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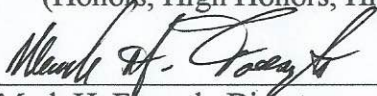
Mechanisms of *Helicobacter pylori* Adhesion Regulation and Impacts on Host Immune Response

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

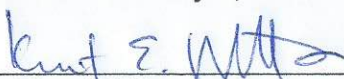
by

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Accepted for Honors
(Honors, High Honors, Highest Honors)



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Mechanisms of *Helicobacter pylori* Adhesion Regulation and Impacts on Host Immune Response

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A thesis submitted in partial fulfillment of the requirements of the degree of Bachelor of Science with Honors in Biology from The College of William & Mary in Virginia.

Williamsburg, Virginia

May 2016

Abstract

Helicobacter pylori is a gram-negative bacterium that colonizes the human gastric mucosal layer of 50% of the world's population. *H. pylori* utilizes a variety of adhesin proteins to adhere to the gastric epithelial layer, allowing the bacterium to successfully colonize its host, gain access to nutrients, and persist even during gastric mucosal shedding. The present study investigates transcriptional regulation of adhesin-encoding genes *sabA* and *hopZ* in the *H. pylori* strain J99. Several adhesin-encoding genes, including *sabA* and *hopZ*, possess a repeating homopolymeric nucleotide tract within their promoter region and a poly-cytosine-thymine (poly-CT) tract downstream of the translational start site. Strain J99's *sabA* promoter homopolymeric tract consists of 18 thymines, whereas the *hopZ* promoter has a poly-adenine tract composed of 14 adenines. Both *sabA* and *hopZ* are phase-off, i.e., full-length protein cannot be synthesized, in the wild-type based upon poly-CT tract repeat lengths of 8 and 6 respectively. We used site directed mutagenesis to extend and truncate the poly-thymine or poly-adenine tract to determine whether altering lengths of these homonucleotide tracts affects transcription frequency of *sabA* or *hopZ*. Using qRT-PCR, we found that extending or truncating the poly-thymine tract of *sabA* or the poly-adenine tract of *hopZ* by five thymines or adenines respectively increases transcription frequency of these adhesin-encoding genes. In addition, alterations in the poly-CT tract of *sabA* to switch phase status to phase-on led to significant increases in transcription frequency of *sabA*. However, phase-on and poly-T tract extension mutations did not have synergistic effects on *sabA* transcription frequency. Phase-on mutations, but not poly-thymine tract mutations, increased *H. pylori*'s adhesion to human gastric epithelial cells. Not only did the phase-on mutants increase *H. pylori* adhesion, but also induced more inflammatory cytokine interleukin-8 (IL-8) production by the human gastric epithelial cells. Overall, the present study

examines different mechanisms of *H. pylori* adherence regulation at the genetic level and effects on the host inflammatory immune response.

Introduction

Helicobacter pylori

Helicobacter pylori is a gram-negative bacterium that colonizes the human gastric mucosal layer of 50% of the world's population, making it one of the most prevalent human bacterial infections (Brown, 2000). Early research linked *H. pylori* infection to the development of ulcers, gastritis, and gastric adenocarcinoma (Marshall & Warren, 1984; Blaser *et al.*, 1995). Since then, many studies have provided overwhelming evidence supporting *H. pylori*-specific induction of these gastric pathologies. As a result, the World Health Organization classified *H. pylori* as the sole bacterial Class I carcinogen, meaning there is sufficient evidence of carcinogenicity in animal models and humans (World Health Organization, 2015).

H. pylori has co-evolved with humans for tens of thousands of years (Atherton & Blaser, 2009). It was first discovered and characterized by Australian scientists, Barry Marshall and Robin Warren, in 1982. They observed the consistent presence of this bacterium in the stomachs of patients who exhibited gastritis and peptic ulcers (Marshall & Warren, 1984). Twenty-three years later, Marshall and Warren won the Nobel Prize for their discovery of *H. pylori* and its link to the development of gastric inflammation.

The link between *H. pylori* and gastritis, peptic ulcer disease, and gastric cancer sparked great interest in understanding how *H. pylori* can elicit disease and how the infection is transmitted. Several clinical studies demonstrated that *H. pylori* infection in children is more likely to occur when one or both parents of the child are infected (Malaty *et al.*, 1991; Oderda *et al.*, 1991). *H. pylori* is generally believed to be transmitted via a fecal-oral or oral-oral route,

usually from mother to child (Brown, 2000; Oshio *et al.*, 2009). *H. pylori* transfer via a fecal-oral route is evidenced by increased *H. pylori* infection prevalence in developing countries with limited access to clean water (Aziz *et al.*, 2015).

Marshall and Warren's discovery of *H. pylori* within the stomach was challenged because the highly acidic environment of the stomach was thought to create an inhospitable environment for bacteria. Later studies investigated the question of how *H. pylori* survives in such a highly acidic environment. A few years after the bacteria's discovery, researchers found that *H. pylori* is able to hydrolyze urea via a cytoplasmic protein called urease (Moblely *et al.*, 1988). Although urease is characterized as a cytoplasmic protein, it is also localized outside of or associated with the *H. pylori* outer membrane (Dunn & Phadnis, 1998). By hydrolyzing urea, *H. pylori* generates ammonia, which surrounds the bacterium in a basic microenvironment, protecting the bacterium from the harsh, acidic environment of the stomach (Chen *et al.*, 1997).

Pathogenicity

Although 50% of the world's population is infected with *H. pylori*, not all those infected develop *H. pylori*-associated pathologies (Brown, 2000). Genetic analysis of *H. pylori* present in patients with gastric cancer revealed the frequent presence of an island of DNA within these *H. pylori* isolates not frequently present in isolates of *H. pylori* from patients without Peptic Ulcer Disease (PUD) or gastric cancer (Ali *et al.*, 2005; Mattar *et al.*, 2007). This island of DNA, termed a pathogenicity island (PAI) encodes a Type IV Secretion System (T4SS), and was named the cytotoxin-associated gene pathogenicity island (*cagPAI*). The *cagPAI* also encodes a toxic effector protein, CagA. Interestingly, the origins of the *cagPAI* are unknown.

Since the discovery of this pathogenicity island, several studies have demonstrated ulcer, gastritis, and gastric adenocarcinoma development correlated significantly with *cagPAI*-positive

H. pylori infection in different populations (Blaser *et al.*, 1995). A major conclusion from these studies underscores the idea that patients infected with *cagPAI* positive *H. pylori* strains are more likely to develop gastric cancer than those patients infected with *cagPAI* negative strains or not infected by *H. pylori* at all (Blaser *et al.*, 1995).

CagA Signaling

Mounting evidence regarding the involvement of CagA in the development of gastric cancer led researchers to investigate further the CagA signaling pathway within the gastric epithelial cells and its potential effects on oncogenes and immune system response.

CagA contains unique C-terminal glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) repeated motifs (Müller, 2012). Schematically, EPIYA motifs are divided into distinct repeat regions A, B, and C (Figure 1). Eastern strains of *H. pylori* often do not possess a C repeat region, and instead possess a D repeat region (Müller, 2012). Upon translocation via the T4SS encoded by the *cagPAI* from *H. pylori* to host epithelial cell, CagA is first phosphorylated at a tyrosine residue within the EPIYA-C motif by the host c-Src kinase early in infection (Müller, 2012). This phosphorylation event primes CagA to be subsequently phosphorylated by another host kinase, c-Abl, at a different EPIYA motif site (Müller, 2012) (Figure 1). Phosphorylated CagA activates host intracellular SHP-2 tyrosine phosphatase that subsequently activates the Ras/Erk pathway as well as dephosphorylates Focal Adhesion Kinase (FAK), thus inducing morphological elongation of the host epithelial cell (Müller, 2012). However, unphosphorylated CagA can affect other signaling pathways such as STAT3 and PAR1b to disrupt tight junctions between gastric epithelial cells (Müller, 2012). Overall, the injection of CagA into host epithelial cells can elicit a variety of responses due to alterations in signaling cascades.

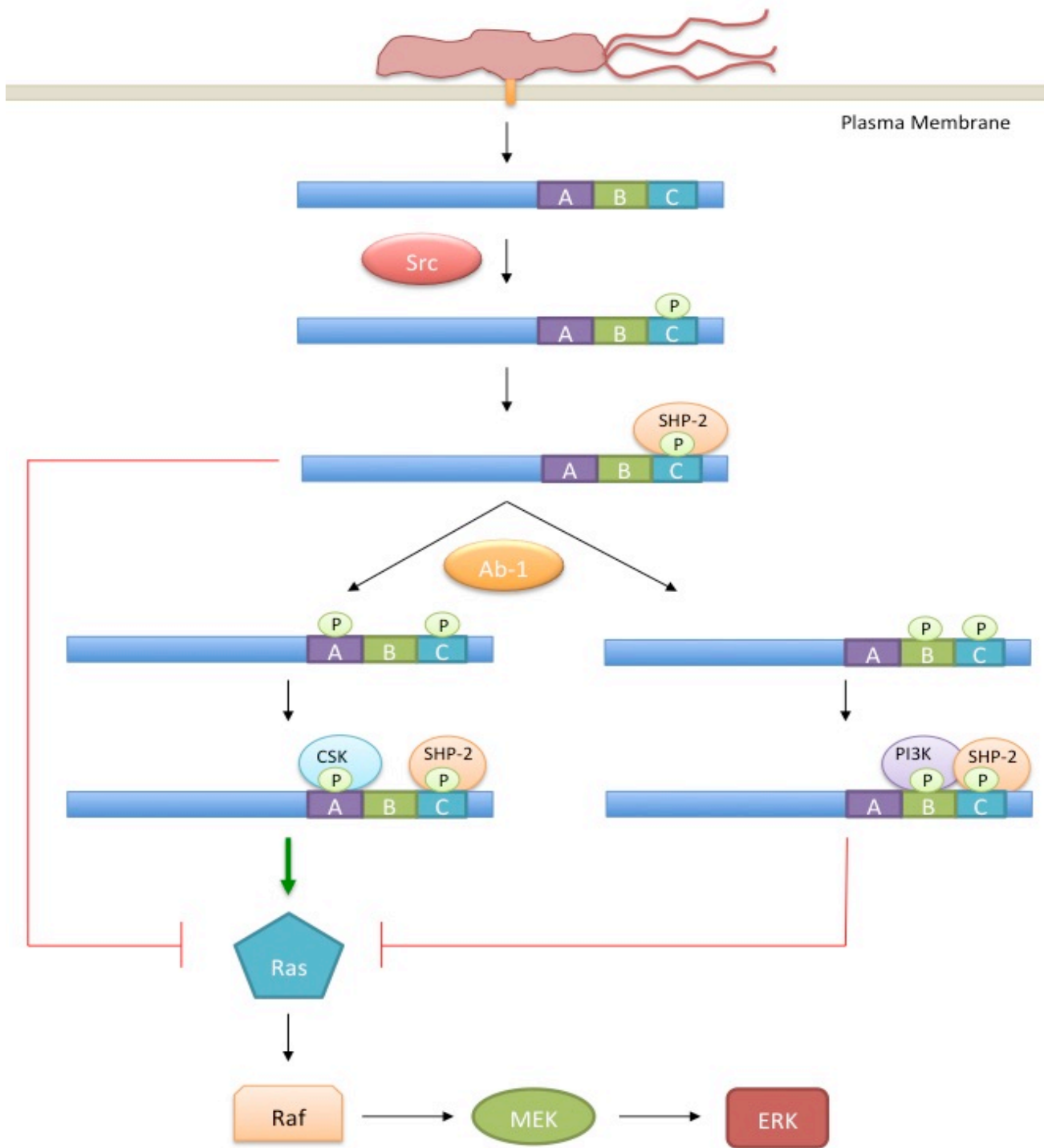


Figure 1: CagA phosphorylation pathways. CagA is phosphorylated by host Src and Ab1 kinases at different EPIYA motifs upon translocation. Only certain combinations of EPIYA phosphorylation patterns can activate the Ras-Raf pathway (Adapted from Müller, 2012).

***H. pylori* Induces Interleukin Expression**

Correlational studies demonstrated that gastric epithelial cells in patients infected with *cagPAI* positive strains of *H. pylori* tend to express more interleukins, such as interleukin-8 (IL-8) and interleukin-6 (IL-6) (Brandt *et al.*, 2005; Nakagawa *et al.*, 2013). Both IL-8 and IL-6 act as inflammatory chemokines that help recruit leukocytes to the site of *H. pylori* infection (Ataie-Kachoie *et al.*, 2014; Harada *et al.*, 1994, Brandt *et al.*, 2005, Nakagawa *et al.*, 2013). In addition to these functions, IL-6 promotes the maturation of B cells (Ataie-Kachoie *et al.*, 2014).

Although IL-8 and IL-6 expression is usually beneficial to the host as it triggers an immune response to infection, high expression levels of IL-8 and IL-6 are associated with the promotion of gastric tumorigenesis and metastasis (Kitadai *et al.*, 1998; Lee *et al.*, 2013; Zhu *et al.*, 2014, Kinoshita *et al.*, 2013).

Previous studies on colorectal and breast cancer, demonstrated that tumor cells overexpress IL-8, which was quantified in patient samples and cell lines using Southern Blots and quantitative polymerase chain reaction (qPCR) (Ning *et al.*, 2011; Freund *et al.*, 2009). This overexpression of IL-8 has also been linked to epithelial and tumor cell proliferation, and even resistance to chemotherapy (Waugh & Wilson, 2008; Ning *et al.*, 2011). IL-8 overexpression has been associated with increased angiogenesis within the stomach, which increased likelihood of tumor cell proliferation, cell movement, and metastasis (Kitadai *et al.*, 1998; Lee *et al.*, 2013). Mesenchymal stem cells (MSCs) present in gastric cancer activate neutrophils through IL-6 signaling via STAT3 and promote the differentiation of these MSCs into cancer-associated fibroblasts (CAFs) (Zhu *et al.*, 2014). Increased IL-6 production and signaling through STAT3 can promote tumorigenesis (Kinoshita *et al.*, 2013), as evidenced by impaired *H. pylori*-induced gastric tumor development in IL-6 knockdown mice. Overall, the increased angiogenesis and

tumor formation as a result of hyper-interleukin production can increase the severity of gastric cancer and likelihood of tumor metastasis (Kitadai *et al.*, 1998; Lee *et al.*, 2013; Zhu *et al.*, 2014, Kinoshita *et al.*, 2013). Subsequent investigations on the molecular mechanisms behind *H. pylori*'s induction of interleukins have proposed different pathways such as CagA-dependent and/or T4SS-dependent pathways.

One such pathway is the CagA-independent, *cagPAI*-dependent induction of interleukin-8 (IL-8) (Gorrell *et al.*, 2013; Boonyanugomol *et al.*, 2013). *Cag* positive strains of *H. pylori* utilize CagL, a structural protein within the T4SS, to interface with gastric epithelial cells (Cover, 2012). Association of CagL with its receptor integrin $\alpha 5\beta 1$ induces IL-8 production *in vitro* (Yeh *et al.*, 2013). CagL achieves this by initiating the $\alpha 5\beta 1$ -Src-Ras-Raf-ERK pathway, which leads to the activation of NF- κ B, an essential transcription factor for IL-8 expression (Gorrell *et al.*, 2013; Schlaepfer & Hunter, 1998) (Figure 2).

On the other hand, *H. pylori* also utilize a CagA-dependent pathway for increased IL-8 production, most notably later in the infection time course. Similarly to studies done with CagL, translocation of functional CagA has been linked to increased activation of NF- κ B (Brandt *et al.*, 2005). In addition, CagA-dependent IL-8 production is dependent on functional small GTPase protein Ras and mitogen-activated kinases Raf and MEK, thus suggesting that CagA induces IL-8 through a Ras-Raf-Mek-Erk-NF- κ B pathway (Brandt *et al.*, 2005).

Several studies have linked the presence of CagA with increased IL-8 production at later points (Sokolova *et al.*, 2013). However, other recent studies demonstrated that it is in fact the presence of a functional T4SS that is the essential feature in increased IL-8 production rather than the presence of functional CagA at earlier time points (Sokolova *et al.*, 2013). A study by Sokolova, *et al.*, in 2013, demonstrated that infection with either a wild-type *H. pylori* strain or a

cagA mutant strain induced phosphorylation and degradation of IκBα, an inhibitory protein of NF-κB. On the other hand, the *virB7* mutant strain of *H. pylori*, which is unable to synthesize the pilus of the T4SS, did not exhibit phosphorylation and degradation of IκBα. These results indicate that the release of NF-κB as a result of IκBα phosphorylation and subsequent degradation of IκBα is T4SS-dependent and CagA-independent in earlier time points (Sokolova *et al.*, 2013).

A recent study investigated the role of microRNAs (miRNA) in host interleukin production in response to *H. pylori* infection (Cheng *et al.*, 2015). miRNAs are small regulatory

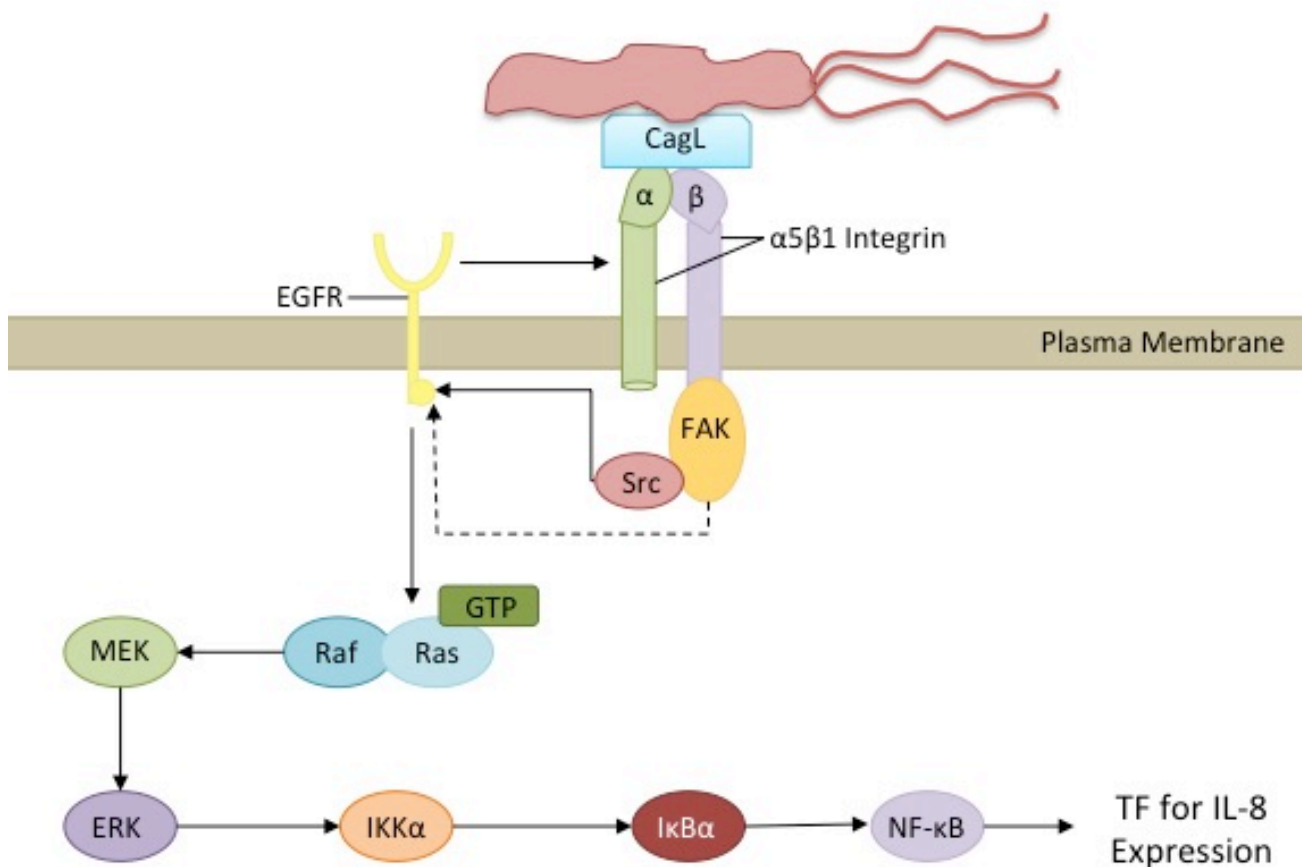


Figure 2: CagL-Dependent CagA-Independent IL-8 Activation. α5β1-Src-Ras-Raf-ERK pathway is essential in CagL-dependent and CagA-independent induction of IL-8 expression (Adapted from “Qiagen- ERK Signaling,” n.d. and Smith & Marshall, 2010).

RNAs that promote the degradation of target mRNAs post-transcriptionally. Previous studies have demonstrated that infection with *cag* positive strains of *H. pylori* induces overexpression of interleukin-6 (IL-6) (Cheng *et al.*, 2015), which allows for the recruitment of leukocytes to the site of *H. pylori* infection to help clear it (Scheller *et al.*, 2011). Gastric epithelial cell miRNA-155 and miRNA-146b, which are negative regulators of IL-6, are upregulated when gastric cells are infected with *cag* positive *H. pylori* (Cheng *et al.*, 2015). Upregulation of these miRNAs was demonstrated through integrative analyses of gastroduodenal ulcer biopsies. In order to analyze the effects of miRNA-155 and miRNA-146b, AGS cells were transfected with these miRNAs and then co-cultured with *cag* positive *H. pylori*. IL-6 expression was then analyzed using Western Blotting and immunohistochemistry (Cheng *et al.*, 2015). Through an undiscovered mechanism, *H. pylori* promotes the expression of miRNA-155 and miRNA-146b, which leads to a decrease in IL-6 expression (Cheng *et al.*, 2015). These findings further our understanding of how *H. pylori* can impair host immune response in chronic infection.

***H. pylori* Promotes Oncogenesis**

As previously discussed, *cagPAI* positive *H. pylori* strains have been frequently linked to the development of ulcers, gastritis, and gastric cancer (Blaser *et al.*, 2005). Various studies investigated the impact of CagA and the T4SS on oncogenic targets within host cells subjected to *H. pylori* infection. One protein marker used in the diagnosis and prognosis of gastric cancer is osteopontin (Chang *et al.*, 2015). Osteopontin is a protein involved in immune response, inflammation, and apoptosis regulation (Chang *et al.*, 2015). Increased levels of osteopontin (OPN) lead to inhibition of apoptosis, which can contribute to the development of cancer. In one study, CagA significantly increased the expression of intracellular osteopontin (iOPN), as quantified by Western Blotting, in human gastric epithelial cells (Chang *et al.*, 2015). In

addition, the functionality and adhesion of the T4SS via adhesion protein CagL was also required for this increase in iOPN (Chang *et al.*, 2015). Increase of iOPN is specifically associated with increased production of IL-8 as demonstrated by the decrease in IL-8 protein when gastric epithelial cells were treated with small interfering RNA (siRNA) targeting iOPN. Additionally, accumulation of β -catenin, a protein involved in epithelial cell adhesion, results from increased production of IL-8, as demonstrated by similar techniques (Chang *et al.*, 2015).

In addition to affecting osteopontin expression, the CagA can also impact DNA copy number and transcriptional expression of certain oncogenes, such as the gene for human epidermal growth factor receptor-2, otherwise known as *HER-2* (Shim *et al.*, 2014). *HER-2* encodes for a membrane tyrosine kinase receptor (Sun *et al.*, 2015; Guitierrez & Schiff, 2011). Its activation has been linked to tumor cell proliferation (Sun *et al.*, 2015; Guitierrez & Schiff, 2011). Patients with breast cancer exhibit an overexpression of *HER-2*, demonstrating the role of *HER-2* in the development of cancer (Gutierrez & Schiff, 2011). *H. pylori* can trigger the overexpression of *HER-2* in human gastric epithelial cell lines via induction of increased *HER-2* copy number, quantified by RT-PCR and immunoblotting, as a result of CagA translocation (Shim *et al.*, 2014). Although the exact mechanism has not been fully elucidated, one study (Shim *et al.*, 2014) found that CagA translocation in conjunction with exposure to hydrogen peroxide led to an increase in reactive oxygen species (ROS) and *HER-2* copy number, transcription, and translation. However, when exposed to radical scavenger TEMPOL (Chatterjee *et al.*, 2000), the increased amounts of ROS and *HER-2* copy number were reverted (Shim *et al.*, 2014). These findings suggest that increased *HER-2* copy number may be a result of CagA's induction of ROS.

CagA and CagL can work in concert to promote oncogenesis within the gastric microenvironment. One particular CagL amino acid polymorphism Y58/E59 is associated with increased CagA phosphorylation, downstream IL-8 expression, integrin protein $\beta 1$ activation, and integrin protein $\alpha 5$ expression (Yeh *et al.*, 2013). Although studies demonstrate a variety of consequences associated with this polymorphism, there are currently no studies explaining why these specific amino acid substitutions elicit these changes. Yeh and colleagues' study observed most CagA phosphorylation between a pH of 5-7. However, lowest CagA phosphorylation occurred at more acidic conditions with a pH of 4.4 (Yeh *et al.*, 2013). The CagL Y58/E59 mutant demonstrated higher amounts CagA phosphorylation and translocation into epithelial cells and subsequent increased IL-8 production (Yeh *et al.*, 2013). Integrin $\beta 1$ and $\alpha 5$ were found to be activated and expressed more at a neutral pH in CagL Y58/E59 mutants (Yeh *et al.*, 2013). These key changes are essential in understanding the persistence of oncogenesis due to *H. pylori*. Chronic *H. pylori* infection can lead to de-acidification of the stomach microenvironment (Zhao *et al.*, 2003) and, therefore, increased expression and activation of key proteins involved in oncogenesis pathways.

***H. pylori* Adhesion**

In vivo, *H. pylori* exists both bound to gastric epithelial cells or actively navigating within the gastric mucus layer. *H. pylori* adherence to gastric epithelial cells increases the bacterium's access to nutrients from host cells and prevents clearance of the bacterium when the mucus layer is shed. However, adherence may be detrimental to the bacterial population if the host exhibits a strong inflammatory immune response to the presence of *H. pylori*.

H. pylori utilizes a variety of outer membrane proteins, called adhesins, to bind to gastric epithelial cells. These proteins include BabA, SabA, AlpA, AlpB, OipA, HopZ, and HomB.

BabA, which binds to Lewis b antigens presented on gastric epithelial cells, was one of the first and most well characterized adhesin proteins of *H. pylori*. Expression of the *babA* gene at the transcriptional and translational level is regulated via phase variation resulting from polymorphisms of the poly-cytosine-thymine (poly-CT) dinucleotide tract within the 5' coding region. Due to its repetitive nature, the poly-CT dinucleotide tract is susceptible to slip-strand mispairing which is an error that results during genome replication. Errors that result from slip-strand mispairing can lead to extensions or truncations of repetitive nucleotide tracts, which can therefore alter the frame and thus the expression or “*phase*” status of transcribed mRNA from the gene. *babA* has two paralogous genes, *babB* and *babC*, which are hypothesized to originate from duplication events of the *babA* gene. As a result, *babA* expression is also regulated via ectopic gene conversion with these slightly divergent paralogous genes. Interestingly, adherence mediated by BabA has been shown to potentiate the Type IV Secretion System (T4SS) encoded by the *cag* pathogenicity island (Ishijima, 2011).

Sialic acid binding protein A, otherwise known as SabA, is another adhesin protein that shares some characteristics with BabA. SabA binds sialyl-dimeric-Lewis x antigens present on gastric epithelial cells as well as sialylated structures on mucins, neutrophils, and erythrocytes (Oleastro & Menard, 2013). Like *babA*, the *sabA* gene is regulated via phase variation of a poly-CT tract through a slip-strand mispairing mechanism and ectopic gene conversion with its paralogous gene *sabB* of unknown function (Goodwin *et al.*, 2008; Talarico *et al.*, 2012). In addition, *sabA* expression is repressed by phosphorylated ArsR protein in response to increased acidity signaled through the ArsRS two-component signal transduction (TCST) system (Goodwin *et al.*, 2008).

Hypothetical outer membrane protein Z, or HopZ, has been recently identified as an adhesin, but its receptor has yet to be determined. Again, similarly to the regulation of *babA* and *sabA*, *hopZ* expression at the transcriptional and translational levels is regulated via slip-strand mispairing phase variation of a poly-CT tract within its coding region (Kennemann *et al.*, 2012). Other possible mechanisms of *hopZ* expression regulation have yet to be elucidated.

Because *H. pylori* adhesion can be a benefit or a detriment to the bacterium's fitness depending on the environment, tight regulation of adhesin protein expression is essential. Interestingly, the promoter regions of several adhesin and putative adhesin genes contain a unique homopolymeric tract composed of either thymines or adenines. For example, in one of the first strains of *H. pylori* to be completely genetically characterized (Alm *et al.*, 1998), J99, *sabA* contains a poly-thymine (poly-T) tract composed of 18 tandem thymines from the -50 to -33 relative to the transcriptional start site, whereas *hopZ* contains a poly-adenine (poly-A) tract composed of 14 adenines from -37 to -24. Similarly, putative adhesin *sabB* also possesses a poly-T tract of 10 thymines from -42 to -33. The conserved presence of these repetitive homopolymeric tracts within the promoters of putative adhesin protein-encoding genes suggests that these tracts may play a role in the regulation of adhesin gene expression.

H. pylori is a complex bacterium that has evolved over thousands of years to colonize the human stomach and persist throughout the host's lifespan. In some cases, the presence of *H. pylori* can be detrimental to the host, causing inflammation, PUD, gastritis, and/or gastric cancer. Understanding the regulation of *H. pylori* adherence to the gastric epithelium and implications of increased adherence on host health will elucidate *H. pylori*'s ability to persist in the hospitable environment of the stomach and elicit disease.

Research Goals and Experimental Methods

H. pylori adherence is critical to successful colonization of the host and persistent infection. The adhesin-encoding *sabA* gene is an excellent model for studying the genetic regulation of *H. pylori* adherence because the SabA protein is very well-characterized (Yamaoka, 2008; Pang *et al.*, 2013; Aspholm *et al.*, 2006) and many of the *sabA* promoter and phase variable motifs are observed in other putative adhesin-encoding genes (Alm *et al.*, 1998; Tomb *et al.*, 1997). Although *H. pylori* colonization is beneficial to the bacterium, this may elicit abnormal gastric pathologies within the host stomach, often initiated by the toxic effector protein CagA. The main goals of this study are to examine common regulatory mechanisms of adhesin-encoding gene expression and investigate the effects of increased *H. pylori* adherence on host cell inflammatory response. The main regulatory motifs this study focuses upon include homo-polymeric tracts within the promoter region and poly-CT tracts downstream of the transcriptional and translational start sites. Mutations were created in the homo-polymeric and poly-CT tracts of adhesin-encoding genes *sabA* and *hopZ* as well as putative adhesin-encoding gene *sabB*. Gene transcription of these mutant *H. pylori* strains was quantified using qRT-PCR. Adhesion assays were also conducted to determine differences in *H. pylori* mutant adhesion to gastric epithelial cells. Following these experiments, enzyme-linked immunoabsorbent assays were performed to quantify and compare gastric epithelial cell inflammatory cytokine IL-8 production in response to increased *H. pylori* adherence. Overall, this study explores expression regulation of *H. pylori* adhesin-encoding genes and effects of hyper-adherence on host inflammatory immune response in an *in vitro* model.

Methods

H. pylori Culture

H. pylori strain J99 was cultured on tryptic soy agar II with 5% sheep's blood (BBL™) for 24-48 hours at 37°C in an ambient air/5% CO₂ atmosphere. Liquid cultures of *H. pylori* were grown in sulfite-free Brucella broth supplemented with 10% newborn calf serum, or 1X cholesterol (Gibco® by Life Technologies™), and 20µg vancomycin/mL shaking at 150 rpm.

AGS Cell Culture

AGS cells were a gift from Timothy Cover of Vanderbilt University Medical Center. Cells were grown in RPMI Medium 1640 supplemented with L-glutamine (Gibco® by Life Technologies™), HEPES (Gibco® by Life Technologies™), 10% fetal bovine calf serum (FBS), and penicillin/streptomycin (P/S). Cultures were grown at 37°C in an ambient air/5% CO₂ atmosphere on either 6-well tissue culture treated plates (CytoOne®) or tissue culture flasks (PRIMARIA™).

Cloning of *sabA* plasmids

A 603 base pair amplicon including the 3' end of *jhp0663*, the poly thymine (poly-T) tract, -35/-10 putative promoter sites, transcriptional start site, and 5' coding region of *sabA* (*jhp0662*) was amplified using oligonucleotide primers HP0726 Fwd and *sabA*PolyT.R (Table 1) from *H. pylori* strain J99. The amplicon was initially cloned into pCR® 4-TOPO® (Invitrogen), sub-cloned into the *EcoRI* site of pBlueScript SK+ vector (Stratagene), and designated *psabA*.

To generate the control plasmid, the construct upon which all subsequent mutations were made, a chloramphenicol acetyltransferase (CAT) gene from *Campylobacter coli* was cloned as a *SmaI/EcoRV* fragment (Wang & Taylor, 1990) from pBSC103 (Ando *et al.*, 1990) into a blunt-ended *MluI* site at the end of *jhp0633*, the gene immediately upstream of *sabA*. The resulting

control plasmid, selected for resistance to 25µg chloramphenicol/ml in *E. coli* DH5α, was designated *psabA.T18* (Table 2). The orientation of the chloramphenicol resistance gene in this construct was confirmed by sequencing.

The poly-T tract region of *sabA* within the control plasmid *psabA.T18* was mutated to varying T tract lengths using mutagenic primers *sabA.5T.F*, *sabA.5T.R*, *sabA.less5T.F*, and *sabA.less5T.R* (Table 1). Mutagenic oligonucleotides were used with the GeneArt® Site-Directed Mutagenesis System and Accu Prime™ Pfx polymerase (Life Technologies). Mutagenic primers were designed to match the sequence of the poly-T tract site and surrounding sequence, however modifications in the number of T's were introduced, adding or subtracting five T's. The primers were designed according to the manufacturer's specifications.

For the second series of mutagenesis experiments introducing alternate sequence extensions, the forward and reverse primers introducing AAAAA, GGGGG, or the random sequence ACTAG upstream and immediately adjacent to the poly-T tract were designated *sabA.5A.F*, *sabA.5A.R*, *sabA.5G.F*, *sabA.5G.R*, *sabA.Ran.F*, and *sabA.Ran.R* respectively (Table 1). Mutagenesis was carried out according to the manufacturer's protocol using 40 ng of *psabA.T18* per 50µl reaction. The mutagenesis product was analyzed by agarose gel electrophoresis, and underwent a recombination reaction and transformation into DH5α-T1^R *E. coli* as suggested by the manufacturer's protocol.

Plasmids were generated with variant poly-T tract lengths of T13 and T23. In the process of creating plasmids with the expected new length of thymines, alternate length polymorphisms were isolated as well, possibly due to oligonucleotide poly-T tract length polymorphisms generated during oligonucleotide synthesis. Thus, three additional plasmids were isolated and confirmed via sequencing reactions and amplified fragment length polymorphism to have lengths

of T17, T16, and T22 (Table 2). They too were subsequently recombined onto *H. pylori* strain J99 genome via allelic exchange to further characterize the role of the poly-T tract in *sabA* expression.

For additional mutagenesis experiments, plasmids were created to represent three different extended poly-T tracts; the T18 tract extended by the nucleotides AAAAA (*psabA.A5*), the random nucleotides ACTAG (*psabA.Ran*), or the nucleotides GGGGG (*psabA.G5*) at the 5' end of the poly-T tract (Table 2). The mutated poly-T tract regions of all plasmids were confirmed via sequencing reactions performed using the Big Dye® Sequencing Kit (Applied Biosystems), using the primer CAT Fwd (Table 1).

To examine whether phase variation and polymorphisms in the poly-T tract length have an additive effect on *sabA* transcription frequency, plasmids were created to turn the *sabA* gene phase-on through altering the poly-CT tract or turn the *sabA* gene phase-on and increase the promoter poly-T tract by five thymines. A mutagenic primer, *sabA.On* (Table 1) was designed to change the poly-CT tract length from a wild-type phase-off length of 8 CT's to 7 CT's, turning the *sabA* gene phase-on. This primer was used in QuikChange Lightning Multi System (Agilent) mutagenesis reactions with the *psabA.T18* and *psabA.T23* plasmids described previously. Mutagenesis reaction products were used to transform XL10-Gold Competent cells, which were selected for chloramphenicol resistance. The mutated poly-CT tract regions of phase-on plasmids were confirmed via sequencing reactions performed using the Big Dye® Sequencing Kit (Applied Biosystems), using the primer CAT Fwd (Table 1). Although the mutagenic primer was designed to decrease the poly-CT tract to a length of 7 CT's, selected colonies from mutagenesis reactions had poly-CT tract lengths of 10 CT's. This CT mutation in *psabA.T18* and *psabA.T23*,

despite differing from the mutagenic primer, still turned the *sabA* gene phase-on without any other addition mutations.

Cloning of *hopZ* plasmids

Using primers *hopZ.F2* and *hopZ.R2* (Table 1), a 853 bp amplicon, including the 3' end of the upstream gene, promoter sequence of *hopZ*, transcriptional start site of *hopZ*, and 5' coding region of *hopZ*, was isolated using PCR from *H. pylori* strain J99. The resulting amplicon was cloned into a pCR® 4-TOPO® vector, confirmed through sequencing using the Big Dye® Sequencing Kit (Applied Biosystems) and *hopZ.F2* primer, and designated *phopZ* (Table 2).

To create a *hopZ* control plasmid, similar to the one used in of *sabA* experiments, we designed a mutagenic primer *hopZ.BglIII* to insert a *BglIII* site 152 bp upstream of the transcriptional start site of *hopZ* and performed mutagenesis using GeneArt® Site-Directed Mutagenesis System and Accu Prime™ Pfx polymerase (Life Technologies). Mutagenesis reactions were cloned into DH5α-T1^R *E. coli* as suggested by the manufacturer's protocol and selected for ampicillin resistance on Luria broth (LB) plates containing 100 micrograms/mL ampicillin. Plasmids isolated from successfully transformed *E. coli* were digested using *BglIII* restriction enzyme and associated buffer. Similarly to methods used in *sabA* cloning, a CAT gene originating from *Campylobacter coli* (Wang & Taylor, 1990), was ligated into the *BglIII* site of the digested *hopZ* plasmid using T4 ligase. Ligation products were used to transform DH5α *E. coli* and colonies were selected for chloramphenicol resistance on LB plates containing 25µg chloramphenicol/mL chloramphenicol. Insertion and forward orientation of the CAT gene was confirmed via sequencing reactions with *hopZ.F2* primer (Table 1). The resulting control plasmid was successfully generated and named *phopZ.A14* (Table 2).

Mutagenic primers inserting (*hopZ.A19F* and *hopZ.A19R*) or deleting (*hopZ.A9F* and *hopZ.A9R*) five adenines (Table 1) were used to mutate *phopZ.A14* using site-directed mutagenesis. Again, we transformed DH5 α *E. coli* and colonies were selected for chloramphenicol resistance. Insertion and deletions in the poly-A tract were confirmed via sequencing reactions and were designated as *phopZ.A19* and *phopZ.A9* respectively (Table 2).

Cloning of *sabB* plasmids

Primers *jhp0658.F* and *sabB.Rev* were used to isolate a 1658 bp amplicon from *H. pylori* strain J99 using PCR. Amplicon includes the promoter region of *sabB*, including the 3' end of gene *jhp0658*, the poly-T tract from positions -42 to -33, the transcriptional start site of *sabB*, poly-CT tract, and 5' coding region of *sabB*. This amplicon was cloned into the pCR[®] 4-TOPO[®] vector and cloning reaction was used to transform DH5 α *E. coli* and were selected for resistance to ampicillin. Plasmids were isolated from ampicillin-resistant *E. coli* colonies, sequenced using *jhp0658.F* primer (Table 1) to confirm the successful insertion of the *sabB* amplicon into the plasmid, and designated *psabB* (Table 2).

A mutagenic primer containing a *Bam*HI restriction site from positions +676 to +711 relative to the transcriptional start site of the amplicon (Table 1) was designed and used to introduce a *Bam*HI restriction site into *pSabB* via site-directed mutagenesis using the QuikChange Lightning Multi System (Agilent). Mutagenesis reaction products were used to transform XL10-Gold Competent cells, which were selected for ampicillin resistance. Introduction of a *Bam*HI site was confirmed by *Bam*HI digestion and by sequencing.

A gene cassette containing the chloramphenicol acetyl transferase and *rdxA* gene (CAT-*rdxA*) was isolated from pMM674 (Loh *et al.*, 2011), a gift from Mark McClain of Vanderbilt University Medical Center, using *Bam*HI restriction digest. Digestion reaction of the plasmid

was run on a gel via gel electrophoresis, and the CAT-*rdxA* cassette was isolated by gel purification. *psabB* was digested with *Bam*HI restriction enzyme and the CAT-*rdxA* cassette was ligated in using T4 ligase. This plasmid is designated *psabB*.CAT-*rdxA*. *psabB* was mutated using mutagenic primers designed to delete or insert five thymines from the poly-T tract, *sabB*.T5 or *sabB*.T15 respectively, or insert one CT in the poly-CT tract (*sabB*.On) (Table 1). Plasmids are designated *psabB*.T5, *psabB*.T15, and *psabB*.On respectively (Table 2).

Plasmid Purification and Isolation

5mL of liquid LB/chloramphenicol + ampicillin were inoculated with a single isolated colony from the antibiotic plates. Liquid culture incubated at 37° C shaking at 225rpm for 16-24hours. Cells were spun down at 4360 rpm. Cells were lysed and plasmids were isolated using Midsci Mini Hi-Speed Plasmid Kit. Concentrations of plasmid dsDNA were quantified using a nanophotometer (Implen).

H. pylori Transformation

Desired *H. pylori* strain to be transformed was grown from freezer stock for 3-5 days and passed onto 4 blood agar plates to grow for 24-36 hours. Cells were harvested in 1mL 0.9% NaCl saline solution and pelleted at 6000 rpm. *H. pylori* cells were resuspended in 100 microliters of sulfite-free brucella broth with 10% NCS and vancomycin. Transforming plasmid were heat-sanitized at 80° C for 20 minutes to reduce contamination. 7-10 µg of plasmid were added to resuspended *H. pylori* cells. Mixture was spotted over 5 blood agar plates and incubated in 37° C, 5% CO₂ for 4 hours. Spots were spread and plates were incubated for 37° C, 5% CO₂ for 24 hours. *H. pylori* was then harvested and passed onto sulfite-free brucella agar plates containing 10% newborn calf serum and the selection antibiotic chloramphenicol (10 µg/ml). Plates were incubated at 37° C, 5% CO₂ for 3-5 days.

Genomic DNA Extraction

H. pylori was grown for 24-48 hours in standard conditions. Cells were harvested in 1mL saline and spin down at 6000rpm. gDNA extraction via Promega Wizard Genomic DNA Extraction Kit was performed according to manufacturer's suggested protocol. Cells were lysed using provided nuclei lysis solution. Proteins were precipitated and separated via centrifugation. Supernatant containing gDNA was transferred into isopropanol and mixed until it precipitated. gDNA was washed using 70% ethanol and rehydrated in rehydration buffer provided.

Amplified Fragment Length Polymorphism (AFLP) Analysis

To quantify the degree of slipped-strand mispairing and the variation in poly-T length found in the J99 wild-type, J99 T18 control mutant and J99 *sabA* poly-T variant populations of *H. pylori*, amplified fragment length polymorphism (AFLP) was conducted by a modification of the protocol of Hallinger *et al.* (Hallinger *et al.*, 2012). Briefly, an oligonucleotide primer pair bracketing the poly-T tract was synthesized with a VIC tag on the 5' end of the reverse primer (Applied Biosystems/Life Technologies). Primers used in all AFLP were *sabA* IG Fwd and *sabA* IG Rev (Table 1). Amplicons generated were diluted by a factor of 50 and analyzed by ABI 3100 Automated Fluorescent DNA Sequencer (ABI) using a Liz 300 molecular weights standard set and data analyzed using GeneScan (Life Technologies).

RNA Extraction

H. pylori strains of interest were grown on TSA II with 5% sheep blood (Benton-Dickinson) at 37°C in an ambient air/5% CO₂ atmosphere. Broth cultures were grown in Sulfite-Free Brucella Broth (SFBB) with 10% newborn calf serum (Gibco/BRL), or 1X cholesterol (Gibco® by Life Technologies™), and 20µg vancomycin/mL until the cells reached an OD₆₀₀ of 0.8-1.6. For RNA extraction, 1x10⁹ cells were harvested at 3300 g for 5 minutes and the pellets

were resuspended in 1 mL Tri Reagent (Ambion) prior to RNA extraction. Total RNA was extracted from each cell pellet according to the manufacturer's protocol for the MagMAX™-96 for Microarrays Total RNA Isolation Kit (Life Technologies) and the AM1839 Spin Program on a MagMAX™ Express Magnetic Particle Processor (Life Technologies). Purified RNA concentrations were analyzed on a P360 Nanophotometer (Implen) and frozen at -80°C .

cDNA was synthesized from 1 μg of purified RNA samples using iScript reverse transcriptase (Bio-Rad), following the manufacturer's cDNA synthesis protocol. cDNA was diluted 1:10 and used for qRT-PCR.

qRT-PCR

The expression of *H. pylori* *sabA* and *hopZ* was compared to the housekeeping gene *ftsZ* (jhp0913) encoding the cell division protein FtsZ using a TaqMan® Gene Expression assay (Life Technologies) performed on the Applied Biosystems StepOne™. The assay was carried out according to the manufacturer's protocol using custom TaqMan Gene Expression assays, including the *sabA*.Taq, *hopZ*.Taq, and *ftsZ*.Taq probes (Table 1). Assays for each strain and each gene were run in technical triplicate, and experiments were repeated three times. Relative expression of genes among the various mutants was calculated using the $2^{\Delta\Delta\text{Ct}}$ method as described by Livak and Schmittgen (Livak & Schmittgen, 2001) and processed using DataAssist™ software (Applied Biosystems).

Adhesion Assay

2.5×10^5 AGS cells were grown in each well of a 6-well plate for 24 hours. Medium was removed and washed with antibiotic-free RPMI 1640 1X (Gibco® by Life Technologies™) supplemented with 1X HEPES (Gibco® by Life Technologies™) and 10% FBS three times. *H. pylori* suspended in supplemented RPMI was introduced to the washed AGS cells at a

multiplicity of infection of 100:1. AGS cells were co-cultured with bacteria for 5 hours shaking at 50 rpm in standard conditions.

Medium was removed and collected followed by three washes with supplemented RPMI. AGS cells were lysed using 1 mL of PBS/0.1% saponin for 15 minutes at standard conditions shaking at 50 rpm. Lysates were collected and serially diluted to 10^{-7} . Dilutions were spotted onto blood agar plates and incubated for 5 days at standard conditions. After 5 days, colony-forming units per mL (CFUs/mL) were calculated.

Enzyme-Linked Immunosorbent Assay (ELISA)

2.5×10^5 AGS cells were grown in each well of a 6-well plate for 24 hours. Medium was removed and washed with antibiotic-free RPMI 1640 1X (Gibco® by Life Technologies™) supplemented with 1X HEPES (Gibco® by Life Technologies™) and 10% FBS three times. *H. pylori* suspended in supplemented RPMI was introduced to the washed AGS cells at a multiplicity of infection of 100:1. AGS cells were co-cultured with bacteria for 5 hours shaking at 50 rpm in standard conditions.

Medium was removed and centrifuged at 6000rpm. Supernatant was collected for use in ELISA. 96-well plate (BioLegend®) was coated with human IL-8 capture antibody (ELISA MAX™ DELUXE by BioLegend®) overnight at 37°C. ELISA was performed according to manufacturer's suggested protocol (antibody (ELISA MAX™ DELUXE by BioLegend®). Plates were blocked using 1X Assay Diluent (ELISA MAX™ DELUXE by BioLegend®), following a series of washes with a 1X PBS 0.05% Tween-20 wash buffer, cell medium samples were added to appropriate wells and incubated for 2 hours. Wells were washed with wash buffer and IL-8 detection antibody (ELISA MAX™ DELUXE by BioLegend®) was added. Again, wells were washed with wash buffer, incubated with Avidin-HRP antibody (ELISA MAX™

DELUXE by BioLegend®), washed again, and incubated with Substrate Solution C (ELISA MAX™ DELUXE by BioLegend®) in the dark. Reactions were stopped using 2N sulfuric acid. Plate was read at 450nm and 570nm using a microplate reader (Synergy HT by BioTek®) and analyzed using Gen5 v2.05 software. Using IL-8 standards as references, absorbance readings were converted to concentration of IL-8.

Results

Multiple alleles of *sabA* exist within a population due to differing poly-T tract lengths

Plasmid constructs with modifications to the poly-T tract length were designed and introduced into *H. pylori* J99 strains. In order to examine allelic variation in the thymine repeat tract at the *sabA* locus within *in vitro* populations of *H. pylori* strain J99 and isogenic poly-T tract indel mutants, we employed Amplified Fragment Length Polymorphism (AFLP) analysis. Results indicated the presence of multiple length polymorphisms within the *sabA* poly-T repeat tract amplicons from wild-type and all indel mutants. While each *sabA* poly-T tract indel mutant population contained multiple sub-populations possessing slightly different poly-T lengths based upon AFLP analyses, each of these *H. pylori* J99 mutant strains had a clear dominant population with a particular modified poly-T tract length. The wild-type *H. pylori* J99 containing 18 thymines was measured as having a dominant sub-population with an amplicon of 230 base pairs (Alm *et al.*, 1999). DNA sequencing of this locus confirmed the creation of *H. pylori* J99 mutants with varying poly-T tract lengths. In addition, the length variation of amplicons possessing the poly-T tract of the *sabA* promoter within wild type and mutant *H. pylori* populations, demonstrated by AFLP, supports the hypothesis of slipped-strand mispairing events during DNA replication. In fact, our data suggests that this activity may increase with the increasing length of the repetitive tract. When we experimentally increased the length of the tract

from 18 to 23, AFLP analyses showed a less dominant size variant in the population and more measurable length polymorphisms.

Transcription of *sabA* and *hopZ* is Regulated by Homopolymeric Tract Length

To examine the effects of *sabA* promoter poly-T tract length on *sabA* transcription, *H. pylori* strain J99 was transformed with plasmids containing mutated poly-T tract lengths. RNA was isolated from poly-T tract J99 mutants (Table 3), converted to cDNA, and used in qRT-PCR

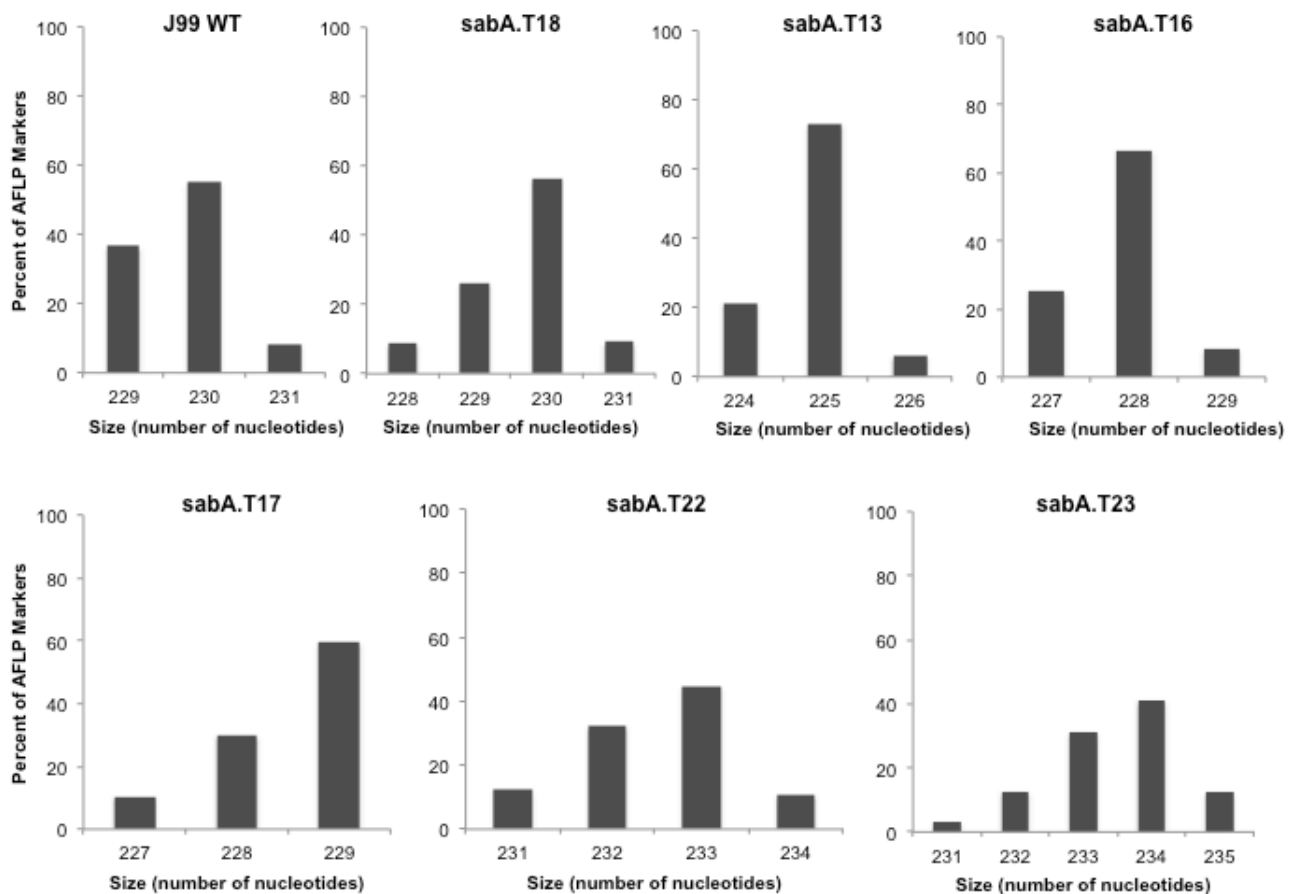


Figure 3: Amplified Fragment Length Polymorphism (AFLP) analysis of *H. pylori sabA* poly-T tract mutants. AFLP analysis was used to quantify variations in the *sabA* poly-T tract containing amplicons from *H. pylori* J99 and poly-T tract mutants. The amplicon generated using primers *sabA* IG Fwd and *sabA* IG Rev (Table 1) is predicted to be 230 bp based upon the annotated sequence of *H. pylori* strain J99 where the *sabA* poly-T tract possesses 18 thymines (Alm *et al.*, 1998).

to quantify *sabA* transcription in each J99 mutant. Insertion of the CAT gene, via the *psabA.T18* control plasmid (Table 1), into J99 did not affect transcription of *sabA* ($p > .05$) as determined by a Welch's unpaired t-test of unequal variance using the program R Studio (Figure 4A). Thus, *sabA* transcriptional differences are solely due to changes in the length of the poly-T tract. Mutations altering the poly-T tract to a length of 16, 17, or 22 thymines did not significantly alter *sabA* transcription ($p > .05$) (Figure 4B). Notable however was the 5.9-fold increase ($p = 0.0013$) of *sabA* expression in the *H. pylori* J99 strain *sabA.T13* when compared to *H. pylori* J99 strain *sabA.T18*, and the 10-fold increase ($p = 0.00086$) in *sabA* expression in the *H. pylori* J99 strain *sabA.T23* when compared to *H. pylori* J99 strain *sabA.T18* (Figure 4B).

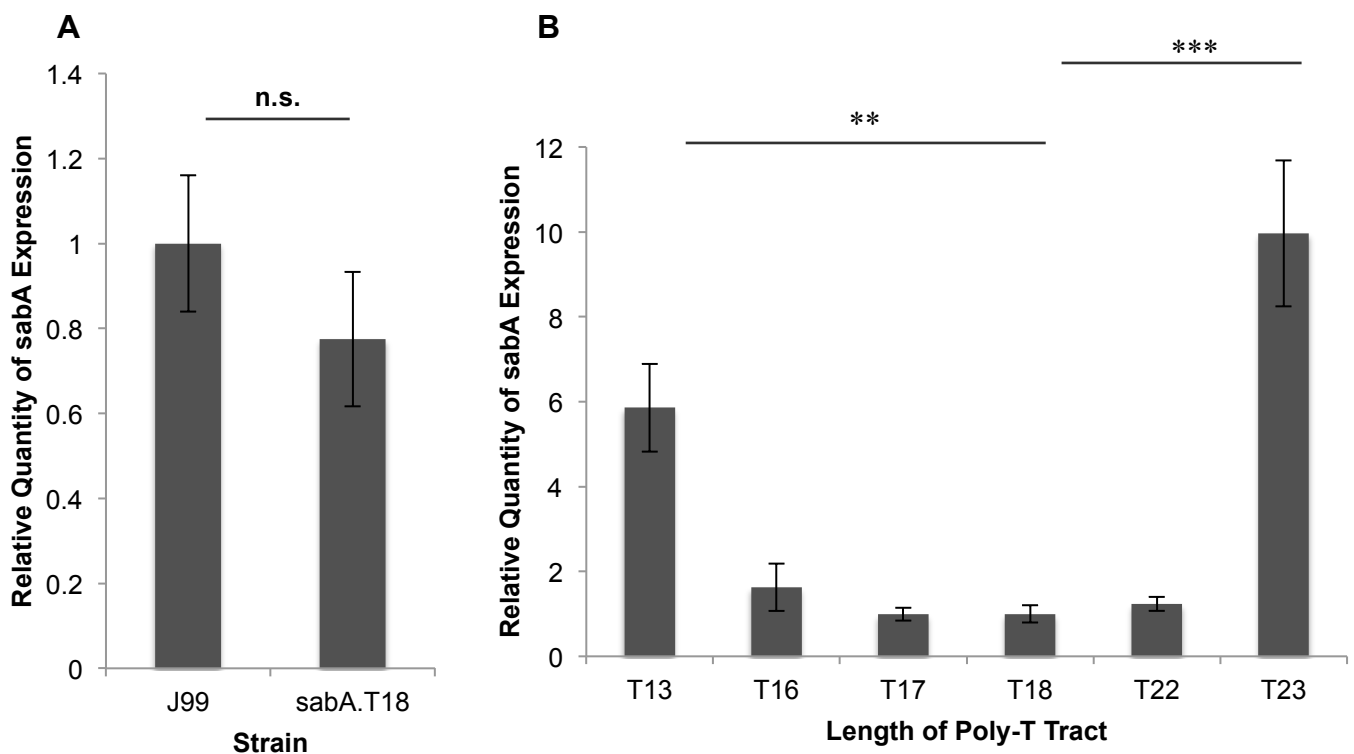


Figure 4: *sabA* promoter poly-T tract length affects transcription frequency.

(A) Relative *sabA* gene expression levels of *H. pylori* wild-type J99 (set to 1) in comparison to mutant control strain *sabA.T18* containing a CAT gene upstream of the *sabA* promoter sequence. (B) Relative *sabA* gene expression levels of *H. pylori* *sabA* poly-T tract mutants. Mutant control strain *sabA.T18* is set to 1. n.s. = $p > .05$, ** = $p < .01$, *** = $p < .001$ as determined by a Welch's unpaired *t*-test of unequal variance. Error bars show standard deviation.

Using similar methods, J99 was transformed with mutant plasmids containing extension or truncation mutations in the poly-A tract to lengthen or shorten the tract by five adenines. Comparable to experiments done with *sabA*, insertion of the CAT gene upstream of *hopZ*, via the *hopZ.A14* control plasmid, did not significantly impact the gene's expression ($p > .05$) as determined by a Welch's unpaired *t*-test of unequal variance using the program R Studio (Figure 4A). However, there was a 2.6-fold ($p = 0.00977$) or 2.2-fold ($p = 0.002205$) increase in *hopZ* transcription when the poly-A tract was truncated or extended by five adenines respectively when compared to the *H. pylori* J99 *hopZ.A14* strain (Figure 5B).

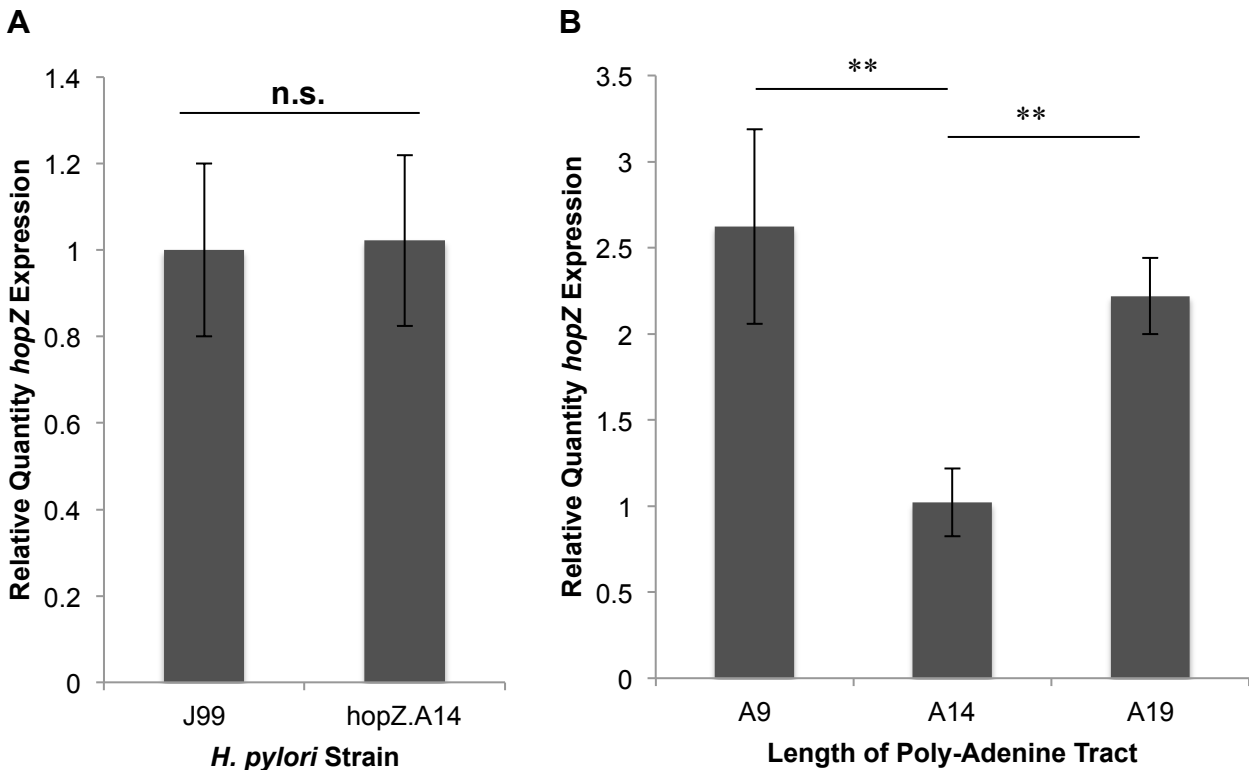


Figure 5: *hopZ* promoter poly-A tract length affects transcription frequency.

(A) Relative *hopZ* gene expression levels of *H. pylori* wild-type J99 (set to 1) in comparison to mutant control strain *hopZ.A14* containing a CAT gene upstream of the *hopZ* promoter sequence. (B) Relative *hopZ* gene expression levels of *H. pylori* *hopZ* poly-A tract mutants. Mutant control strain *hopZ.A14* is set to 1. n.s. = $p > .05$, ** = $p < .01$ as determined by a Welch's unpaired *t*-test of unequal variance. Error bars show standard deviation.

Poly-T and poly-A tracts are rigid and their presence affects the bendability of DNA (Suter *et al.*, 2000). The rigidity and bendability of certain regions of DNA have been found to influence proteins' ability to loop DNA (Laurens *et al.*, 2012). The formation of DNA loops is essential in processes such as DNA replication and transcription regulation. Thus, changes in sequences that modify the rigidity and bendability of sections of DNA influence the energetics of loop formation associated with these essential processes (Laurens *et al.*, 2012). We hypothesize that indels within the poly-T or poly-A tract in the promoter region of *sabA* or *hopZ* in *H. pylori* respectively may influence the ability for proteins to loop the DNA and ultimately affect RNA polymerases' ability to bind or for other protein-DNA interactions to take place. Homopolymeric tracts similar to the poly-T and poly-A tracts in *H. pylori* were found in a variety of prokaryotic systems (Orsi *et al.*, 2010). The overwhelming presence of homopolymeric tracts across prokaryotic taxa suggests that these tracts have been beneficial to these organisms. A study done by Wernegreen (Wernegreen *et al.*, 2010) proposed that these homopolymers are advantageous because they are mutational hotspots where slippage can help eliminate and resurrect gene function.

We sought to test our revised hypothesis through a subsequent qRT-PCR experiment with the poly-T tract of the *sabA* promoter. An algorithm created by Vlahovicek *et al.* (Vlahovicek *et al.*, 2003) predicts a sequence of adenine or thymine nucleotides to be non-curved, and of limited bendability while sequences with higher percentages of guanine and cytosine allow for more curvature and flexibility in DNA topology. To begin to address the role of curvature of DNA in the expression of *sabA*, we designed and created a series of *H. pylori* J99 *sabA* mutants that contained the wild-type *sabA* poly-T tract length of 18 nucleotides but now extended, either by five adenines (*sabA.A5*), five guanines (*sabA.G5*), or the random series of five nucleotides

ACTAG (*sabA*.Ran) (Table 3). In *sabA*.A5, *sabA* expression increased 11-fold compared to *sabA* expression in *sabA*.T18 ($p = 1.6 \times 10^{-5}$). This increased *sabA* expression was comparable to *sabA*.T23 mutant strain whose poly-T tract had been extended by five thymines (Figure 6). This result is perhaps not surprising as the extended tract still consists of A-T base pairs. Strikingly however, no significant increase ($p > 0.05$) in *sabA* expression occurred when the poly-T tract was extended by five guanines or by the random series of five nucleotides, ACTAG (Table 3) (Figure 6). Similar statistics and results were reproduced in the additional two biological replicates.

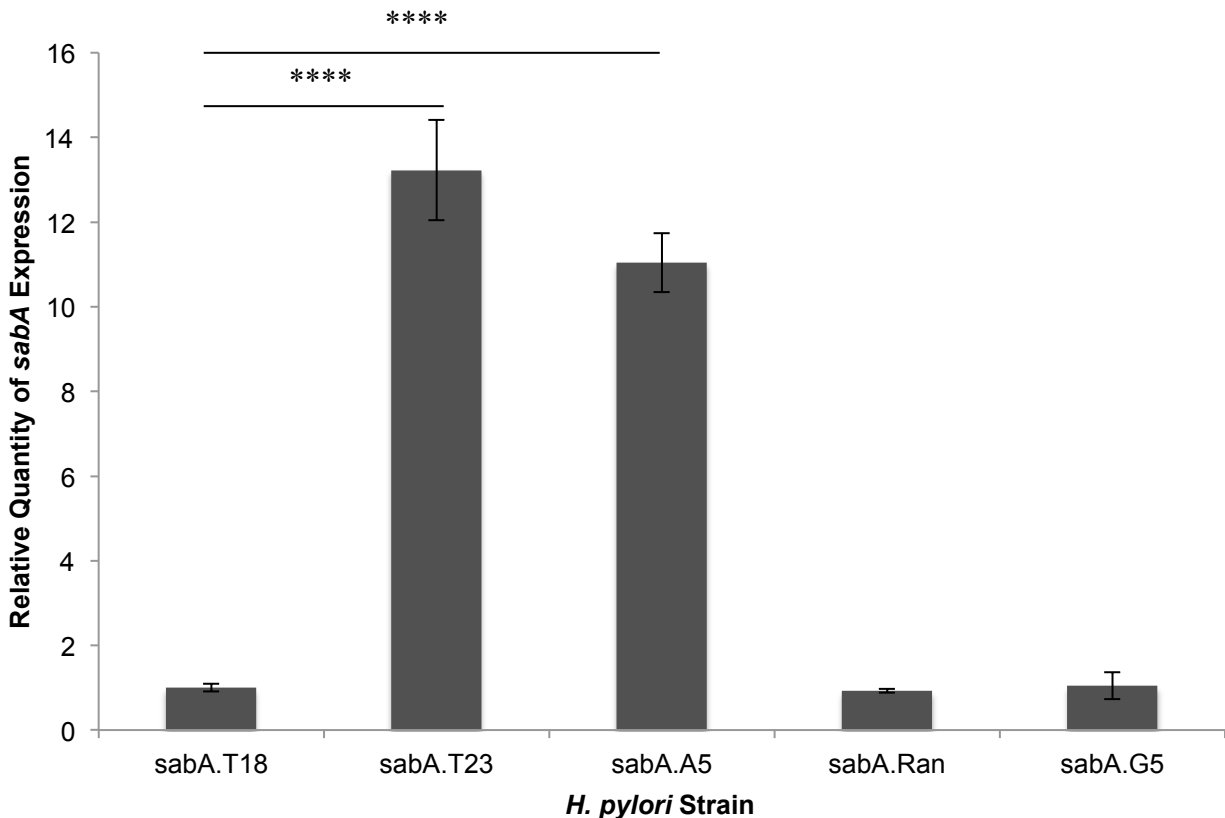


Figure 6: *sabA* expression in *H. pylori* J99 and J99 *sabA* poly-T mutants (Non-thymine extensions). Quantitative real time PCR was used to determine the relative expression of *sabA* in *H. pylori* J99 as compared with mutants containing a poly-T tract with various five nucleotide insertions upstream and adjacent to the poly-T tract. The data shown here is representative of the results obtained in three independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch's unpaired *t*-test of unequal variance with *sabA*.T18 as the control (**** = $p \leq .0001$).

These results support the hypothesis that the nature of the adenine-thymine tract, as compared to one possessing guanine-cytosine base pairs or a random series of base pairs, results in changes in DNA topology and has a major effect on *sabA* expression. The intrinsic curvature of poly-A tracts in the *ureR- ureD* intergenic region in *Proteus mirabilis* is bound in an *E. coli* model system by both UreR and H-NS. Thus, proteins binding to the intrinsic curvature of this poly-A tract affects *ureR* transcription (Poore & Mobley, 2003). We hypothesize that the proper bending and curvature of the DNA in and around the *H. pylori sabA* poly-T tract may allow RNA polymerase to bind to the promoter optimally, or allow an upstream activator site and its bound transcription factor to approach RNA polymerase closely enough to affect transcription initiation. It is not a simple case of changing the distance between the binding site for a trans-acting factor and the promoter that affects *sabA* expression, rather it is the topology of the DNA that allows for modulation of *sabA* expression.

Another potential means of the altered *sabA* promoter activity associated specifically with such an A/T homopolymeric tract could be via increased stability of RNA polymerase association with the promoter sequences mediated through the poly-T tract. Consensus UP elements have been identified that are A/T rich and located just upstream of -35 promoter elements (Estrem *et al.*, 1998; Estrem *et al.*, 1999). These sequences are capable of increasing RNA polymerase affinity for promoters by interacting with C-terminal domains of RNA polymerase α subunits. This may explain the similarity of the results of this study on *sabA* promoter activity in the native organism, *H. pylori*, with those of Kao *et al.* (Kao *et al.*, 2012) examining similar *sabA* poly-T tract length changes in the heterologous host, *E. coli*. The use of poly-A/T tracts in association with *H. pylori* *omp* genes is quite widespread (Alm *et al.*, 1998;

Tomb *et al.*, 1997). This may be a common means of modulating promoter activities in a bacterium such as *H. pylori* that has a paucity of transcription factors.

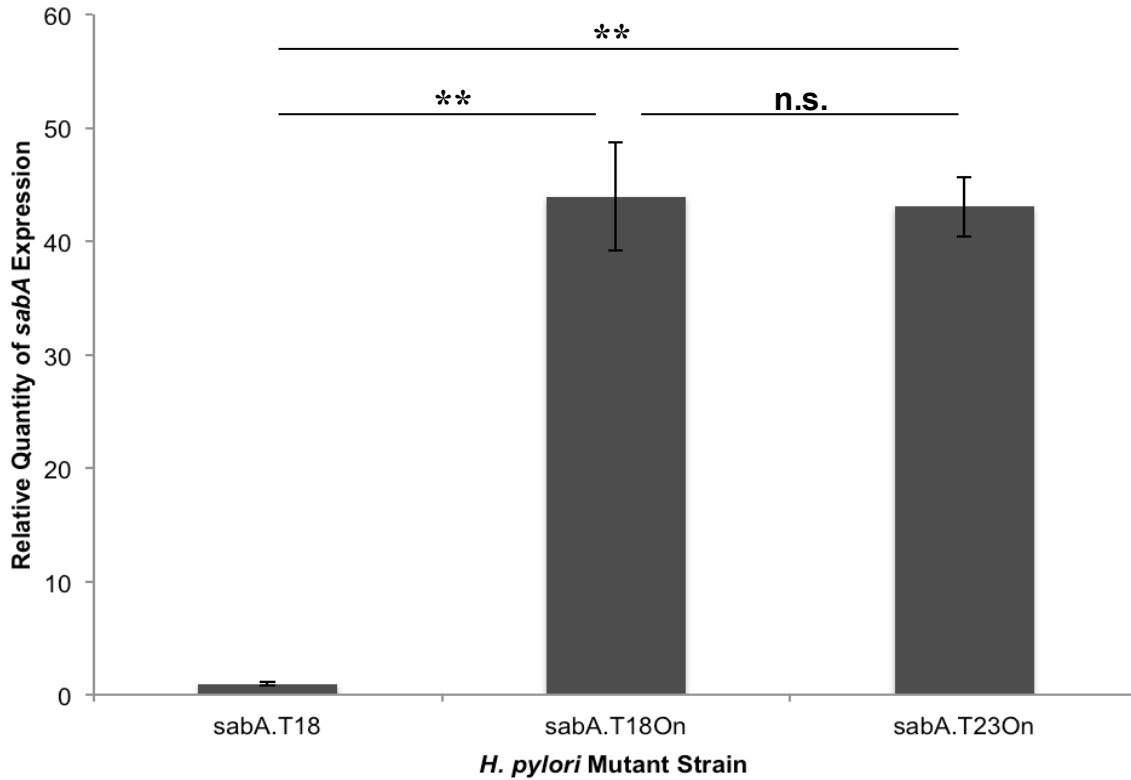


Figure 7: Phase-on status and poly-T tract extensions do not have synergistic effects on *sabA* transcription frequency. Quantitative real time PCR was used to determine the relative expression of *sabA* in *H. pylori* control strain *sabA.T18* as compared with mutants containing phase-on mutation and/or a poly-T tract five thymine extensions. The data shown here is representative of the results obtained in three independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch's unpaired *t*-test of unequal variance with *sabA.T18* as the control (** = $p \leq .01$, n.s. = $p > .05$).

Previous research in our lab suggests that transcription and translation of *sabA* is regulated via phase variation of the poly-CT as evident by differential *sabA* expression in strains with varied poly-CT tract lengths (Goodwin *et al.*, 2008). In the current study, we showed five thymine extensions or truncations in the poly-T tract of the *sabA* promoter significantly increases

transcriptional frequency of *sabA* independent of phase status. To determine whether five thymine extensions of the poly-T tract and altered phase status of *sabA* have additive effects in increasing *sabA* transcriptional frequency, new *sabA* phase-on mutants were created. Wild-type J99 *sabA* possess a phase-off poly-CT tract of 8 CT's. To switch *sabA* phase-on, the poly-CT tract was extended to 10 CT's. The first phase-on mutant (*sabA*.T18On) had a promoter region poly-T tract length of 18, as in the wild type *H. pylori* strain J99, whereas a second phase-on mutant (*sabA*.T23) had an extended poly-T tract length of 23 thymines (Table 3). mRNA extracted from mutant *H. pylori* strains and gene expression of *sabA* was quantified using qRT-PCR (Figure 5). *sabA*.T18On had a statistically significant 44-fold increase in *sabA* transcription frequency in comparison to the control strain *sabA*.T18 ($p = .004$) as determined by a Welch's unpaired *t*-test. Similarly, *sabA*.T23On had a 43-fold increase in *sabA* transcription frequency when compared to the control ($p = .001$). Interestingly, there was no significant difference in *sabA* transcription frequency between *sabA*.T18On and *sabA*.T23On ($p > .05$), suggesting that there are no additive effects of increased poly-T tract length and phase-on status on *sabA* transcription (Figure 7).

As previously mentioned wild-type J99 *sabA* possesses poly-CT tract length of 8 CT's that causes a frameshift resulting in a premature translational stop codon on nascent mRNA (Alm *et al.*, 1998; Tomb *et al.*, 1997). Although phase variation is traditionally thought to only result in translation termination, it can also influence transcription termination as well (Adhya *et al.*, 1974; Peters *et al.*, 2011; Boudvillain *et al.*, 2013). Other Gram-negative bacteria, such as *Escherichia coli*, possess RNA stem-loop structures that aid in transcription termination (Uptain & Chamberlin, 1997). Unlike other Gram-negative bacteria, *H. pylori* has very few identified stem-loop sequences within the *H. pylori* genome (Tomb *et al.*, 1997). These results suggest that

H. pylori transcription termination is Rho-dependent (Washio *et al.*, 1998). Rho is a transcription termination factor protein that is able to attach to the 5' end of nascent mRNA. Rho moves along nascent mRNA in the 5' to 3' direction until it reaches RNA polymerase, causing it to dissociate from the gene 3' end (Adhya *et al.*, 1974; Peters *et al.*, 2011; Boudvillain *et al.*, 2013). Rho-dependent RNA polymerase dissociation occurs at the end of a gene when the gene is phase-on because ribosome-mRNA association interferes with Rho's movement along the mRNA. However, if a gene is phase-off, especially if the stop codon occurs early within the coding region of the mRNA, ribosomes frequently dissociate from the mRNA, allowing Rho to move uninterrupted, causing premature dissociation of RNA polymerase from the gene. Thus, phase variation does not only affect complete translation, but also affects complete transcription (Adhya *et al.*, 1974; Peters *et al.*, 2011; Boudvillain *et al.*, 2013).

Although we observe an increase in transcription frequency when the poly-T tract is extended by five thymines (Figure 4B) in a *sabA* phase-off strain, poly-T tract length does not seem to affect transcription frequency of a *sabA* phase-on strain (Figure 7). We speculate that this is indicative of a potential threshold for the frequency of RNA polymerase binding to the *sabA* promoter and that phase-on status of *sabA* causes this frequency to reach its maximal threshold. Therefore, any alterations to the *sabA* promoter poly-T tract is not able to further increase transcription frequency.

Increased *sabA* Transcription Increases *H. pylori* Adherence to Gastric Epithelial Cells

After demonstrating that altering the poly-T tract and poly-CT tract of *sabA* affects the gene's transcription frequency, we wanted to investigate whether or not increased transcription leads to increased *H. pylori* adherence to gastric epithelial cells. *H. pylori* strains *sabA*.T18, *sabA*.T18On, *sabA*.T23, or *sabA*.T23On were co-incubated with the AGS gastric

adenocarcinoma cell line at a multiplicity of infection (MOI) of 100:1. Adherent cells were collected and plated in serial dilutions. After 5 days, colony-forming units were counted. The adherence of *H. pylori* strains *sabA.T18* and *sabA.T23* did not significantly differ ($p > .05$) despite increased *sabA* transcription in strain *sabA.T23* (Figure 6). More notably, *H. pylori sabA.T18On* had a 2.1-fold increase in adherence when compared to *sabA.T18* ($p = 0.035$) and a 2.7-fold increase when compared to *sabA.T23* ($p = 0.004$). In addition, *sabA.T23On* had a 3.2-fold increase in adherence when compared to *sabA.T18* ($p = 0.042$) and a 8.7-fold increase when compared to *sabA.T23* ($p = 0.029$) (Figure 6). Interestingly, *sabA.T23On* and *sabA.T18On* did not significantly differ in adhesion ($p > 0.05$) (Figure 6). This data suggests that although the poly-T tract increases *sabA* transcription, only phase variation via the poly-CT influences *H. pylori* adherence mediated by SabA protein. These results were consistent with our findings that the independent effects of phase status and poly-T tract mutations on *sabA* transcription frequency are not additive. Adhesion experiments were conducted in technical triplicate and experiments were repeated three times.

Despite the 44-fold increase of *sabA* transcription frequency in *sabA* phase-on strains, adherence only increased by around 2-fold in phase-on strains *sabA.T18On* and *sabA.T23On*. There are several possible explanations for this observation. First, increase in *sabA* transcription frequency does not necessarily indicate increase in SabA protein. Thus, post-translational regulation of SabA may decrease the amount of outer-membrane SabA. Ongoing research in our lab is examining whether or not increased transcription frequency of *sabA* results in increased SabA protein product. Second, SabA-mediated *H. pylori* binding to gastric epithelial cells is dependent upon the presence of SabA receptor sialyl-dimeric-Lewis x antigens present on gastric

epithelial cells. Thus, the 2-fold increase in *H. pylori* adherence resulting from a 44-fold increase *sabA* transcription may be a result of saturated SabA receptors on the gastric epithelial cells.

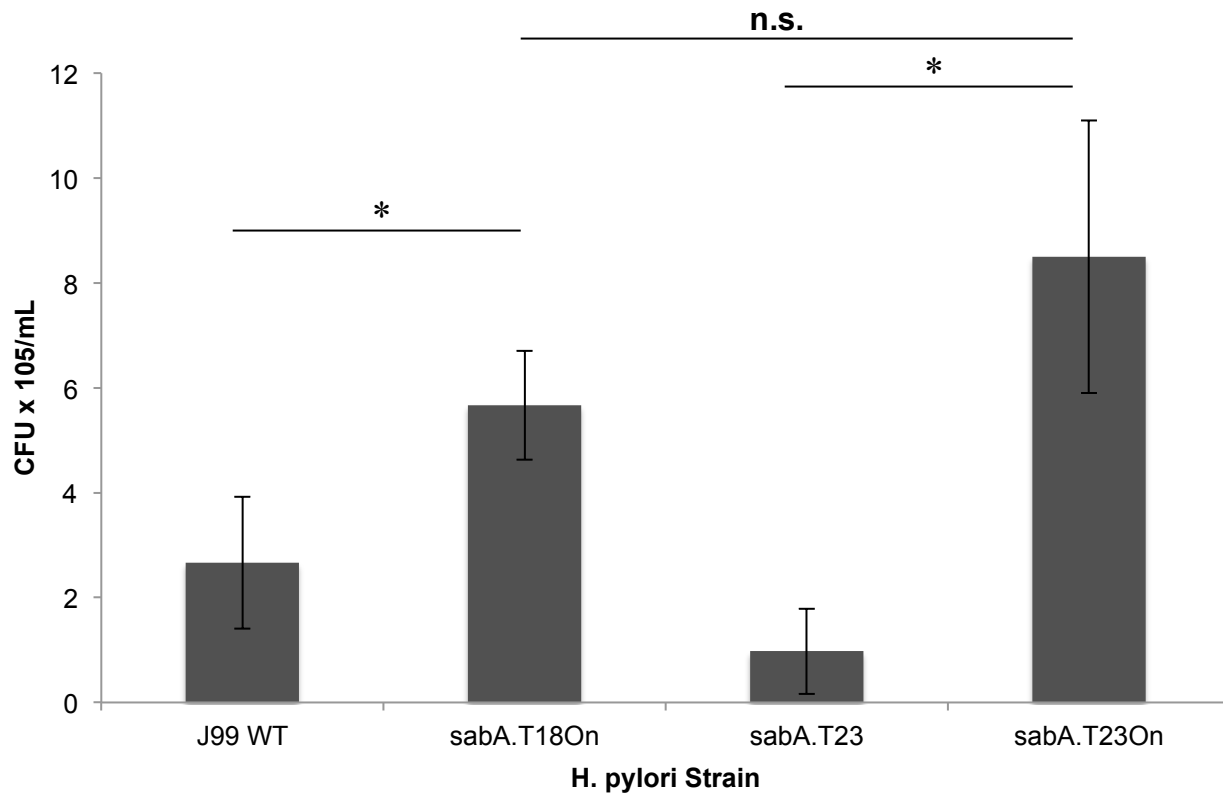


Figure 8: Phase variation but not poly-T tract length of *sabA* influence *H. pylori* adherence. Adhesion assays were used to determine whether increased *sabA* transcription led to increased adherence to AGS cells. Adhesion phenotypes of *H. pylori* strains J99 WT, *sabA*.T18On, *sabA*.T23, and *sabA*.T23On were compared. The data shown here is representative of the results obtained in three independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch's unpaired *t*-test of unequal variance (* = $p \leq 0.05$, n.s. = $p > 0.05$).

Increased *H. pylori* Adhesion via SabA Phase Variation Does Not Affect Epithelial IL-8 Production

As previously mentioned, the model strain of *H. pylori* utilized in these experiments was the *cagPAI*-positive strain J99, which is able to translocate the toxic effector protein CagA via a

T4SS into gastric epithelial cells causing downstream increased IL-8 production. Because phase variation of the *sabA* gene significantly increased number of *H. pylori* cells adhered to one AGS cell, we hypothesized that infection of AGS cells with *sabA* phase-on variants would increase subsequent epithelial cell IL-8 production mediated by increased interaction of the T4SS with AGS cells and perhaps increased translocation of CagA protein into the epithelial cells.

Culture medium was collected after a 5 hour infection of AGS cells with a *sabA* phase-off (*sabA.T18*) or phase-on (*sabA.T18On*) *H. pylori*. ELISA was conducted with undiluted medium. Experiments were conducted in technical triplicate and repeated for three biological replicates. AGS cells infected with *sabA.T18On* exhibited a 20% increase in IL-8 production in comparison to cells infected with *sabA.T18*. Differences in IL-8 production in these two infections were analyzed using a Welch's unpaired *t*-test. Although there was a 20% increase in IL-8 production in AGS cells treated with *sabA.T18On*, these results were not statistically significant ($p > .05$).

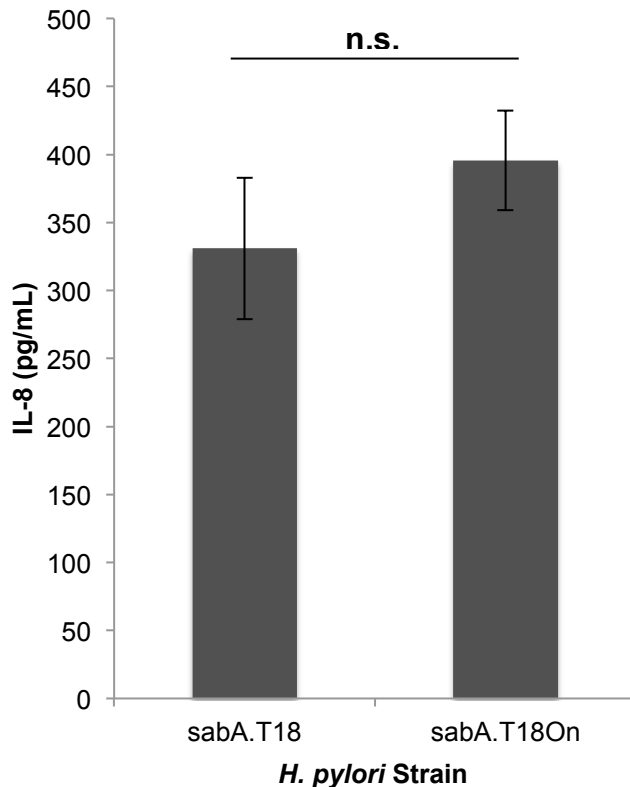


Figure 9: Increased adhesion via SabA does not affect gastric epithelial cell IL-8 production.

Cell medium from AGS cells infected with phase-off *sabA.T18* or phase-on *sabA.T18On* *H. pylori* strains was collected after a 5 hour infection and used in an ELISA to detect AGS cell IL-8 production. IL-8 production from AGS cells infected with *sabA.T18* or *sabA.T18On* were compared. The data shown here is representative of the results obtained in three independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch's unpaired *t*-test of unequal variance (n.s. = $p > 0.05$).

The original hypothesis suggests that with increased adhesion mediated by SabA would induce a higher T4SS-dependent epithelial cell immune response. However, our results suggest otherwise. As previously mentioned, IL-8 production can be triggered by a variety of pathways, such as CagA-dependent NF- κ B activation and CagA-independent CagL-dependent NF- κ B activation. Although we observed increased *H. pylori* adherence mediated by SabA, it remains uncertain whether or not adherence is influencing these epithelial cell inflammatory pathways at all. Further experimentation examining SabA-mediated adhesion on CagA translocation and NF- κ B activation need to be conducted to come to any conclusions regarding the effect of non-*cagPAI* adhesins on host immune response.

Discussion

H. pylori utilizes a wide variety of adhesin proteins to mediate the bacterium's attachment to the gastric epithelial layer. By adhering to gastric epithelial cells, *H. pylori* is able to extract nutrients from the host through disruption of the tight junctions of the epithelial cells (Testerman & Morris, 2014). However, adherent *H. pylori* can trigger toll like receptor (TLR) signaling, thus invoking a host immune response. Bacteria closest to the epithelium are more susceptible to clearing from this response (Smith, 2014). *H. pylori* faces these major problems regarding the tradeoffs of adherence, which was coined the "adherence dilemma" (Moore *et al.*, 2011). The present study investigates multiple regulatory mechanisms *H. pylori* utilizes to regulate adhesin-encoding gene expression and effects of these mechanisms at the phenotypic level. Our results show that adhesin-encoding genes *sabA* and *hopZ* are regulated via promoter homopolymeric tract length polymorphisms. Homopolymeric tracts vary naturally within a population of *H. pylori*. Extensions and truncations of these tracts by five thymines or adenines significantly increases transcription frequency of *sabA* and *hopZ*. Although the mechanism

behind why these polymorphisms increase transcription frequency is unknown, we hypothesized that these changes may alter DNA topology to facilitate RNA polymerase binding, as previously demonstrated in studies in *H. pylori ureR* gene regulation (Poore & Mobley, 2003).

The gene *sabA* has a homolog gene *sabB* that arose during a duplication event in *H. pylori*'s genetic history (Kawai *et al.*, 2011). SabB protein function is unknown; however, because of shared sequence between *sabA* and *sabB*, we hypothesized that SabB may act as an adhesin. Interestingly, the promoter of *sabB* also contains a poly-T tract with a poly-CT tract downstream of the transcriptional and translational start sites (Alm *et al.*, 1998; Tomb *et al.*, 1997). Mutant plasmids were constructed to extend or truncate the poly-T tract by five thymines or turn the *sabB* gene phase on by extending the poly-CT tract from wild-type 9 CT's to 10 CT's. However, transformations of *H. pylori* with mutant plasmids were unsuccessful and further attempts are required to fully research *sabB* regulation and SabB protein function.

In addition to *sabA*'s transcription regulation via poly-T tract length, transcription frequency of *sabA* is influenced by phase variation determined by poly-CT tract length. Our results demonstrated that *H. pylori* strains with phase-on *sabA* have significantly higher transcription frequency in comparison to strains with phase-off *sabA*. Interestingly, five-thymine extension of the poly-T tract and phase-on status of *sabA* did not have additive effects on transcription frequency. We speculate that this is indicative of a potential threshold for the frequency of RNA polymerase binding to the *sabA* promoter.

In agreement with these results, we found that only phase-on status of *sabA* increased *H. pylori* adherence to gastric epithelial cells independent of poly-T tract length. However, turning the *sabA* gene phase-on, while increasing transcription frequency by more than 44-fold, only led to a 2-fold increase in adhesion. This suggests that adhesin protein expression may be regulated

at the translational level as well or that the SabA receptors on the gastric epithelial cells were saturated. Ongoing studies by others in our lab are designed to develop means (Flag epitope tagging of SabA) to allow examination of post-transcriptional regulation.

We hypothesized that the observed increase in adherence, and therefore increased number of *H. pylori* cells bound to one gastric epithelial cell, facilitating association of CagL with its receptor, thus increasing the amount of translocated CagA protein and signaling into the epithelial cell, thus inducing increased inflammatory response via increased IL-8 production. Contrary to our hypothesis, preliminary ELISAs showed no significant difference in epithelial cell production of IL-8 in response to SabA-mediated hyper-adherent *H. pylori* infection. These results suggest that SabA-mediated hyper-adherence of *H. pylori* may not increase translocation of CagA as originally hypothesized. To further investigate SabA-mediated adhesion and host immune response, studies examining the effect of phase variation and poly-T tract length on SabA protein levels, CagA translocation into gastric epithelial cells, and additional trials on gastric epithelial cell IL-8 production need to be conducted.

As previously mentioned, *H. pylori* utilizes a wide variety of adhesin proteins. Interestingly, many of these adhesin-encoding genes possess similar promoter and regulatory elements, such as homopolymeric tracts and dinucleotide tracts (Alm *et al.* 1998; Tomb *et al.*, 1997). In a previous study with clinical isolates, AlpA and AlpB were produced at a constant rate, but all other outer membrane proteins (OMPs) were produced at highly variable rates ranging from 35% to 73%. This result indicates that variable expression of OMPs such as SabA, along with other adhesin proteins, is important in functional adaptation to the individual host or gastric niche (Odenbreit *et al.*, 2009). The present study focuses primarily on *sabA* and *hopZ*, but the transcriptional and translational regulatory mechanisms of several adhesin-encoding genes

such as *hopD* and *oipA* remain understudied. Because the presence of promoter homopolymeric tracts and downstream dinucleotide tracts is widespread in *H. pylori* adhesin-encoding genes, the present study provides a platform for the study of other key *H. pylori* adhesins.

Previous studies have connected the importance of *H. pylori* adherence to induction of host immune response (Ishijimi *et al.*, 2011; Oleasetro *et al.*, 2008). In particular, SabA is a key protein of interest. SabA-positive status of *H. pylori* is positively correlated with the formation of gastric pathologies in Western *H. pylori* strains (Yamaoka *et al.*, 2002). In 2005, Unemo *et al.* proposed a potential mechanism explaining this observation. Their study demonstrated that mutant and wild-type *H. pylori* strains lacking SabA had no neutrophil-activating capacity, suggesting that SabA adhesion to sialylated neutrophil receptors plays an essential initial role in the adherence and phagocytosis of the bacteria. This further supports an argument for the critical role of adhesins as a virulence factors in disease pathogenesis (Unemo *et al.*, 2005). Extensive research on adhesins BabA and SabA within the past twenty years has only scratched the surface of understanding how *H. pylori* adhesin regulation and function can impact infection. As previously mentioned, many *H. pylori* adhesins have yet to be fully investigated. Furthering our understandings of *H. pylori* adhesion regulation will allow us to better understand the persistence of *H. pylori* infection and virulence.

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Table 1: Primers

Primer Name	5' to 3' Sequence
HP0726 Fwd	TACGCAATCTTGTGGAGTCC
sabAPolyT. R	CGCCGATTTGATGCCCGCGCTCAC
sabA.5T.F	TACCCCAAATC TTTTTTTTTTTTTTTTTTTTTTTTTTT GAA ATCCAATAA
sabA.5T.R	TTATTGGATTTCAAAAAAAAAAAAAAAAAAAA AAAAA GATTTTGGGGTA
sabA.less5T. F	TACCCCAAATCTTTTTTTTTTTTTTTGAAATCCAATAA
sabA.less5T. R	TTATTGGATTTCAAAAAAAAAAAAAGATTTTGGG GTA
sabA.5A.F	TACCCCAAATC AAAAA TTTTTTTTTTTTTTTTTTTTTTTGA ATCCAAT
sabA.5A.R	ATTGGATTTCAAAAAAAAAAAAAAAAAAAA TTTTTGA TTTTGGGGTA
sabA.5G.F	TACCCCAAATC GGGGG TTTTTTTTTTTTTTTTTTTTTTGA AATCCAAT
sabA.5G.R	ATTGGATTTCAAAAAAAAAAAAAAAAAAAA CCCCCGA TTTTGGGGTA
sabA.Ran.F	TACCCCAAATC ACTAG TTTTTTTTTTTTTTTTTTTTTTGA AATCCAAT
sabA.Ran.R	ATTGGATTTCAAAAAAAAAAAAAAAAAAAA CTAGTGA TTTTGGGGTA
sabA.On	AAAAGACAATTCTACTCTCTCTCTCTCTCGCTTCATCGCTCTT
CAT Fwd	CTTGAAACCCAGGACAATAAC
SabASpecific. R	TAAAGAGCTATTGACCAGCTC
SabA.IG.Fwd	GTTTGGCTTTATTCCCATG
SabA.IG.Reverse	VIC – GTCTTTTCATAAAATGTTCTT
hopZ.F2	CGTGGTCGTGAATGAAGTGC
hopZ.R2	GCCAATTAGCCCTTGCGTGT
hopZ.BglIIF	CCTTTTGGGGGTTTTTATTAGATCTAACCGCTCGTTTTTAAAAAC
hopZ.BglIIR	GTTTTTAAAAACGAGCGGTTAGATCTAATAAAAAACCCCAAAGG
hopZ.A9F	CTAAATTTTCTCAAATGACAAAAAAAAAACGATTTCATGCTACAATGC T
hopZ.A9R	AGCATTGTAGCATGAAATCGTTTTTTTTTTGTCATTGGAGAAAATTTAG
hopZ.A19F	CTAAATTTTCTCAAATGACAAAAAAAAAAAAAAAAAAAAACGATTTCATGCTACAATGCT
hopZ.A19R	AGCATTGTAGCATGAAATCGTTTTTTTTTTTTTTTTTTTGTGTCATTGGAGAAAATTTAG
Jhp0658.F	TGGCGGATTTTGAAGCCCGCAA
sabB.R	TCTTAGCCACGCTTAAAGCC
sabB.BamHI	CGCGCAACAGCTCATGGATCCAATCGAACAGACCAA
sabB.T5	AAACACCCAAAATCTTTTTGAAATCCAAAAAATT
sabB.T15	AAACACCCAAAATCTTTTTTTTTTTTTTTTGAATCCAAAAAATT
sabB.On	AAAAGACAATTCTACTCTCTCTCTCTCTCTCTCGCTTCATCGCTCT

	T
<i>ftsZ</i> .Taq.F	TGAGCGGCATTTCTACGATTATCAC
<i>ftsZ</i> .Taq.R	CGCTCTTTAAATCGGCAAAATCAAC
<i>ftsZ</i> .Taq	FAM—CAAACCCGGTAATATC—MGB-NFQ
<i>sabA</i> .Taq.F	GATCAGTATCGTTATTTAGAGAAAGCCTATTTGA
<i>sabA</i> .Taq.R	ACCTCCTGTCTGTAAGGGTTAGTAG
<i>sabA</i> .Taq	FAM—CAATGCGGGTAAAACG—MGB-NFQ
<i>hopZ</i> .Taq.F	ACTTGAGCTAGCCGATCAAATGAAA
<i>hopZ</i> .Taq.R	GGCAAGCTGCCAAGTAATTTGTG
<i>hopZ</i> .Taq	FAM—TCCCAAGCCAATTTAT—MGB-NFQ

Table 2: Plasmids

Plasmid Name	Description
<i>psabA</i>	pBlueScript SK+ vector containing a 603 base pair amplicon including the 3' end of <i>jhp0663</i> , the poly thymine (poly-T) tract, -35/-10 putative promoter sites, transcriptional start site, and 5' coding region of <i>sabA</i> of <i>H. pylori</i> strain J99.
<i>psabA.T18</i>	<i>psabA</i> containing <i>Campylobacter coli</i> forward-oriented chloramphenicol acetyltransferase (CAT) gene upstream of <i>sabA</i> promoter sequence.
<i>psabA.T13</i>	<i>psabA.T18</i> with promoter poly-T sequence composed of 13 thymines.
<i>psabA.T16</i>	<i>psabA.T18</i> with promoter poly-T sequence composed of 16 thymines.
<i>psabA.T17</i>	<i>psabA.T18</i> with promoter poly-T sequence composed of 17 thymines.
<i>psabA.T122</i>	<i>psabA.T18</i> with promoter poly-T sequence composed of 22 thymines.
<i>psabA.T23</i>	<i>psabA.T18</i> with promoter poly-T sequence composed of 23 thymines.
<i>psabA.A5</i>	<i>psabA.T18</i> with promoter poly-T extended by 5 adenines at the 5' end.
<i>psabA.Ran</i>	<i>psabA.T18</i> with promoter poly-T extended by random five nucleotide sequence ACTAG at the 5' end.
<i>psabA.G5</i>	<i>psabA.T18</i> with promoter poly-T extended by 5 guanines at the 5' end.
<i>psabA.T18On</i>	<i>psabA.T18</i> with <i>sabA</i> poly-CT tract length of 10 CTs.
<i>psabA.T23On</i>	<i>psabA.T23</i> with <i>sabA</i> poly-CT tract length of 10 CTs.
<i>phopZ</i>	pCR® 4-TOPO® vector containing a 853 bp amplicon, including the 3' end of the upstream gene, promoter sequence of <i>hopZ</i> , transcriptional start site of <i>hopZ</i> , and 5' coding region of <i>hopZ</i> of <i>H. pylori</i> strain J99.
<i>phopZ.A14</i>	<i>phopZ</i> containing <i>Campylobacter coli</i> forward-oriented chloramphenicol acetyltransferase (CAT) gene upstream of <i>hopZ</i> promoter sequence.
<i>phopZ.A9</i>	<i>phopZ.A14</i> with promoter poly-A sequence

	composed of 9 adenines.
<i>phopZ.A19</i>	<i>phopZ.A14</i> with promoter poly-A sequence composed of 19 adenines.
<i>psabB</i>	pCR® 4-TOPO® vector containing a 1658 bp amplicon including the promoter region of <i>sabB</i> , including the 3' end of gene <i>jhp0658</i> , the poly-T tract from positions -42 to -33 the transcriptional start site of <i>sabB</i> , poly-CT tract, and 5' coding region of <i>sabB</i> of <i>H. pylori</i> strain J99.
<i>psabB.CATrdxA</i>	<i>psabB</i> with CAT-rdxA cassette cloned into a BamHI restriction site ___bp downstream of <i>sabB</i> transcriptional start site
<i>psabB.T5</i>	<i>psabB</i> with promoter poly-T sequence composed of 5 thymines
<i>psabB.T15</i>	<i>psabB</i> with promoter poly-T sequence composed of 15 thymines
<i>psabB.On</i>	<i>psabB</i> with poly-CT sequence composed of 10 CTs.
<i>pΔrdxA</i>	pCR® 4-TOPO® vector containing an amplicon of the <i>H. pylori</i> strain 26695 <i>rdxA</i> gene with a deletion mutation within the <i>rdxA</i> coding region.

Table 3: *H. pylori* strains

<i>H. pylori</i> Strain	Description
<i>sabA</i> .T18	J99WT containing CAT gene upstream of <i>sabA</i> promoter with a poly-T tract length of 18 thymines
<i>sabA</i> .T13	J99WT containing CAT gene upstream of <i>sabA</i> promoter with a poly-T tract length of 13 thymines
<i>sabA</i> .T16	J99WT containing CAT gene upstream of <i>sabA</i> promoter with a poly-T tract length of 16 thymines
<i>sabA</i> .T17	J99WT containing CAT gene upstream of <i>sabA</i> promoter with a poly-T tract length of 17 thymines
<i>sabA</i> .T22	J99WT containing CAT gene upstream of <i>sabA</i> promoter with a poly-T tract length of 22 thymines
<i>sabA</i> .T23	J99WT containing CAT gene upstream of <i>sabA</i> promoter with a poly-T tract length of 23 thymines
<i>sabA</i> .A5	J99WT containing CAT gene upstream of <i>sabA</i> promoter with a poly-T tract extended at the 5' end by 5 adenines
<i>sabA</i> .Ran	J99WT containing CAT gene upstream of <i>sabA</i> promoter with a poly-T tract extended at the 5' end by nucleotides ACTAG
<i>sabA</i> .G5	J99WT containing CAT gene upstream of <i>sabA</i> promoter with a poly-T tract extended at the 5' end by 5 guanines
<i>sabA</i> .T18On	<i>sabA</i> .T18 with <i>sabA</i> poly-CT tract length of 10 CTs
<i>sabA</i> .T23On	<i>sabA</i> .T23 with <i>sabA</i> poly-CT tract length of 10 CTs
<i>hopZ</i> .A14	J99WT containing CAT gene upstream of <i>hopZ</i> promoter with a poly-A tract length of 14 adenines
<i>hopZ</i> .A9	J99WT containing CAT gene upstream of <i>hopZ</i> promoter with a poly-A tract length of 9 adenines
<i>hopZ</i> .A19	J99WT containing CAT gene upstream of <i>hopZ</i> promoter with a poly-A tract length of 19 adenines