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Repetitive Sequence Variations in the Promoter Region of the Adhesin-Encoding Gene *sabA* of *Helicobacter pylori* Affect Transcription

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The pathogenesis of diseases elicited by the gastric pathogen *Helicobacter pylori* is partially determined by the effectiveness of adaptation to the variably acidic environment of the host stomach. Adaptation includes appropriate adherence to the gastric epithelium via outer membrane protein adhesins such as SabA. The expression of *sabA* is subject to regulation via phase variation in the promoter and coding regions as well as repression by the two-component system ArsRS. In this study, we investigated the role of a homopolymeric thymine [poly(T)] tract −50 to −33 relative to the *sabA* transcriptional start site in *H. pylori* strain J99. We quantified *sabA* expression in *H. pylori* J99 by quantitative reverse transcription-PCR (RT-PCR), demonstrating significant changes in *sabA* expression associated with experimental manipulations of poly(T) tract length. Mimicking the length increase of this tract by adding adenines instead of thymines had similar effects, while the addition of other nucleotides failed to affect *sabA* expression in the same manner. We hypothesize that modification of the poly(T) tract changes DNA topology, affecting regulatory protein interaction(s) or RNA polymerase binding efficiency. Additionally, we characterized the interaction between the *sabA* promoter region and ArsR, a response regulator affecting *sabA* expression. Using recombinant ArsR in electrophoretic mobility shift assays (EMSA), we localized binding to a sequence with partial dyad symmetry −20 and +38 relative to the *sabA* +1 site. The control of *sabA* expression by both ArsRS and phase variation at two distinct repeat regions suggests the control of *sabA* expression is both complex and vital to *H. pylori* infection.

Helicobacter pylori is a Gram-negative bacterium that colonizes the human gastric epithelium and infects more than half the world's population. Individuals are most frequently colonized during childhood, and without treatment, infection persists for the lifetime of the infected host. Though the majority of colonized individuals remain asymptomatic, *H. pylori* is the causative agent of chronic active gastritis, peptic ulcer disease, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (1–3).

Successful colonization of the stomach and the onset of disease are determined largely by interactions between the host and *H. pylori* (1, 4–6). The abilities of *H. pylori* to adapt to various acidic environments and to adhere to the gastric epithelium play substantial roles in colonization and long-term persistence in the human stomach (7). Adherence prevents clearance of *H. pylori* during gastric mucous shedding and ensures that nutrients from damaged epithelial cells are consistently available to the bacterium (8).

Several putative adhesins have been identified in *H. pylori*, most of which are members of the large outer membrane protein (OMP) family. BabA is a major adhesin and binds the Lewis b (Le^b) antigen expressed in the gastric mucosa (7). Another outer membrane protein adhesin, SabA (jhp0663 [HP0725]), facilitates adhesion by binding to glycosphingolipids displaying a sialyl-dimeric Lewis X antigen, the expression of which is increased in gastric epithelial cells during inflammation. Additionally, dynamic adaptation in sialyl-binding properties of SabA during persistent infection has been observed and may allow *H. pylori* to specialize for individual host variation in mucosal glycoprotein sialylation. Variations in sialyl-binding properties of SabA also may allow for tropisms in local areas of inflamed tissue, as inflammation often results in changes in mucosal sialyated glycoconjugates (9). Thus, the ability of *H. pylori* to adhere to sialylated glycoconjugates may contribute to virulence by facilitating persistent infection in the face of the host's inflammatory response (10).

Several studies have revealed the regulation of *sabA* expression in *H. pylori* to be very complex (11–13). Transcription of *sabA* is regulated via phase variation mediated by changes in dinucleotide repeats near the 5′ end of the coding sequence (11). Additional layers of expression control are mediated by environmental signals acting through the two-component signal transduction system ArsRS (11). A recent study by Talarico et al. (12) showed that recombination between the *sabA* locus and its paralogous locus, *sabB*, affects *sabA* expression significantly. Finally, recent evidence has shown that *sabA* and *babA* are coregulated with the host expression of mucins, to which *H. pylori* adheres in the gastric mu-

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cosal epithelium (14). Thus, the regulation of *sabA* is complex and multidimensional.

Phase variation is mediated by slipped-strand mispairing leading to insertions or deletions (indels) within repetitive DNA tracts during replication. When an indel-modified tract arises in the coding region of the gene, these indels result in an altered reading frame (15). *sabA* contains a dinucleotide cytosine-thymine repeat in the 5′ coding region, and changes in the CT tract length were shown in a previous study from our laboratory to result alternatively in a truncated or full-length SabA protein, as indicated by altered binding to AGS cells *in vitro* (11).

There is a second repetitive sequence associated with *sabA*: a homopolymeric thymine [poly(T)] tract in the promoter region. The *H. pylori* strain J99 contains a poly(T) tract of 18 nucleotides in length situated −50 to −33 relative to the *sabA* transcriptional start site mapped by Sharma et al. (16). A recent study by Kao et al. (13) compared the *sabA* expression of several distinct clinical isolates of *H. pylori* to that of variant poly(T) tracts in the *sabA* promoter region, suggesting that differing poly(T) tract lengths change *sabA* gene expression levels. However, the observed differences in *sabA* expression also could be affected by other differences between the genomes of these diverse *H. pylori* isolates. In addition, Kao et al. constructed an *Escherichia coli lux* reporter system to measure the effect of experimentally manipulated *sabA* poly(T) tract lengths, again showing changes in *sabA* expression as a function of the poly(T) tract length (13). This result is interesting, as *sabA* expression in *E. coli* was altered in the absence of any *H. pylori* transcription factors or *H. pylori* RNA polymerase. This suggests the effect of poly(T) tract length lies in DNA topology or flexibility rather than any specific *H. pylori* regulatory systems. However, the experimental alterations in the *sabA* poly(T) tract within *H. pylori* itself were not performed, and our current study aimed to elucidate whether changes in this homopolymeric thymine tract impact *sabA* transcription in its native setting.

While phase variation of *sabA* allows population-level control of gene expression, *H. pylori* also regulates gene expression at the individual cell level using a small number of two-component signal transduction (TCST) systems. This allows adaption to environmental changes at the transcriptional level (17–23). When the histidine kinase protein of a TCST encounters the appropriate stimulus, it undergoes autophosphorylation; subsequently, the phosphoryl group is transferred to a response regulator. The resulting conformational change results in altered binding of the response regulator to cognate sites on the genome and modified expression of genes in the TCST regulon (24). Previous studies of the HP0165-HP0166 TCST in *H. pylori* strain 26695 determined that changes in environmental pH acted as a stimulus for this TCST; thus, it was redesignated *arsRS* (acid-responsive signaling) (18). A previous study in our laboratory comparing the genome-wide transcriptional profiles between wild-type strain 26695 *H. pylori* and an isogenic *arsS* histidine kinase mutant found *sabA* was derepressed in the mutant, suggesting that ArsR is a repressor of *sabA* (11, 19). Our laboratory had previously demonstrated a significant role for ArsRS in the expression of *sabA* (11, 19), and our current study used electrophoretic mobility shift assays (EMSA) to localize the binding of recombinant ArsR.

In the present study, we hypothesized that indels in this poly(T) tract affect *sabA* expression levels by affecting the efficiency of transcription of *sabA*, thereby acting as a second method of phase variation regulation in addition to the poly(CT) tract. Thus, SabA expression may be regulated both transcriptionally and translationally by two distinct phase variation events. We hypothesized that phase variation in the poly(T) tract results in physical changes in the length of the promoter control region that result in a change in DNA topology and affect the *trans*-acting regulatory protein interaction(s) governing *sabA* expression (25, 26).

Our findings regarding phase variation and signal transduction provide new insights into the complex genetic mechanisms used by *H. pylori* and lead to a better understanding of how *H. pylori* adapts to sustain a persistent infection despite a robust inflammatory and immune response from the host.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* strain J99 was grown on Trypticase soy agar with 5% sheep blood (TSA II; BD) at 37°C in an ambient air-5% CO_2 atmosphere. Broth cultures were grown in sulfite-free brucella broth (SFBB) with 5% newborn calf serum (Gibco/BRL).

Cloning. A 603-bp amplicon, including the 3′ end of jhp0663 (HP0726), the poly(T) tract, −35/−10 putative promoter sites, transcriptional start site, and 5′ coding region of *sabA* (jhp0662 [HP0725]) was amplified using oligonucleotide primers HP726 Fwd and sabAPolyT.R from *H. pylori* strain J99 (see Table S1 in the supplemental material). This corresponds to positions 743281 to 743845 of the *H. pylori* J99 genome. The amplicon was initially cloned into pCR 4-TOPO (Invitrogen) and then subcloned into the EcoRI site of the pBlueScript SK+ vector (Stratagene). The resulting plasmid was designated pVKC2 (see Table S2).

To generate the control plasmid, the construct upon which all subsequent mutations were made, a chloramphenicol acetyltransferase (CAT) gene from *Campylobacter coli* (27) was cloned as a SmaI/EcoRV fragment from pBSC103 (28) into a blunt-ended MluI site at the end of jhp0633, the gene immediately upstream of *sabA* (Fig. 1; also see Table S2 in the supplemental material). The resulting control plasmid, selected for resistance to 25 μg chloramphenicol/ml in *E. coli* DH5α, was designated pVKC4.T18 (see Table S2). 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 pVKC4.T18 was mutated to various T tract lengths using mutagenic primers PolyT.Fwd.5T, PolyT.Rev.5T, PolyT.Fwd.less5T, and PolyT.Rev.less5T (see Table S1 in the supplemental material). 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 alternative sequence extensions, the forward and reverse primers introducing AAAAA, GGGGG, or the random sequence ACTAG upstream and immediately adjacent to the $poly(T)_{18}$ tract were designated PolyT.Fwd.5A, PolyT.Rev.5A, PolyT.Fwd.5G, PolyT.Rev.5G, PolyT.Fwd.Ran, and PolyT.Rev.Ran, respectively (see Table S1). Mutagenesis was carried out according to the manufacturer's protocol using 40 ng of pVKC4.T18 per 50-μl reaction mix. 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 13 and 23 residues (see Table S2 in the supplemental material). In the process of creating plasmids with the expected new length of thymines, alternative-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 (AFLP) to have lengths of 17, 16, and 22 residues (see Table

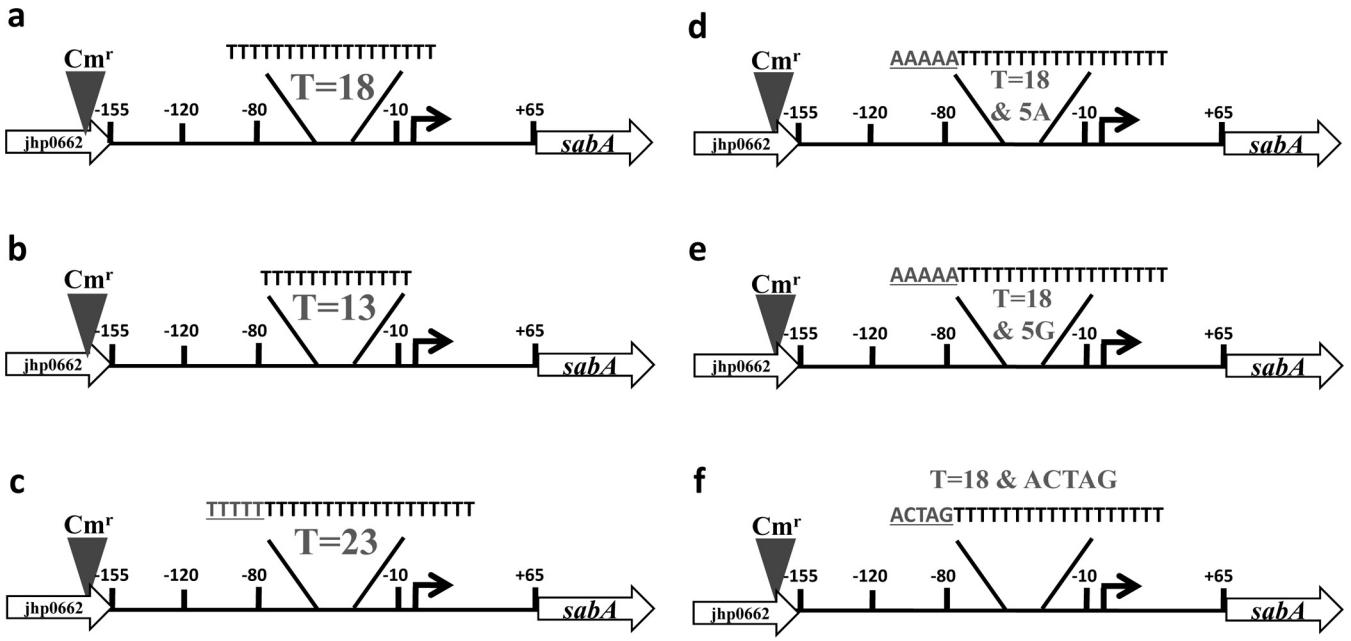

FIG 1 Mutational strategy to examine the role of a polythymine [poly(T)] tract in the *in vivo* expression of *sabA* in *H. pylori* strain J99. (a) Plasmid constructs containing the jhp0662-*sabA* region were marked for allelic replacement in *H. pylori* J99 by the addition of the chloramphenicol resistance gene into the MluI site in jhp0662 and moved into *H. pylori* J99 to create the control mutant possessing a wild-type poly(T) tract length (T = 18). (b to f) All subsequent mutations were created using the control mutant plasmid. Numbers are nucleotide positions relative to the transcriptional start point (16), shown as a bent arrow. The number of tandem thymines is indicated in each mutant, and added nucleotides are indicated by gray and underlining.

S2). They too were recombined subsequently 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 poly(T)$_{18}$ tract extended by the nucleotides AAAAA (pVKC4.A5), the random nucleotides ACTAG (pVKC4.Ran), or the nucleotides GGGGG (pVKC4.G5) (see Table S2 in the supplemental material). The mutated poly(T) tract regions of all plasmids were confirmed via sequencing reactions performed using the BigDye sequencing kit (Applied Biosystems) with the primer CAT Fwd (see Table S1).

Natural transformation of and allelic exchange in *H. pylori*. The *H. pylori* strain J99 (see Table S2 in the supplemental material) was used as the recipient in natural transformation experiments to select for allelic exchange, resulting in resistance to 10 μg chloramphenicol/ml and the exchange of the wild-type allele for mutant alleles of the *sabA* promoter region. Freezer stocks of *H. pylori* J99 were grown for 24 to 36 h on TSA II plates containing 5% sheep blood (Becton-Dickinson) at 37°C in a 5% CO_2 atmosphere. Cells were harvested into SFBB plus 5% newborn calf serum (NCS). The tubes were centrifuged for 2 min at 3,300 × *g*, and the supernatant was removed. The pellet was resuspended in 100 μl SFBB with NCS and 20 μg vancomycin per ml and spotted on sheep blood agar plates. Ten μg of pVKC4.T18 (control) or poly(T) tract mutant plasmids was spotted onto the *H. pylori* J99 cell pad, the plate was incubated at 37°C and 5% CO_2 for 4 h, and the cells spread over each plate and grown overnight. The cells were harvested from each blood agar plate and transferred to SFBB–5% NCS plates containing 10 μg chloramphenicol/ml. These plates were incubated for 3 to 5 days at 37°C and 5% CO_2 until colonies appeared. Mutant strains of *H. pylori* J99 were designated by the number of thymines present in the poly(T) tract: *sabA* poly(T)$_{13}$, *sabA* poly(T)$_{16}$, *sabA* poly(T)$_{17}$, *sabA* poly(T)$_{18}$, *sabA* poly(T)$_{22}$, and *sabA* poly(T)$_{23}$ (see Table S2). For additional mutagenesis experiments, mutant strains of *H. pylori* J99 were designated by the five-nucleotide extension upstream and adjacent to the wild-type poly(T) tract length of 18 thymines: *sabA* A5, *sabA* G5, and *sabA* Ran (see Table S2).

To ensure that the allelic exchange of pVKC4.T18 or plasmids bearing mutated poly(T) tracts targeted *sabA* rather than its paralog, *sabB*, primers were designed such that an amplicon could be achieved from the *sabA* locus but not from the *sabB* locus. The primers for amplification of the region from putatively recombinant *H. pylori* J99 *sabA* mutants were CAT Fwd, which lies within the chloramphenicol acetyltransferase gene, and SabASpecific.R, which represents a sequence unique to *sabA* and absent from the paralog *sabB* (see Table S1 in the supplemental material). The amplification and subsequent sequence analysis showed that all recombination had successfully targeted *sabA*.

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*, AFLP was conducted by a modification of the protocol of Hallinger et al. (29). 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 analyses were *sabA* IG Fwd and *sabA* IG Rev (see Table S1 in the supplemental material). Amplicons generated were diluted by a factor of 50 and analyzed by an ABI 3100 automated fluorescent DNA sequencer (ABI) using a Liz 300 molecular weight standard set, and data were analyzed using GeneScan (Life Technologies).

Quantitative real-time PCR (qRT-PCR). *H. pylori* wild-type strain J99, the J99 T18 control mutant, and J99 poly(T) variants were grown on TSA II (BD) at 37°C in an ambient air-5% CO_2 atmosphere. Broth cultures were grown in SFBB with 5% newborn calf serum (Gibco/BRL) until the cells reached an optical density at 600 nm (OD_{600}) of 0.8 to 1.6. For RNA extraction, 1 × 10^9 cells were harvested at 3,300 × *g* for 5 min, and the pellets were frozen at −80°C. Samples were suspended 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 (Life Technologies) total RNA isolation kit 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) by following the manufacturer's cDNA synthesis protocol. The resulting cDNA was used in qRT-PCR. The expression of *H. pylori sabA* was compared to that of 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 system. The assay was carried out according to the manufacturer's protocol using custom TaqMan gene expression assays, including the *sabA* and *ftz* probes and forward and reverse primers (see Table S1 in the supplemental material). Assays for each strain and each gene were run in technical triplicate, and experiments were repeated three times. The Relative expression of *sabA* among the various mutants was calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (30) and processed using DataAssist software (Applied Biosystems).

Recombinant ArsR production and purification. The response regulator ArsR was produced recombinantly in *E. coli* M15/pREP4 (see Table S2 in the supplemental material). Briefly, *arsR* was amplified from *H. pylori* using primers arsRFwd.Bam and arsRRev.HindIII (see Table S1). This amplicon, encoding the entire *arsR* coding sequence, was cloned as a BamHI/HindIII fragment in pQE30 (Qiagen), a 6× His-tagging expression plasmid (see Table S2). Soluble ArsR was expressed in and extracted from *E. coli* M15/pQE30-26695*arsR*+pREP4 according to the native batch purification protocol of the manufacturer (Qiagen). Cells of *E. coli* M15 carrying pQE30 were cultured in 250 ml LB Amp Kan broth (LB containing 100 μg/ml ampicillin and 50 μg/ml kanamycin) in a baffled flask. This culture was grown at 37°C with shaking to an OD_{600} of 0.5 to 0.7. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and the culture was returned to the incubator. Cell pellets were resuspended in 2 ml NPI-10 lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) per gram. Lysozyme was added to a final concentration of 1 mg/ml cell suspension, and samples were incubated for 30 min on ice. The resuspended cells then were sonicated at maximum power on ice in six 10-s bursts with a cooling period between each burst. Twelve units of Benzonase nuclease (Novagen) per ml of expression culture was added to each sonicated sample, and the mixtures were incubated for 15 min on ice. The lysates then were centrifuged for 30 min at 10,000 × *g* and 4°C to pellet the cell debris. The supernatant, which is the soluble fraction, was removed and stored at 4°C. The time point that produced the largest amount of the desired protein in the soluble fraction was selected as the expression time for subsequent purifications.

ArsR was purified on 5-ml-bed-volume drip columns containing 3 ml 50% Ni-NTA-agarose slurry according to the manufacturer's protocol (Qiagen). During purification, a 20-μl aliquot of each fraction was taken for SDS-PAGE, which was performed to check the quality of the purification and to determine which elution fractions to save for use in EMSA. Recombinant ArsR was concentrated to 1 mg/ml using a 10K Microsep column according to the manufacturer's instructions (Pall Life Sciences), glycerol was added to 10% by volume, and aliquots were frozen for subsequent use in DNA binding assays.

EMSA. Biotin-labeled *sabA* probes used in DNA binding experiments were amplified from *H. pylori* strain J99 genomic DNA using oligonucleotides sabA4Bio and sabAProbeRev (see Table S1 in the supplemental material) to amplify a 156-bp *sabA* probe. The same 156-bp region was amplified for specific competition using a nonbiotinylated version of the forward oligonucleotide (sabA4-F) rather than the biotin-labeled version. A truncated, 96-bp version of the *sabA* probe was generated in both biotinylated and unlabeled versions using either sabA5Bio or sabA5-F oligonucleotides (see Table S1), respectively, paired with sabAProbeRev. All probes were gel purified before use in EMSA.

EMSAs were carried out according to a procedure adapted from the LightShift EMSA optimization kit protocol (Thermo) and the ArsR EMSA protocol of Loh et al. (20). Recombinant ArsR (0.2 to 0.4 nmol) was incubated in a binding reaction mixture containing 10 mM Tris-HCl, 50 mM KCl, 1 mM dithiothreitol (DTT), 2.5% glycerol, 1 mM $MgCl_2$, 0.05% NP-40, 2 mg poly(dI-dC), 500 ng unlabeled probe (for specific competition reactions), and 500 ng Epstein Barr virus nuclear extract (Sigma) (for nonspecific competition reactions). This mixture was incubated for 10 min at room temperature. One microliter (1 to 2 ng) labeled *sabA* probe then was added to each binding reaction and incubated for 20 min at room temperature. Reactions were electrophoresed immediately on native 6% PAGE gels, blotted onto Zeta probe membranes (Bio-Rad), and cross-linked via UV irradiation. The imaging procedure followed Thermo Scientific's chemiluminescent nucleic acid detection module and was visualized using X-ray film.

RESULTS AND DISCUSSION

Multiple alleles of *sabA* exist in *H. pylori* J99 and *sabA* poly(T) mutants due to phase variation in poly(T) tract. Plasmid constructs with modifications to the poly(T) tract length were designed and introduced into *H. pylori* J99 strains (Fig. 1A to C). 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 AFLP analysis. Results indicated the presence of multiple-length polymorphisms within the *sabA* poly(T) repeat tract amplicons from the wild type and all indel mutants (Fig. 2). While each *sabA* poly(T) tract indel mutant population contained multiple subpopulations possessing slightly different poly(T) lengths based on AFLP analyses, each of these *H. pylori* J99 mutant strains had a clear dominant population with a particular modified poly(T) tract length. Wild-type *H. pylori* J99, containing 18 thymines, was measured as having a dominant subpopulation with an amplicon of 230 bp (31). DNA sequencing of this locus confirmed the creation of *H. pylori* J99 mutants with various poly(T) tract lengths (data not shown). 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 suggest that this activity increases 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 (Fig. 2).

Polythymine tract length in *sabA* promoter region affects expression. In order to understand the effects of poly(T) length variation on transcriptional activity of the *sabA* locus, we conducted qRT-PCR on *H. pylori* strain J99 and the mutant J99 strains with variant poly(T) tract lengths (Fig. 3). The J99 WT strain and $poly(T)_{18}$ control mutant performed similarly with no significant statistical difference ($P > 0.05$), as determined by a Welch unpaired *t* test of unequal variance using the program R Studio, indicating that the addition of the chloramphenicol acetyltransferase gene, used as a marker in the mutant strains, did not have a significant impact on *sabA* expression (see Fig. S1 in the supplemental material). Notable, however, was the 5.9-fold increase ($P = 0.0013$) in *sabA* expression in the *H. pylori* J99 mutant containing 13 thymines compared to the $poly(T)_{18}$ control mutant and the 10-fold increase ($P = 0.00086$) in *sabA* expression in the *H. pylori* J99 mutant containing 23 thymines compared to the $poly(T)_{18}$ control mutant. In contrast, mutants with a poly(T) tract length of 16, 17, 18, and 22 showed less striking changes of no statistical significance ($P > 0.05$) in the associated *sabA* expression compared to that of the $poly(T)_{18}$ control mutant. Similar results and statistical significances were reproduced in the two additional biological replicates. This suggested that when the length of the

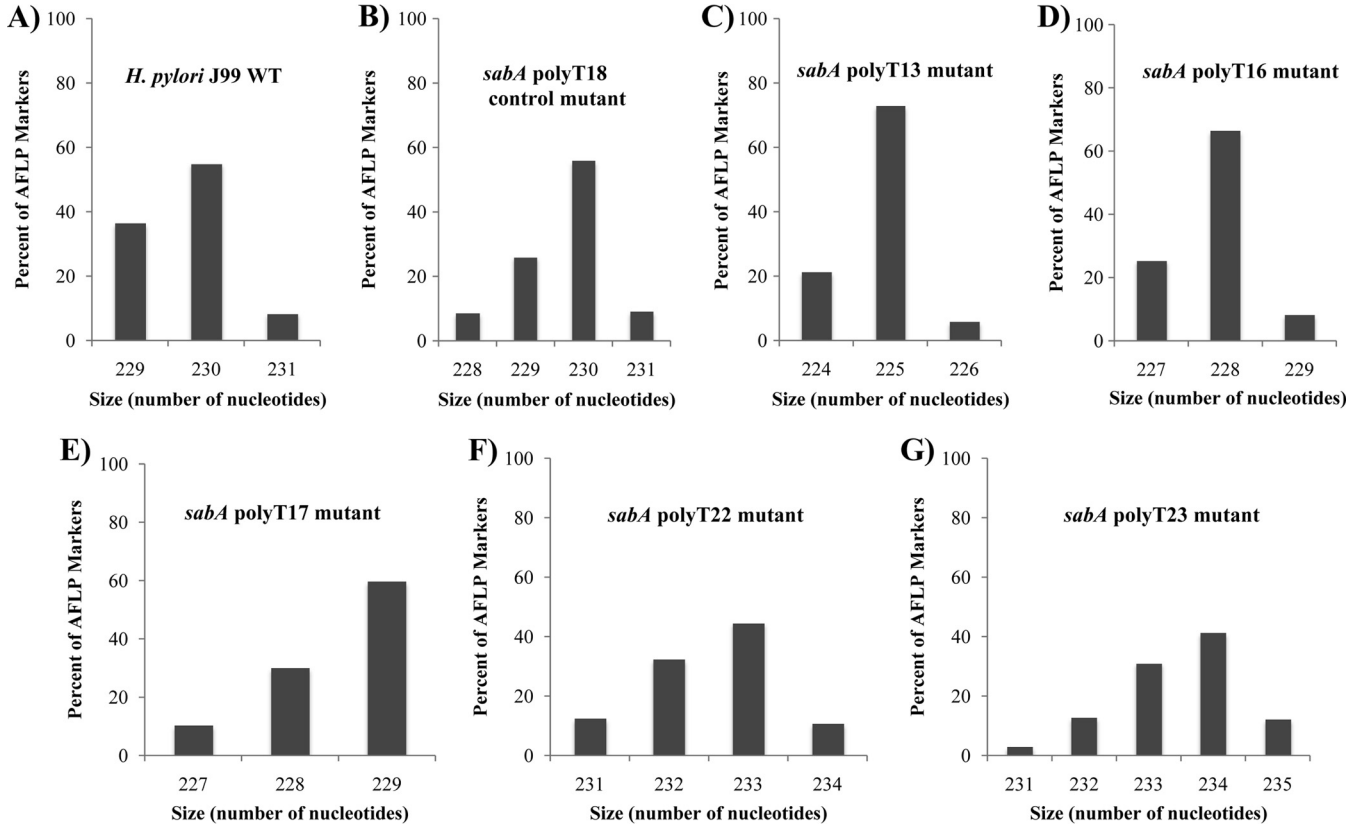

FIG 2 AFLP analysis of *H. pylori sabA* poly(T) tract mutants. AFLP analysis was used to quantify variations in the *sabA* poly(T) tract containing amplicons from *H. pylori* J99 and poly(T) tract mutants. The amplicon generated using primers *sabA* IG Fwd and *sabA* IG Rev (see Table S1 in the supplemental material) is predicted to be 230 bp based on the annotated sequence of *H. pylori* strain J99, where the *sabA* poly(T) tract possesses 18 thymines (31). Shown are WT *H. pylori* J99 (A) and the *sabA* poly(T)$_{18}$ control mutant (B), *sabA* poly(T)$_{13}$ mutant (C), *sabA* poly(T)$_{16}$ mutant (D), *sabA* poly(T)$_{17}$ mutant (E), *sabA* poly(T)$_{22}$ mutant (F), and *sabA* poly(T)$_{23}$ mutant (G).

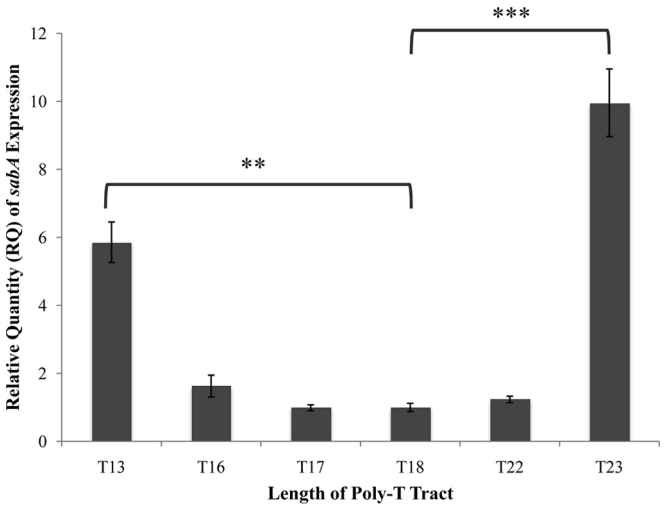

FIG 3 *sabA* expression in *H. pylori* J99 and J99 *sabA* poly(T) tract length variants. Quantitative real-time PCR was used to determine the relative expression of *sabA* in *H. pylori* J99 compared to that in mutants containing poly(T) tracts of various thymine lengths. The data are a representative trial of the results obtained from three independent experiments, each conducted in technical triplicate. Error bars show standard errors of the means. Statistics were calculated using a Welch's unpaired *t* test with *sabA* poly(T)$_{18}$ as the control (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$).

poly(T) tract is increased or decreased by five nucleotides, corresponding to a half turn of the DNA helix, there is a maximal differential effect on *sabA* expression.

In light of these results, we first hypothesized that altering the length of the tract by a half turn of the DNA would place the binding site for a *trans*-acting factor on the opposite face of the DNA, rendering it more or less likely to interact with the RNA polymerase. However, a 2012 study by Kao et al. (13) showed an effect of *H. pylori sabA* poly(T) variation on *lux* reporter expression in *E. coli*, where *H. pylori* regulatory proteins or RNA polymerases are absent. Thus, considering our data for *H. pylori* and the results of Kao et al. (13) for *E. coli*, we hypothesized that the length of the poly(T) tract has a significant impact on the topology or flexibility of the DNA in the *sabA* promoter region. Alternatively, there may be a shared transcriptional factor found in both *E. coli* and *H. pylori* that recognizes such poly(T) tracts mediating similar effects on the *sabA* promoter activity in both organisms.

Poly(T) tracts are rigid, and their presence affects the bendability of DNA (25). The rigidity and bendability of certain regions of DNA have been found to influence proteins' ability to loop DNA (26). 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 (26). We hypothesize that indels within

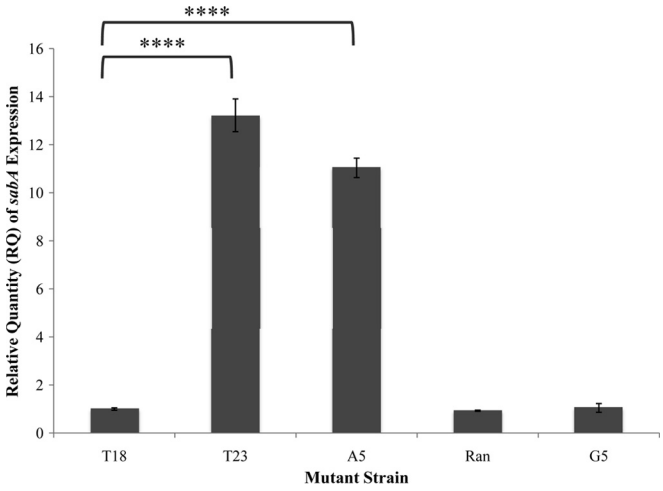

FIG 4 *sabA* expression in *H. pylori* J99 and J99 *sabA* poly(T) mutants (nonthymine extensions). Quantitative real-time PCR was used to determine the relative expression of *sabA* in *H. pylori* J99 compared to that in mutants containing a poly(T) tract with various five-nucleotide insertions upstream and adjacent to the poly(T) tract. The data shown here are representative of the results obtained from three independent experiments, each conducted in technical triplicate. Error bars show standard errors of the means. Statistics were calculated using a Welch's unpaired *t* test of unequal variance with *sabA* poly(T)$_{18}$ as the control (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$). Schematic representations of these mutants are shown in Fig. 1.

the poly(T) tract in the promoter region of *sabA* in *H. pylori* influence the ability of 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) tract in *H. pylori* were found in a variety of prokaryotic systems (32). The overwhelming presence of homopolymeric tracts across prokaryotic taxa suggests that these tracts have been beneficial to these organisms. A study done by Wernegreen et al. (33) 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. An algorithm created by Vlahovicek et al. (34) predicts a sequence of adenine or thymine nucleotides to be noncurved 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 were extended, either by five adenines, five guanines, or a random series of five nucleotides (ACTAG) (Fig. 1d to f). In the mutant containing a poly(T) tract extended by five adenines, *sabA* expression increased 11-fold compared to *sabA* expression in the poly(T)$_{18}$ control mutant ($P = 1.6 \times 10^{-5}$). This increased *sabA* expression was comparable to that of the T23 mutant strain which had been extended by five thymines (Fig. 4). Perhaps this result is 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 (Fig. 4). Similar statistics and results were reproduced in the additional two biological replicates.

These results support the hypothesis that the nature of the adenine-thymine tract, 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, binding of proteins to the intrinsic curvature of this poly(A) tract affects *ureR* transcription (35). We hypothesize that the proper bending and curvature of the DNA in and around the *H. pylori sabA* poly(T) tract allows 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 mRNA synthesis 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 is 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 (36, 37). 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*, to those of Kao et al. (13), who examined 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 (31, 38). This may be a common means of modulating promoter activities in a bacterium, such as *H. pylori*, that has a paucity of transcription factors.

Determining the ArsR binding site and its relationship with the poly(T) tract. Previously studies from our laboratory demonstrated that the two-component system ArsRS affects the expression of *sabA* (11, 19). We now sought to characterize the location of the ArsR binding site in order to determine its proximity to the phase-variable *sabA* promoter region poly(T) tract. EMSA to locate ArsR binding sites relative to the polythymine tract determined that the binding site was, in fact, downstream of the poly(T) tract and the transcriptional start site. This suggests that ArsR functions as a *sabA* repressor by preventing passage of the RNA polymerase independent of the poly(T) tract (Fig. 5). Although it appears that ArsR is not involved in regulation of *sabA* expression via the poly(T) tract, the localization of recombinant ArsR (rArsR) determined in this study is consistent with the established role of ArsR as a repressor of *sabA* expression (11, 19). Both transcriptional control via phase variation at the poly(T) tract in the *sabA* promoter region as well as transcriptional control via ArsRS-mediated two-component signal transductions are layered on the translational phase variation mediated by the homopyrimidine dinucleotide repeat, CT, found near the 5′ end of the *sabA* protein-coding sequence (11). The location of the ArsR binding site itself (between −20 and +38 relative to the transcriptional start site) further supports its role as a repressor of *sabA* expression (19), as binding here likely prevents open complex formation by preventing RNA polymerase from binding the *sabA* promoter efficiently. Adding further to the complexity of *H. pylori*'s control of *sabA* expression is the 2012 study by Talarico et al.

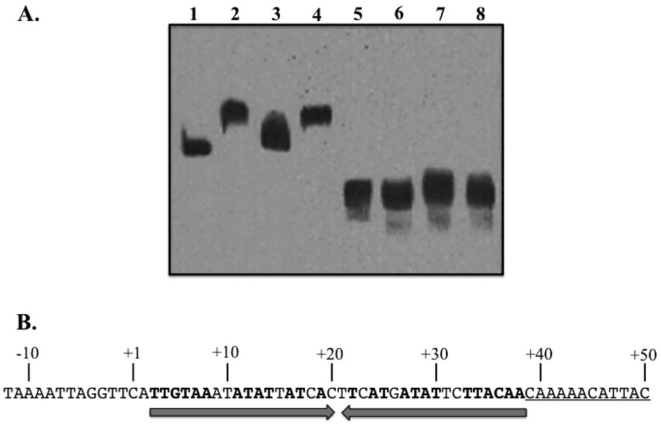

FIG 5 ArsR binds a sequence downstream of the poly(T) tract associated with the *sabA* promoter. (A) Nested biotin-labeled probes of the *sabA* promoter region were used in EMSA to localize the ArsR binding site. A 156-bp *sabA* probe (−20 to +135) was electrophoresed alone (lane 1), with rArsR (lane 2), with rArsR and a 500-fold excess of unlabeled probe as a specific competitor (lane 3), and with rArsR and a 500-fold excess of Epstein-Barr virus nuclear antigen DNA as a nonspecific DNA competitor (lane 4). rArsR-DNA binding reactions in lanes 5 to 8 were run identically except for the use of a 5′-truncated (to +39 to +135) *sabA* probe to demonstrate the loss of rArsR binding with the loss of the upstream 60 bp. (B) Proposed binding site of ArsR in the 5′ untranslated region of *sabA*. Converging arrows indicate a region of partial dyad symmetry within the region bound by rArsR. The underlined sequence is the 5′ end of the truncated probe used in lanes 5 to 8 in panel A.

(12) that demonstrated strong host selection to maintain *sabA* as opposed to frequent loss *in vitro* and that via recombination, *sabA* could be duplicated as a means to increase SabA levels and increase adherence to host tissue.

rArsR bound the 156-bp *sabA* probe (−20 to +135), the longer of the probes utilized in EMSAs to localize the ArsR binding site (Fig. 5, lanes 1 to 4), and failed to bind the truncated *sabA* probe (+39 to +135) (Fig. 5A, lanes 5 to 8), indicating the binding site for ArsR lies between −20 and +38 relative to the *sabA* transcriptional start point. A region of partial dyad symmetry lies at +3 to +38, and this sequence may be responsible for the mobility shift seen in Fig. 5 (lanes 2 and 4).

In the complex interplay between *H. pylori* and its host, SabA is a key protein of interest. Although other adhesins, such as BabA, are important in the initial stages of infection, studies have shown that the regulation of SabA expression becomes key once host inflammation increases, as the expression of *sabA* is rapidly modulated in response to the changing conditions of the stomach (39).

Additionally, a common feature of *H. pylori*-induced gastritis is an infiltration of neutrophils into the gastric mucosa. A 2005 study by Unemo et al. (40) 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 SabA as a virulence factor in disease pathogenesis (40).

Investigating *sabA* regulation mediated via phase variation provides a platform for the study of other key *H. pylori* adhesins. *H. pylori* is equipped with a variety of outer membrane proteins, including the Hop (*Helicobacter* outer membrane porin) members, Hor (Hop-related protein) members, and the OMPs AlpA, AlpB, BabA, SabA, and HopZ. Much about their functions remains undiscovered (41). Repetitive nucleotide tracts are found in association with numerous outer membrane protein genes, both in the coding sequence and associated with promoter regions. HopZ (jhp0007 [HP0009]) is regulated by both a phase-variable CT repeat in the coding sequence (42) and a polyadenine tract associated with the promoter. Examining the sequenced genomes of *H. pylori* shows there are at least eight other outer membrane proteins with a poly(T) or poly(A) tract of at least 10 nucleotides or longer in the associated promoter regions; thus, it is possible that there are similarities in the genetic regulation of many OMPs.

There are few pieces of data suggesting the direct effect of adhesins on signaling pathways, but there is consensus on the role of adhesins to mediate a tight interaction between *H. pylori* and the host target cell, allowing additional bacterial factors to interact with their corresponding receptors (6). In a previous study with clinical isolates, AlpA and AlpB were produced at a constant rate, but all other OMPs were produced at highly variable rates, ranging from 35% to 73%. This result indicates that SabA is not the only outer membrane protein whose function is in close adaptation to the individual host or gastric niche (41).

Homopolymeric tracts and dinucleotide tracts are abundant in the coding sequence of *H. pylori* genes, as they are in several other mucosal pathogens (38, 43). In *Helicobacter canadensis*, an emerging human pathogen, 29 annotated coding regions were determined to be associated with variable-length homopolymeric tracts, and 21 of the repeat-associated coding regions were determined to be potentially transcriptionally or translationally phase variable (43). Another example illustrates that mutations in homopolymeric tracts within the promoter gene of a symbiont can affect host fitness: a single-base polymorphism of a gene coding for a heat shock protein in the obligate bacterial symbiont *Buchnera* has the potential to allow pea aphid populations to adapt quickly to prevailing conditions (44).

There may be more layers of complexity to the regulation of *sabA*. While this study and those of others have begun to characterize the relationship between SabA and the TCS ArsRS, there may yet be other regulatory proteins affected by the changing DNA topology resulting from increases and decreases in the *sabA* poly(T) tract length. The results of our study will aid in a more complete understanding of *sabA* regulation in *H. pylori* and the further study of other adhesins, as well as in the understanding of adaptive evolution in pathogens.

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