against the wild-type strain to determine if there is differential functionality in *hsdM*1-truncated and *hsdM*1-repaired. We are currently anticipating the results of the methylome sequencing in the final week of May 2022, so we cannot present or discuss these findings yet.

Conclusion

In the human stomach pathogen *Helicobacter pylori* strain 26695, the Type I DNA methyltransferase *hsdM*1 (HP0463) is regulated by the main acid-sensing mechanism of the cell, the two-component system ArsRS. *hsdM*1 is repressed under pH neutral conditions and induced under acidic conditions. *hsdM*1 acid response is unique among the Type I DNA methyltransferases and two selected Type II methyltransferases in *H. pylori* 26695. In addition, *hsdM*1 and *hsdM*2 (HP0850) seem to be under the regulation of the quorum sensing protein LuxS. This is consistent with the data that expression of Type I MTases is reduced upon resuspension in new media which lacks the extracellular quorum sensing molecule AI-2 that is produced by LuxS. The selected Type II MTases, *M.HpyAI* (HP1208) and *M.HpyAII* (HP1368), do not follow the observed patterns and did not exhibit any significant changes in gene expression. This indicates that our findings are *hsdM*1 and Type I methyltransferase-specific and allows us to use the Type II methyltransferase as a control for any global effects the experimental procedure may cause. The methylome sequencing data will allow us to determine the effects of differential methyltransferase expression on the methylome. The ability of restriction-modification systems to regulate the genome affords *H. pylori* methylome plasticity, and this study helps further elucidate the mechanisms this system. Methylome plasticity may increase genetic diversity and genome plasticity to aid *H. pylori* in colonizing the harsh and volatile environment of the human gastric epithelium.

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Supplementary Figures

Figure S1. **ThermoFisher TaqMan Assay Sequences**

gyrB - consensus
Fwd 5'AAAGCCAGAGAGCTTACAAGGAAAA
Rev. 3' CGCCCTCCACTAAAAAGATTTCACT
Reporter 5' FAM TTGCCTGGAAAATTAG 3' NQR

HP0463_hsdM- type I restriction enzyme M protein (*hsdM*1)
F- ATGAGCCGACTAGAAATGTCAAAATCT
R- CCTATTTGGTGGGCTAATGCCATT
Reporter- CCTGTGCCTGCACTTG

HP0850 - type I restriction enzyme M protein (*hsdM*2)
F- AAAGTGTTAGGCGATAAAAATGTCTCAAAAG
R- GGCAAAGGTTGTAAGTGGTCAAATT
Reporter- TCTTGCCCAAAATACC

HP1403 - type I restriction enzyme M protein (*hsdM*3)

F- CGCGCGCCAGAAAGG

R- AGCGGTTTTATTGCCGTCTTTTT

Reporter- CCTTGCTCGCATCTAT

HP1208 - ulcer associated adenine specific DNA methyltransferase

F- GCCTTAAAAAAAGCGCTCAAAAAGA

R- TGCTGATACACTTCGTTAGCGTTTA

Reporter- AAAGGCGCTGATTTTG

HP1368 - type IIS restriction enzyme M2 protein (mod)

F- ACATGCTAAAAAACAAACCTAAAATGTTCTTACT

R- GGGTAGCTCCCAAACATCAATCTTT

Reporter- ACGCGCAAATCCCAC

Figure S2: *H. pylori* 26695 Type I Restriction-Modification Systems

Locus Tag	**Gene Name**	**Protein Class**
HP0464	*hsdR*1	Restriction Endonuclease
HP0463	*hsdM*1	DNA Methyltransferase
HP0462	*hsdS*1b	Specificity Subunit
HP0846	*hsdR*2	Restriction Endonuclease
HP0850	*hsdM*2	DNA Methyltransferase
HP0848	*hsdS*2	Specificity Subunit
HP1402	*hsdR*3	Restriction Endonuclease
HP1403	*hsdM*3	DNA Methyltransferase
HP1404	*hsdS*3b	Specificity Subunit
HP0790	*hsdS*5	Specificity Subunit
HP1383	*hsdS*6	Specificity Subunit

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