The HP0165-HP0166 Two-Component System (ArsRS) Regulates Acid-Induced Expression of HP1186 α-Carbonic Anhydrase in *Helicobacter pylori* by Activating the pH-Dependent Promoter[▽]

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The periplasmic α-carbonic anhydrase of *Helicobacter pylori* is essential for buffering the periplasm at acidic pH. This enzyme is an integral component of the acid acclimation response that allows this neutralophile to colonize the stomach. Transcription of the HP1186 α-carbonic anhydrase gene is upregulated in response to low environmental pH. A binding site for the HP0166 response regulator (ArsR) has been identified in the promoter region of the HP1186 gene. To investigate the mechanism that regulates the expression of HP1186 in response to low pH and the role of the HP0165-HP0166 two-component system (ArsRS) in this acid-inducible regulation, Northern blot analysis was performed with RNAs isolated from two different wild-type *H. pylori* strains (26695 and 43504) and mutants with HP0165 histidine kinase (*ArsS*) deletions, after exposure to either neutral pH or low pH (pH 4.5). ArsS-dependent upregulation of HP1186 α-carbonic anhydrase in response to low pH was found in both strains. Western blot analysis of *H. pylori* membrane proteins confirmed the regulatory role of ArsS in HP1186 expression in response to low pH. Analysis of the HP1186 promoter region revealed two possible transcription start points (TSP$_1$ and TSP$_2$) located 43 and 11 bp 5′ of the ATG start codon, respectively, suggesting that there are two promoters transcribing the HP1186 gene. Quantitative primer extension analysis showed that the promoter from TSP$_1$ (43 bp 5′ of the ATG start codon) is a pH-dependent promoter and is regulated by ArsRS in combating environmental acidity, whereas the promoter from TSP$_2$ may be responsible for control of the basal transcription of HP1186 α-carbonic anhydrase.

Helicobacter pylori is a gram-negative neutralophile that shares some cytoplasmic pH regulatory systems with other neutralophiles, such as *Salmonella enterica* serovar Typhimurium, *Vibrio cholera*, and pathogenic strains of *Escherichia coli* (20, 21, 23). These bacteria can survive in acidic environments by maintaining the cytoplasmic pH at ~4.0 to 5.0 but do not grow and therefore cannot colonize the stomach. However, *H. pylori* has developed additional unique acid acclimation mechanisms that enable this organism not only to survive in but also to colonize the acidic environment of the human stomach (21, 23). This is due to its ability to buffer its periplasmic pH in acidic environments to levels at which both the cytoplasmic pH and membrane potential are compatible with growth (22). Several systems may be involved in maintenance of periplasmic pH which form the basis for acid acclimation and growth in the gastric environment.

The urease system is one of the best-studied processes in *H. pylori* which maintain periplasmic and cytoplasmic pH near neutrality when the organism is exposed to acidity (36, 37). Urease is a nickel-containing hexameric heterodimer, composed of subunits UreA and UreB, which hydrolyzes urea, leading to the formation of ammonia and carbon dioxide, which buffer the cytoplasm and periplasm of the bacterium (34, 39, 40). The enzymatic activity of intrabacterial urease is controlled by the inner membrane pH-gated channel UreI, which

regulates the access of the substrate, urea, to urease in the bacterial cell in response to acidic pH (33, 38, 53). Both urease and the pH-gated channel protein UreI are essential for colonization in several animal infection models (11, 12, 41, 47).

Although these studies have clarified the important role of UreI and urease in the acid acclimation processes of *H. pylori*, it would be extremely unlikely that they are the only means of combating gastric acidity for colonization of the mammalian stomach by the organism (22, 37, 40). Even though urease produces ammonia (NH$_3$), which rapidly diffuses into the periplasm, its pK$_a$ of 9.2 shows that it would be a very weak buffer at the desired relatively neutral periplasmic pH. The other product of urease activity is H$_2$CO$_3$, which is rapidly converted to carbon dioxide (CO$_2$) by the bacterial cytoplasmic β-carbonic anhydrase, allowing rapid diffusion of this gas into the periplasm. The periplasmic α-carbonic anhydrase (HP1186) converts this periplasmic CO$_2$ to HCO$_3^{2-}$, providing a buffer with a pK$_a$ of 6.1. This enzyme has been shown to be important in buffering the bacterial periplasm (22, 42). Wild-type *H. pylori* maintains a periplasmic pH of ~6.1 in the presence of strongly buffered acidic medium between pH 3.5 and 6.2 (38), partially due to the activity of UreI that admits urea into the bacterial cytoplasm (39, 53). However, in an *H. pylori* mutant strain with α-carbonic anhydrase deletion (22), the addition of urea no longer restored membrane potential in acidic medium, as had been found in *ureI* deletion mutants, although UreI and urease remained fully functional in the carbonic anhydrase deletion strains in that there was a normal increase of bacterial urease activity with decreasing pH. At low pH, the elevation of cytoplasmic and periplasmic pH with urea

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was abolished in the absence of α-carbonic anhydrase activity (22). Furthermore, acid survival was significantly impaired, either with deletion of HP1186 or by addition of the carbonic anhydrase inhibitor acetazolamide (22). These results suggest that the role of periplasmic α-carbonic anhydrase in acid acclimation is to generate HCO_3^{2-} in the periplasm both to neutralize entering acid and to buffer the periplasm to a level, pH 6.1 (the pK_a of HCO_3^{2-}), at which a viable inner membrane potential is maintained. Therefore, the periplasmic α-carbonic anhydrase activity of *H. pylori* is essential for acid acclimation.

In recent transcriptome studies, the gene expression of urease (including *ureAB* and *ureIEFGH*), as well as some other ammonia-producing enzymes (including aliphatic amidase *amiE*, asparaginase *ansB*, and arginase *rocF*), which might be considered pH homeostatic genes, were found to be induced upon exposure of *H. pylori* to low pH (5, 26, 49, 55). One of these studies also showed a nearly fivefold increase in expression of HP1186 α-carbonic anhydrase upon exposure to acidic pH, and the low-pH-induced expression was confirmed by real-time PCR analysis (55).

Better understanding of the mechanisms that regulate gene expression in response to the acidic conditions in *H. pylori*, especially for the acid acclimation genes, would eventually facilitate the development of novel eradication therapies for this carcinogenic gastric pathogen. Transcription of the *ureAB* genes was found to be positively regulated by the NikR protein in response to increasing concentrations of Ni^{2+} ions in the surrounding medium (50, 51), through which the environmental acidity might be indirectly sensed (52). More recent studies have shown that transcriptional induction of urease genes (*ureAB* and *ureI*) in response to low pH is mediated mainly by the HP0165-HP0166 two-component system (ArsRS), since the effect was largely abolished in an ArsS (HP0165)-deficient mutant (29, 31, 32). The phosphorylated HP0166 response regulator ArsR (ArsR∼P) was found to bind to extended regions overlapping the P_{ureA} and P_{ureI} promoters (31). In addition, it appeared that ArsRS may also be involved in the pH-responsive transcriptional regulation of some other ammonia-producing genes (*amiE*, *amiF*, and *rocF*) (30). It has been suggested that ArsRS plays a prominent role in the control of acid-responsive gene expression during infection (29, 31, 32). However, very little is known about the regulation of gene expression for HP1186 α-carbonic anhydrase in response to low pH.

The two-component system ArsRS is an acid-responsive signaling system that perceives low-pH stimuli by the periplasmic domain of its sensor histidine kinase (ArsS) and regulates acid-responsive gene expression by the cognate response regulator (ArsR), which directly interacts with the promoter region in *H. pylori* (29–32, 54). A recent electrophoretic mobility shift assay (EMSA) study (54) with a recombinant HP0166-His₆ showed that ArsR binds directly to the promoter region of HP1186 α-carbonic anhydrase, and EMSA studies with a mutated nonphosphorylatable protein (HP0166-D52N) suggested a phosphorylation-dependent binding. Given that the expression of the HP1186 gene is acid inducible and that the phosphorylated form of ArsR can directly bind to the promoter region of HP1186 α-carbonic anhydrase, it is possible that acid-induced expression of HP1186 α-carbonic anhydrase requires the two-component system ArsRS.

In the current study, we demonstrate that the two-component system ArsRS directly regulates low-pH-induced expression of HP1186 α-carbonic anhydrase. Using 5′-rapid amplification of cDNA ends (5′-RACE) and primer extension analysis, we also identify two potential transcriptional start points (TSP_1 and TSP_2) in the 5′-upstream region of the HP1186 α-carbonic anhydrase gene, indicating the presence of two promoters that control the expression of HP1186 α-carbonic anhydrase. We provide evidence that one of the two promoters is pH dependent and controlled by ArsRS in response to low environmental pH, and the other one may be involved in maintaining basal transcription of HP1186 α-carbonic anhydrase.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* strains 26695 and 43504 were obtained from the American Type Culture Collection. An *arsS*-deficient mutant, 26695/HP0165::cat, with an insertion of the chloramphenicol acetyltransferase (*cat*) gene in the HP0165 open reading frame (ORF) (13), was a gift from M. H. Forsyth's laboratory. An *arsS*-deficient mutant, 43504/HP0165::km, and an *H. pylori* strain with deletion of the ArsR-binding site within the promoter region of the HP1186 α-carbonic anhydrase gene were constructed by allelic exchange using a kanamycin resistance gene as described below. Primary plate cultures of *H. pylori* were grown from glycerol stocks on blood agar medium for 2 to 3 days in a microaerobic environment (5% O_2, 10% CO_2, and 85% N_2) at 37°C. In preparation for an experiment, bacteria were scraped from the plates, suspended in 1 mM phosphate HP buffer (138 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, 1 mM glutamine), pH 7.0, and transferred to fresh plates for 24 h. For exposure to experimental low-pH conditions, the overnight cultures of *H. pylori* strains on trypticase soy agar plates supplemented with 5% sheep blood were suspended in brain heart infusion medium (Difco) to an optical density at 600 nm of 0.20 to 0.25. The pH of brain heart infusion medium was adjusted to pH 7.4 or pH 4.5 using concentrated HCl followed by filtration to remove any precipitate. *H. pylori* was then incubated in the presence of 5 mM urea with shaking (120 rpm) under microaerobic conditions at 37°C for 30 min before further extraction of RNA or protein. *Escherichia coli* strains were grown in Luria-Bertani broth. When necessary, antibiotics were added to the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 10 to 30 μg/ml.

Construction of mutant *H. pylori* strains (43054/HP0165:km and 43504/ΔP$_{HP1186}$) by allelic exchange mutagenesis. An isogenic *arsS*-deficient mutant, 43054/HP0165::km, was obtained by transforming *H. pylori* strain 43504 with a pBluescript II vector (Stratagene) carrying the Tn*903* kanamycin resistance gene (45) flanked by a 650-bp fragment comprising the 3′ region of the HP0166 gene and a 463-bp fragment comprising the 5′ region of the HP0163 gene. The DNA fragments were obtained by PCR performed on *H. pylori* chromosomal DNA with primer pairs HP0166-5′p/HP0166-3′p and HP0163-5′p/HP0163-3′p (Table 1), respectively. The resulting mutant had the entire HP0165-0164 coding sequence replaced by the kanamycin resistance gene.

To construct the *H. pylori* strain with deletion of the ArsR-binding site within the promoter region of the HP1186 α-carbonic anhydrase gene, a pBluescript II vector (Stratagene) carrying a kanamycin resistance cassette flanked by a 531-bp fragment comprising 393 bp of the 3′ region of the HP0185 ORF and 138 bp of the intergenic region between HP1185 and HP1186 and a 703-bp fragment comprising 94 bp of the upstream region and 609 bp of the complete ORF of HP1186 was introduced into *H. pylori* strain 43504 by natural transformation. The DNA fragments were obtained by PCR amplified from chromosomal DNA of *H. pylori* 26695 with primer pairs HP1185-5′p/HP1185-3′p and HP1186-5′p/HP1186-3′p (Table 1), respectively. The resulting strain (43504/ΔP$_{HP1186}$) had the ArsR-binding site ranging from position −98 to −136 (with respect to the translational start codon) within the promoter region of the HP1186 α-carbonic anhydrase gene replaced with a kanamycin resistance cassette in a way that the transcriptional direction of the Km gene was divergent from HP1186.

RNA preparation. Total RNA was isolated from *H. pylori* strains using TRIzol reagent (Invitrogen) combined with RNeasy columns (QIAGEN). The bacterial pellet was resuspended in 500 μl of TRIzol reagent (Invitrogen) and lysed at room temperature for 5 min before 100 μl of chloroform was added. After

TABLE 1. Oligonucleotides used in this study

Name	Sequence (5′ to 3′)[a]	Site[b]	Strand	Position[c]
HP0166-5′p	CATGTAACCAATctaGATGAGCCATATACCGGC	XbaI	−	174345–174377
HP0166-3′p	CTTTAAAAAAGATAGAGgtcgacAAAACCCCTTAACTC	SalI	+	173728–173765
HP0163-5′p	CCCGAAAATTTGAGAtctGTGAGCGGAATGAAGGG	BglII	−	172448–172482
HP0163-3′p	GCCCATGGTCGGTaccTTCACAAAAACACAAATCCGC	Acc65I	+	172020–172056
HP1185-5′p	attgggtaccCGCGACACGAATGACTAAAGAAGC	KpnI	+	1255108–1255131
HP1185-3′p	gcaggaattcGGTAACAACTCCCTTAAATTAATGC	EcoRI	−	1255613–1255638
HP1186-5′p	tagtggatccAATCTATTTTTTGTAACTGCGGTCA	BamHI	+	1255678–1255702
HP1186-3′p	tgttgagctcTCACAAACCATGCCACCCCCTCTGT	SacI	−	1256356–1256380
P1186-5′ (479–502)	TTCAAATACGCCGCTTCTAAACCT		+	1255979–1256002
P1186-3′ (875–856)	AACCATGCCACCCCCTCTGT		−	1256356–1246375
P1186-3′ (481–504)	TTAGGTTTAGAAGCGGCGTATTTG		−	1255981–1256004
P1186-3′ (308–327)	TCAGCGCCTACAAGAGAAGC		−	1255808–1255827
P1186-5′ (1–20)	TTAAATCTTAAGGGGGTTGAA		+	1255501–1255520
P0547-5′ (3728–3750)	AGCTAGCCCTGAACCCATTTACG		+	582815–582837
P0547-3′ (4150–4127)	TGCGCAACTATCTTATCATTCACG		−	583214–583237
P0071-5′ (249–272)	GGCAATGCTAGGACTTGTATTGTT		−	75315–75338
P0071-3′ (821–840)	TCACACCCAGTGTTGGATAA		+	74747–74756

[a] Sequences in uppercase letters are derived from the genome sequences of *H. pylori* 26695 (46). Sequences introduced for cloning purposes are given in lowercase letters, and restriction recognition sites are underlined.
[b] Restriction recognition site.
[c] Nucleotide positions refer to the genome sequence of *H. pylori* 26695 (46).

spinning at 12,000 × *g* for 10 min at 4°C, the supernatant was mixed with 250 μl ethanol and applied to an RNeasy spin column (QIAGEN). RNA purification was processed following the manufacturer's instructions (beginning with the application to the column). RNA concentration was quantified by absorbance at 260 nm, and the quality was evaluated by capillary electrophoresis using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay kit (Agilent Technologies).

Northern blot analysis. Fifteen μg of total RNA from different *H. pylori* samples was fractionated on 1% agarose-formaldehyde gels and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH). The RNA on the gel was visualized by ethidium bromide staining and photographed before the RNA was transferred to Nytran membranes.

Internal fragments of the α-carbonic anhydrase (HP1186), *ureI* (HP0071), and *cagA* (HP0547) genes were PCR amplified from genomic DNA of *H. pylori* 26695 with the primer pairs P1186-5′(479–502)/P1186-3′ (875–856), P0071-5′(249–272)/ P0071-3′ (821–840), and P0547-5′ (3728–3750)/P0547-3′ (4150–4127) (listed in Table 1) and used as probes. The probes were radiolabeled with [α-^{32}P]dCTP using a random primer labeling kit (Stratagene, La Jolla, CA) to specific activities of 1×10^8 to 10×10^8 cpm/μg. The blot was hybridized (9) with radiolabeled probe overnight at 65°C in a buffer containing 0.45 M sodium phosphate (pH 7.2), 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin, and 20 mM EDTA. The hybridized blot was washed with 0.1× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C and autoradiographed using a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA). The RNA hybridization bands were normalized based on 16S and 23S rRNA band intensities.

Western blot analysis. To isolate membrane proteins from *H. pylori* strains (wild types and *arsS* deletion mutants) treated with different pHs, the bacteria pellet was resuspended in ice-cold 25 mM sodium phosphate buffer, pH 7.4, and passed three times through a French press at 20,000 lb/in^2. The lysate was centrifuged at 3,000 × *g* for 10 min to pellet any remaining whole cells. The supernatant was removed and centrifuged at 10,000 × *g* for 10 min. This supernatant was layered onto 5 ml of 20% sucrose and centrifuged at 100,000 × *g* for 1 h. The sucrose layer prevented soluble cytoplasmic proteins from contaminating the membrane pellet. The supernatant was removed from the top of the sucrose layer and concentrated by using a centrifugal filter device with a 10-kDa cutoff (Centricon, Inc.). The membrane pellet was resuspended in 25 mM sodium phosphate buffer, pH 7.4. Protein concentration was determined by using bicinchoninic acid (Pierce).

The proteins from the membrane fraction were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 4 to 12% NuPage Bis-Tris gradient gels (Invitrogen) and transferred onto nitrocellulose (Bio-Rad). Western blot analysis was carried out using an antibody against α-carbonic anhydrase (22). Immunolabeling was detected with the SuperSignal West Pico chemiluminescence detection kit (Pierce). A Western blot analysis by a rabbit polyclonal antibody (NCL-HPp) reactive with heat-stable, somatic antigens of the whole *H.*

pylori organism (Novocastra Laboratories Ltd., United Kingdom) with the same preparation of the membrane proteins was used as a loading control.

Characterization of the HP1186 promoter region. The BD Smart RACE cDNA amplification kit (BD Biosciences Clontech, Palo Alto, CA) was used for characterization of the HP1186 promoter region from *H. pylori* by RACE-PCR. According to the manufacturer's manual, 1 μg of total RNA isolated from the *H. pylori* strain 26695 was reverse transcribed with a gene-specific reverse primer, P1186-3′ (481–504) (Table 1), which is 232 bp downstream of the translation start codon ATG in the ORF of the HP1186 gene, and the BD PowerScript reverse transcriptase (RT; BD Biosciences Clontech, Palo Alto, CA), while the BD SMART II A oligonucleotide was also included in the RT reaction mixture. When the RT reaches the 5′ end of the RNA template, the SMART II A oligonucleotide attaches to the first-strand cDNA tail and serves as an extended template for the RT, resulting in a complete cDNA copy of the original RNA with the additional SMART sequence at the 5′ end (24, 56). A nest gene-specific reverse primer, P1186-3′ (308–327) (Table 1), which is 55 bp downstream of the translation start codon ATG in the ORF of HP1186, was used for 5′-RACE-PCR with the SMART universal primer. The RACE-PCR products were cloned into the T/A cloning vector pCR4-TOPO (Invitrogen, Carlsbad, CA). The authenticity was confirmed by DNA sequencing in both directions with T7 and M13 reverse primers.

Primer extension analysis. The gene-specific reverse primer P1186-3′ (308–327), was 5′-end labeled in the presence of [γ-^{33}P]ATP (5,000 Ci/mmol; GE Healthcare Biosciences, Piscataway, NJ) and T4 polynucleotide kinase (Promega, Madison, WI). Labeled oligonucleotide (1 pmol) was then coprecipitated with 35 μg of *H. pylori* total RNA and resuspended in 6 μl of H$_2$O and 5 μl of 2× avian myeloblastosis virus (AMV) primer extension buffer (Primer Extension system; Promega). The reaction mixture was incubated for 20 min at 58°C followed by 10 min at room temperature, then 5 μl of 2× AMV primer extension buffer, 1.4 μl of 40 mM sodium pyrophosphate, and 1 μl of AMV reverse transcriptase (1 U/μl) were added, and reverse transcription in a total volume of 20 μl was carried out by incubating at 42°C for 30 min. The sample was then ethanol precipitated and resuspended in 5 μl of sequencing loading buffer. After denaturing at 90°C for 2 min, samples were subjected to 6% urea-polyacrylamide gel electrophoresis and autoradiographed using a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, CA). To ensure correct mapping of the extension products, the HP1186 promoter region was cloned with the primer pair P1186-5′ (1–20)/P1186-3′ (308–327) (Table 1) from the chromosomal DNA of *H. pylori* 26695 and sequenced in parallel with the same primer used for primer extension.

RESULTS

Low-pH-induced transcription of HP1186 α-carbonic anhydrase is dependent on the two-component system ArsRS. With microarray analysis, the HP1186 α-carbonic anhydrase gene
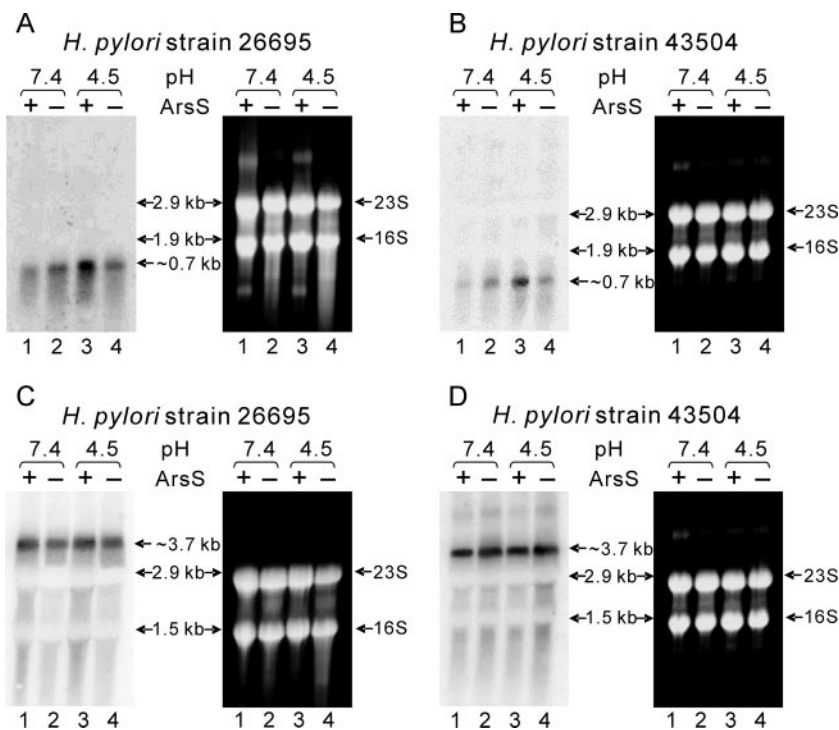
FIG. 1. Low-pH-induced transcription of HP1186 α-carbonic anhydrase is ArsS dependent. Total RNAs were extracted from wild-type strains 26695 (A and C) and 43504 (B and D) and the ArsS mutant strains 26695/HP0165::cat (A and C) and 43504/HP0165::km (B and D), which were exposed to either neutral pH (pH 7.4, lanes 1 and 2) or low pH (pH 4.5, lanes 3 and 4) for 30 min. RNAs were analyzed by Northern blotting with HP1186 α-carbonic anhydrase (A and B) and the HP0547 *cagA* (C and D) gene-specific probes. The same blot with RNAs from *H. pylori* strain 43504 was hybridized twice separately with different probes (B and D). Single transcripts of ∼0.7 kb (A and B) and ∼3.7 kb (C and D) were detected, which correspond to the sizes of HP1186 α-carbonic anhydrase and the HP0547 *cagA* transcripts. Ethidium bromide staining of the gel showed that equal amounts of RNA were analyzed. The size standards are the 16S and 23S rRNA species.

was found to be induced under low-pH conditions (55). In our previous EMSA studies, the promoter region for the HP1186 α-carbonic anhydrase gene was found to interact with HP0166-His₆, likely due to a phosphorylation-dependent binding (54). To investigate the two-component ArsRS system dependency of the low-pH-induced up-regulation on HP1186 α-carbonic anhydrase transcription, Northern blot analysis was performed with equal amounts of total RNAs extracted from *H. pylori* strain 26695 and the *arsS*-deficient mutant 26695/HP0165::cat (13) which had been exposed to either neutral pH (pH 7.4) or low pH (pH 4.5) for 30 min. From the genome organization of *H. pylori* it can be deduced that the HP1186 α-carbonic anhydrase gene is monocistronically transcribed with a 609-bp ORF. Northern blot analysis showed a single band at ∼700 bp (Fig. 1A). In the 26695 wild-type strain, transcription of HP1186 α-carbonic anhydrase was strongly induced at pH 4.5 (a two- to fourfold increase compared to the level at pH 7.4). At neutral pH, the transcription of HP1186 α-carbonic anhydrase in the *arsS*-deficient mutant was slightly higher than in the 26695 wild-type strain. However, no change in transcription level of HP1186 α-carbonic anhydrase in the *arsS*-deficient mutant was observed when the bacteria were exposed to pH 4.5 (Fig. 1A).

In order to understand the low-pH-induced transcription of the HP1186 α-carbonic anhydrase gene in a different *H. pylori* strain, an *arsS*-deficient mutant was constructed in *H. pylori* strain 43504 (43504/HP0165::km). By homologous recombina-

tion with a suicide vector, the entire HP0165/HP0164 coding sequence of *H. pylori* strain 43504 was replaced with a kanamycin resistance gene. Correct replacement of the wild-type sequence with the antibiotic resistance cassette was verified by means of PCR using primers complementary to regions flanking the insertion site (data not shown). A similar Northern blot analysis was performed comparing transcription levels of HP1186 α-carbonic anhydrase from the mutant and wild-type *H. pylori* strain 43504 after exposing the cells to pH 7.4 and pH 4.5. Very similar results were obtained (Fig. 1B) as found in strain 26695 (Fig. 1A). These results suggest that the acid-responsive transcriptional regulation of the HP1186 α-carbonic anhydrase gene is mediated by ArsRS in both strains 26695 and 43504 under our experimental conditions, since the *arsS*-deficient mutants were unable to induce the transcription at pH 4.5 to the levels found in the wild-type strains.

Northern blot analysis on transcription of the *cagA* (HP0547) gene from both *H. pylori* strains was included as a control (Fig. 1C and D) that is pH and ArsS independently transcribed to eliminate the possibility that the observed ArsS-dependent acid-responsive change in HP1186 α-carbonic anhydrase transcription is caused by global changes in transcription activity. Our previous microarray studies have shown that the expression level of *cagA* (HP0547) did not significantly change under the same experimental conditions (55).

Low-pH-induced protein expression of HP1186 α-carbonic anhydrase is dependent on the two-component system ArsRS. To
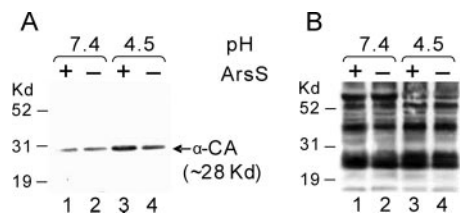
FIG. 2. Western blot analysis of membrane protein from *H. pylori*. Membrane fractions were prepared from wild-type *H. pylori* strain 26695 (lanes 1 and 3) and ArsS mutant strains 26695/HP0165::cat (lanes 2 and 4), which were exposed to either neutral pH (pH 7.4, lanes 1 and 2) or low pH (pH 4.5, lanes 3 and 4) for 30 min. The proteins from the membrane fractions were fractionated by SDS-PAGE and probed with polyclonal antibody against HP1186 α-carbonic anhydrase (A). Western blot analysis of the same preparation of the membrane proteins with a polyclonal antibody (NCL-HPp) against whole *H. pylori* showed that similar amounts of membrane protein from *H. pylori* strains were analyzed (B). The MultiMark MultiColored standard (Invitrogen) was used as a size marker.

confirm the regulatory role of ArsS in HP1186 α-carbonic anhydrase expression in response to low-pH conditions, we carried out Western blot analysis on membrane protein fractions to compare the levels of HP1186 α-carbonic anhydrase protein between the *H. pylori* wild-type strain 26695 and the isogenic mutant strain 26695/HP0165::cat. As shown in Fig. 2A, the protein expression of HP1186 α-carbonic anhydrase in *H. pylori* wild-type strain 26695 was increased by about fourfold at low pH (pH 4.5) compared to the level at pH 7.4. However, no significant increase of protein expression of HP1186 α-carbonic anhydrase at pH 4.5 was detected in *H. pylori* mutant strain 26695/HP0165::cat, suggesting that the protein level of HP1186 α-carbonic anhydrase is up-regulated in response to low-pH conditions, and the low-pH-induced protein expression of HP1186 α-carbonic anhydrase depends on ArsRS. The same preparation of membrane proteins was also used for Western blot analysis with a polyclonal antibody (NCL-HPp) against whole *H. pylori* to ensure that similar amounts of protein samples were loaded (Fig. 2B).

Deletion of the ArsR-binding site in the promoter region of the HP1186 α-carbonic anhydrase gene abolishes low-pH induction of HP1186 transcription. In our previous studies, we identified a binding site for the response regulator ArsR in a

region between 95 bp and 145 bp upstream of the translation start codon (ATG) for the HP1186 gene (54). To further verify the role of the ArsR-binding site in low-pH regulation of the HP1186 promoter, we generated a mutant *H. pylori* strain (26695/ΔP$_{HP1186}$) with a deletion of the ArsR-binding site in the promoter region of the HP1186 α-carbonic anhydrase gene and analyzed the transcription of HP1186 in response to low pH by Northern blotting with an HP1186 probe in this strain. As shown in Fig. 3A, no increase in transcription of HP1186 was observed when the mutant 26695/ΔP$_{HP1186}$ was exposed to pH 4.5 for 30 min, which is similar to what was found for the *arsS*-deficient mutants. As a control, *ureI* (HP0071) transcription, which has been shown to be up-regulated by low pH in an ArsRS-dependent manner (31), retained the low-pH induction in the mutant strain 26695/ΔP$_{HP1186}$ (Fig. 3B). This demonstrates that removal of the ArsR-binding site specifically abolishes the low-pH induction of the HP1186 promoter, confirming the role of the in vitro-identified ArsR-binding site.

Analysis of the promoter region of HP1186 (α-carbonic anhydrase) revealed two possible TSPs. While the binding site for the response regulator ArsR has been identified for the HP1186 gene (54), the promoter region of the HP1186 gene has not been systematically studied. To experimentally identify the transcription start point of the HP1186 α-carbonic anhydrase gene, a reverse primer, P1186-3′ (308-327), which is 55 bp downstream of the translation start codon ATG in the ORF of HP1186, was used for 5′-RACE with RNA isolated from the *H. pylori* 26695 strain. Twenty clones of the 5′-RACE product that had been cloned into the pCR4-TOPO vector were randomly picked, sequenced, and compared with the genomic sequence of the HP1186 gene in *H. pylori* strain 26695 (46). Among the 5′-RACE clones examined, about 55% contained a 5′-RACE product of 99 bp in length, indicating a transcription start point (TSP$_1$) at a G nucleotide located 43 bp 5′ of the translation initiation site (unpublished data). However, about 45% of examined clones contain a 66-bp RACE product, suggesting a possible second transcription start point (TSP$_2$) at a G nucleotide located 11 bp 5′ of the ATG start codon (unpublished data).

A −10 consensus sequence (TA<u>G</u>AAT) was found to be present upstream from each of the two separate TSPs (Fig. 4), indicating that the sites are compatible for binding of the *H.*
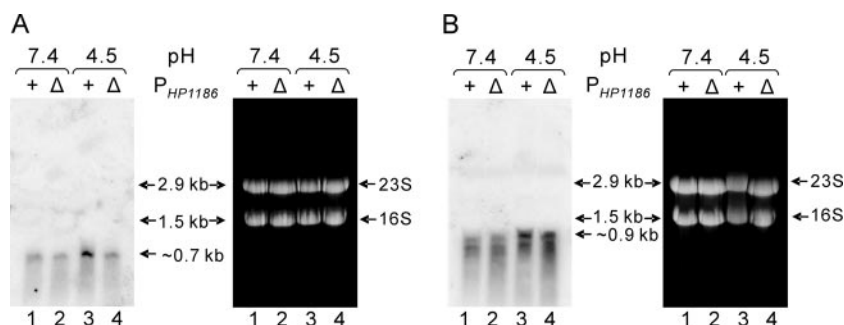

FIG. 3. Deletion of the ArsR-binding site in the promoter region of the HP1186 α-carbonic anhydrase gene abolishes low-pH induction of HP1186 transcription. Total RNAs from wild-type strain 43504 and mutant strain 43504/ΔP$_{HP1186}$, which were exposed to either neutral pH (pH 7.4, lanes 1 and 2) or low pH (pH 4.5, lanes 3 and 4) for 30 min, were analyzed by Northern blotting with HP1186 α-carbonic anhydrase (A) and the HP0071 *ureI* (B) gene-specific probes. The ~0.9-kb band corresponds to the size of the *ureIE* transcript. Ethidium bromide staining of the gel showed that equal amounts of RNA were analyzed. The size standards are the 16S and 23S rRNA species.
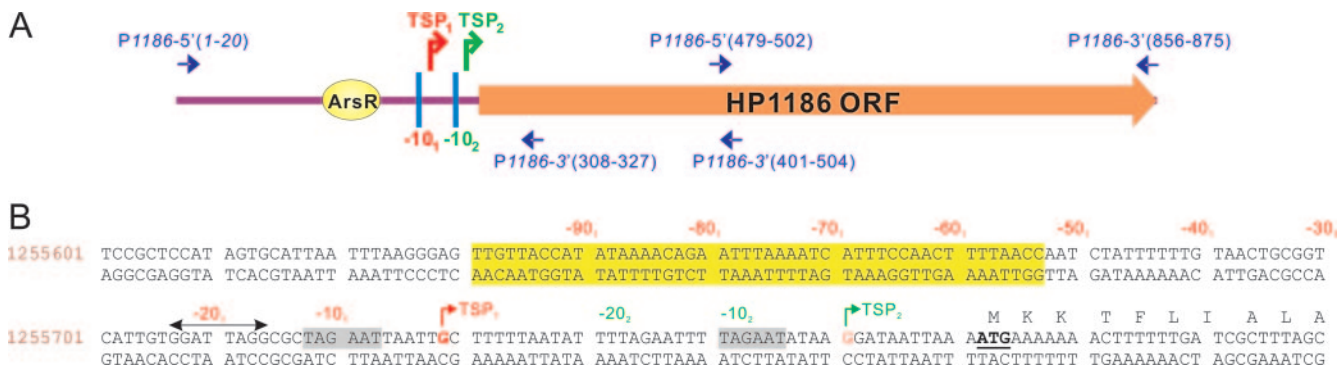
FIG. 4. (A) Schematic representation of the genomic structure of the HP1186 α-carbonic anhydrase gene. The promoter region is shown with two transcription start points (TSP_1 and TSP_2) and their −10 consensus sequences (vertical bars). An HP0166 response regulator binding site is also shown as ArsR. The primers used for preparation of the Northern probe and for 5′-RACE of the HP1186 α-carbonic anhydrase gene are indicated at their corresponding regions of the HP1186 ORF. (B) Genomic sequence of HP1186 α-carbonic anhydrase promoter region. A 200-bp (position 1255601–1255800) 5′-flanking region sequence of the HP1186 α-carbonic anhydrase gene from the *H. pylori* 26695 genome (GenBank accession number NC_000915) (46) is shown. Two transcription start points (TSP_1 and TSP_2) are indicated by bent arrows, and their −10 consensus sequences are highlighted by gray shading. The translational start codon is underlined. The binding site of response regulator ArsR is highlighted in yellow. A mirror repeat (GGATTAGG) is indicated by a double-arrowed line above the double-stranded sequence. Two sets of numbers (in red and green) above the sequence indicate the nucleotide position with respect to the corresponding transcriptional start points (TSP_1 and TSP_2).

pylori σ^{80}. Compared with *E. coli* σ^{70}-recognized promoters (18), this sequence shows one nucleotide mismatch (underlined) in the −10 consensus element sequence (TATAAT). No −35 consensus sequence (TTGACA) (48) with a typical 17 ± 1-bp spacer region resembling that of *E. coli* was identified for either TSP. A mirror repeat (GGATTAGG), possibly representing a site for binding of regulatory elements, was present in the sequence upstream of the −10 region of both TSPs (−15 to −23 of TSP_1 or −47 to −54 of TSP_2).

Our previous EMSA and DNase I footprinting studies (54) had shown that ArsR binds directly to the HP1186 promoter region that is located between −52 and −98 of TSP_1 (Fig. 4). This ArsR-binding region is located immediately upstream of (and may partially overlap with) an AT-rich sequence, possibly a UP element for TSP_1 (between −40 and −60 of TSP_1).

These results suggest that there are two promoters transcribing the HP1186 gene, and both promoters may be regulated by RNA polymerase containing σ^{80}, the *H. pylori* homologue of the vegetative sigma factor σ^{70} from *E. coli*.

Effect of low pH on TSP utilization in the HP1186 promoter. A study by McGowan et al. (25) identified multiple TSPs for several acid-inducible genes in *H. pylori* strain 60190 and suggested the existence of alternative promoters that transduce signals under either neutral or acidic pH conditions in *H. pylori*. In order to investigate the possible relative transcriptional responses from the two identified TSPs of the HP1186 promoter to the low-pH condition that is signaled by the two-component system ArsRS, we analyzed the transcription of HP1186 α-carbonic anhydrase by quantitative primer extension using a reverse primer, P1186-3′ (308-327) and equal amounts (35 μg) of RNA samples from *H. pylori* strains 26695 and 26695/HP0165::cat (13) which had been exposed to either neutral pH (pH 7.4) or low pH (pH 4.5) for 30 min. To ensure correct mapping of the extension products, we cloned and sequenced in parallel the HP1186 promoter region from the chromosomal DNA of *H. pylori* 26695. In agreement with the 5′-RACE results, two extension products were detected from

either the wild-type or the 26695/HP0165::cat RNAs (the transcriptional start sites are unchanged in the HP0165⁻ background), which place the transcription start sites 43 bp and 11 bp, respectively, upstream from the HP1186 translational start codon. As shown in Fig. 5, the TSP_1 (99-bp elongated primer product) was used preferentially at low pH in the wild-type 26695 strain, whereas the low-pH-induced transcription from TSP_1 could not be detected in the histidine kinase ArsS mutant 26695/HP0165::cat. This suggests that the HP1186 promoter with TSP_1 is responsible for pH-dependent regulation signaled by the two-component system ArsSR. The second transcription start point (TSP_2) of the HP1186 promoter (shown as a 66-bp elongated primer product in Fig. 5) appeared unaffected by pH and ArsS, since it was used equally under all pH conditions in both wild-type and 26695/HP0165::cat strains, indicating a possible role in maintaining the basal transcription level of HP1186 α-carbonic anhydrase independently of the acid-responsive signaling system in *H. pylori*.

DISCUSSION

The carbonic anhydrase of the human gastric pathogen *H. pylori* has been studied in some detail because of its possible involvement in acid acclimation that allows colonization and thence virulence (7, 8, 22, 37, 44). These studies have resulted in a large amount of biochemical and physiological data concerning the specific roles of *H. pylori* carbonic anhydrase but have left analysis of the transcriptional regulation of the carbonic anhydrase gene almost untouched. We provide evidence that the HP1186 gene, encoding the periplasmic α-carbonic anhydrase of *H. pylori*, is transcribed by the vegetative sigma factor σ^{80} and regulated by the two-component system ArsSR in response to low-pH conditions.

Evidence demonstrating that ArsRS acts as a transcriptional regulator of α-carbonic anhydrase in response to acidic pH comes from the observation that the mRNA levels of the HP1186 gene were increased about fourfold in wild-type *H.*
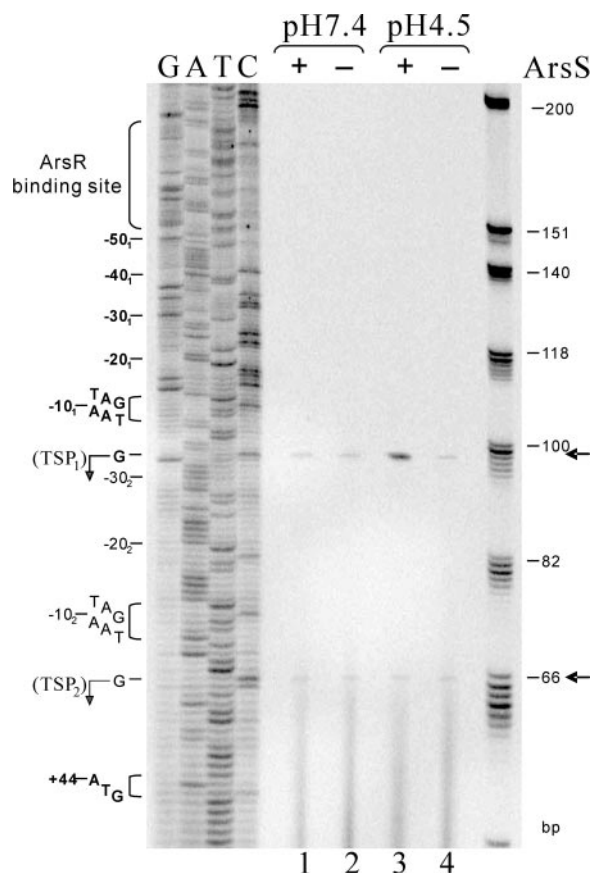
FIG. 5. Primer extension analysis of transcription from the HP1186 α-carbonic anhydrase promoter in *H. pylori* strains exposed to neutral and low pH. Primer extension experiments using the ^{33}P-labeled reverse primer P1186-3′ (308–327) were performed on equal amounts of RNAs isolated from wild-type strain 26695 (lanes 1 and 3) and the ArsS mutant strain 26695/HP0165::cat (lanes 2 and 4), which were exposed to either neutral pH (pH 7.4, lanes 1 and 2) or low pH (pH 4.5, lanes 3 and 4) for 30 min. The same primer used for the primer extension analysis was used for sequencing reactions (lanes G, A, T, and C). The arrows indicate the primer extension products corresponding to TSP_1 and TSP_2. The −10 consensus sequences for each of the TSPs and the nucleotide positions with respect to the corresponding TSPs are given on the left. The binding site for the response regulator ArsR is also marked on the left. The size standards are ^{33}P-labeled ΦX174 HinfI markers provided in the Primer Extension system (Promega).

pylori strains when treated at pH 4.5, whereas no such upregulation of α-carbonic anhydrase mRNA was detected in the ArsS⁻ background (Fig. 1). Western blot analysis of membrane fractions confirmed the regulatory role of ArsS in HP1186 α-carbonic anhydrase expression in response to low-pH conditions (Fig. 2). EMSA and DNase I footprinting experiments with purified HP0166-His₆ (54) showed that the response regulator ArsR protein binds directly to the promoter of the HP1186 gene. When a mutated response regulator, HP0166-D52N, in which the aspartic acid residue at position 52 was replaced with asparagine, was used for EMSA, no binding was observed for the promoter of HP1186 (54), demonstrating that the phosphorylated form of ArsR is necessary for efficient binding of the promoter of HP1186. Consistent with these observations, we also show that deletion of the

ArsR-binding site in the HP1186 promoter region abrogates the acid induction of HP1186 transcription. These data also strongly support the conclusion that the acid-induced activation of the HP1186 promoter is regulated by ArsRS.

The acid-responsive expression of HP1186 α-carbonic anhydrase has been controversial in different studies. Microarray studies by Merrell et al. (26) found a down-regulation of the HP1186 gene at pH 5 after 60 and 120 min of exposure. Other studies (1, 2, 5) did not find a significant change in the expression level of the HP1186 gene in response to low pH. The discrepancies between different studies may be explained by the different bacterial strains used in different studies and the experimental conditions, including the nature of acid exposure, the growth phase, the growth medium used, and the duration of acid exposure, which varied from study to study. Two different *H. pylori* strains (26695 and 43504) have been used in this study, and very similar results were obtained, suggesting that the acid-responsive transcriptional regulation of the HP1186 α-carbonic anhydrase gene is mediated by ArsRS in both 26695 and 43504 strains under our experimental conditions.

Very little is known about the basic processes of gene transcription and transcriptional regulation in *H. pylori*, particularly the regulation of gene expression in response to acid stress. The association of appropriate alternative sigma factors with core RNA polymerase provides a mechanism for cellular responses through redirection of transcription initiation, which leads to the regulated expression of virulence genes and virulence-associated genes in many bacterial pathogens (19). However, an analysis of the *H. pylori* genome performed at The Institute for Genomic Research (46) has shown that this organism probably has only three types of σ factors (σ⁸⁰, σ⁵⁴, and σ²⁸), suggesting that *H. pylori* may use different mechanisms to regulate gene expression in response to environmental changes, such as low pH. Recent studies have shown that the two-component system ArsRS is one of the mechanisms responsible for regulation of many genes in *H. pylori* in response to an acidic environment, which regulates most of the acid acclimation genes identified so far (29-32, 54). In *H. pylori*, the environmental pH is probably sensed directly by the histidines (pK_a, ~5.9) in the periplasmic domain of the histidine kinase sensor protein ArsS, which autophosphorylates in response to pH changes and transfers the phosphoryl group to its cognate response regulator, ArsR. The phosphorylated ArsR may function both as an activator and repressor of pH-responsive target genes by interacting with the promoter regions (30, 32). It is clear from the current study that the phosphorylated ArsR induced by low pH acts as an activator in interacting with one of the promoters of the HP1186 α-carbonic anhydrase gene.

Using 5′-RACE and primer extension assays (Fig. 4 and 5), we found two TSPs for the *H. pylori* HP1186 α-carbonic anhydrase gene, suggesting that two promoters are present for the HP1186 α-carbonic anhydrase gene. The presence of two promoters may allow differential regulation of the gene in response to environmental conditions. The use of multiple promoters therefore provides an efficient means of controlling the expression of a number of genes, particularly when the promoters are differentially utilized. In *Caulobacter crescentus*, two functional promoters (P1 and P2) were found to precede the *dnaK/dnaJ* operon (16). P1 was a heat shock-inducible pro-

moter, with characteristics of a σ^{32} promoter, and P2 was an adjacent σ^{70} promoter. Transcription initiating at the P2 promoter was under temporal control and linked to the onset of DNA replication. McGowan et al. (25) recently identified multiple promoters from several acid-inducible genes in *H. pylori* and found that certain promoters were expressed preferentially under acidic conditions, whereas others either showed maximal expression at pH 7 or showed no differential expression at any pH. These studies provide evidence for pH-dependent promoter regulation in *H. pylori* and demonstrate the existence of alternative promoters that transduce signals under either neutral or acidic pH conditions.

As seen in Fig. 4, the promoters from TSP_1 and TSP_2 are very similar to each other, with an identical -10 element, indicating that both of them may be regulated by RNA polymerase containing σ^{80}, the *H. pylori* homologue of the vegetative sigma factor σ^{70} from *E. coli*. The observation that the promoter from TSP_1 responds strongly in response to low pH even though there is no good -35 sequence for both promoters suggests the involvement of positive transcriptional control by a *trans*-acting factor. The lack of the typical *E. coli* -35 consensus is consistent with previous analysis of transcription in *H. pylori* (4, 14, 25, 28, 43), and this usually provides evidence for a physiological basis for activator function (17). Functioning as an activator, ArsR interacts with a binding site that is located between -52 and -98 of TSP_1 (54), which partially overlaps with a potential UP element (15, 35) found only for TSP_1 (an AT-rich sequence between -40 and -60 of TSP_1). This may lead to different transcription levels of HP1186 α-carbonic anhydrase in response to low-pH conditions.

Similar to class I activators that recruit RNA polymerase via a direct protein-protein interaction (3), it may be that ArsR binds to an upstream location (between -52 and -98) (Fig. 4B) of the HP1186 α-carbonic anhydrase promoter from TSP_1, usually near the carboxy-terminal domain of the RNA polymerase α-subunit (αCTD). This interaction recruits αCTD, and hence the rest of the RNA polymerase, to the promoter (6), and therefore initiates the transcription from TSP_1. Although the location of activator binding at promoters subject to class I activation is variable, because the linker between αCTD and the α-amino-terminal domain is sufficiently flexible to permit the binding of αCTD at different positions, the promoter from TSP_2 appears to be at a location that renders αCTD too distant for ArsR to reach from its binding site (between -84 and -122 of TSP_2) (Fig. 4B).

The discovery of multiple promoters responsible for the expression of HP1186 α-carbonic anhydrase provides new insight regarding the transcriptional organization of the HP1186 α-carbonic anhydrase gene. The presence of the multiple promoters for HP1186 may facilitate differential expression of the HP1186 α-carbonic anhydrase gene in response to environmental pH. Using quantitative primer extension analysis (Fig. 5), we demonstrated that the promoter from TSP_1 is a pH-dependent promoter and is regulated by ArsRS, whereas the promoter from TSP_2 may be involved in control of the basal (or housekeeping) transcription of HP1186 α-carbonic anhydrase or regulated by a different mechanism yet to be identified.

It seems that a basal level of carbonic anhydrase is main-

tained, irrespective of external acidic pH, and this housekeeping function may maintain periplasmic pH within viable limits at around neutral pH between pH 7.0 and 8.0 in the face of basal urease activity. Since *H. pylori* is killed at pH 8.2 (10, 27), bicarbonate generation at pH levels between 7.4 and 8.0 may prevent lethal alkalinization by UreI-independent basal urease activity. However, when faced with an acidic environment, the organism requires higher levels of this periplasmic carbonic anhydrase to maintain its periplasmic pH at levels compatible with not only survival but also growth in the gastric niche it inhabits. With the acid-responsive regulation by the ArsRS two-component system, the combination of urease, UreI, and α-carbonic anhydrase comprise an important component of its acid acclimation mechanisms.

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