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Acid-Induced Activation of the Urease Promoters Is Mediated Directly by the ArsRS Two-Component System of *Helicobacter pylori*

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The nickel-containing enzyme urease is an essential colonization factor of the human gastric pathogen *Helicobacter pylori* which enables the bacteria to survive the low-pH conditions of the stomach. Transcription of the urease genes is positively controlled in response to increasing concentrations of nickel ions and acidic pH. Here we demonstrate that acid-induced transcription of the urease genes is mediated directly by the ArsRS two-component system. Footprint analyses identify binding sites of the phosphorylated ArsR response regulator within the *ureA* and *ureI* promoters. Furthermore, deletion of a distal upstream ArsR binding site of the *ureA* promoter demonstrates its role in acid-dependent activation of the promoter. In addition, acid-induced transcription of the *ureA* gene is unaltered in a *nikR* mutant, providing evidence that pH-responsive regulation and nickel-responsive regulation of the *ureA* promoter are mediated by independent mechanisms involving the ArsR response regulator and the NikR protein.

Helicobacter pylori is the causative agent of chronic type B gastritis and gastric or duodenal ulcers (8, 26). Moreover, infection with *H. pylori* increases the risk of developing gastric malignancies such as adenocarcinoma or mucosa-associated lymphoid tissue lymphoma (34). The ability of the neutralophilic bacterium *H. pylori* to thrive in the low-pH conditions encountered in the human stomach relies on the nickel-containing enzyme urease. Urease, a hexameric heterodimer consisting of the subunits UreA and UreB, hydrolyzes urea, leading to the formation of ammonia and carbon dioxide which buffer the cytoplasm and periplasm of the bacteria (28, 30). The enzymatic activity of urease is controlled by the inner membrane pH-gated channel UreI, which regulates the access of the substrate urea to the bacterial cell in response to acidic pH (29, 41). Both urease and the channel protein UreI proved to be essential for colonization in several animal infection models (16, 18, 24, 31). However, additional mechanisms of pH homeostasis contribute to acid adaptation in *H. pylori* (5, 6).

The urease genes encoding the subunits UreA and UreB, the urea channel UreI, and accessory proteins required for the assembly of nickel into the urease apoenzyme are organized in two transcriptional units comprising *ureAB* and *ureIEFGH*, respectively (1) (Fig. 1). Under standard laboratory growth conditions, basal transcription of the *ureAB* genes is driven by a core promoter consisting of an extended −10 promoter element and a predicted −35 motif of the sequence TTAATC (11). However, transcription of the *ureAB* genes is increased in the presence of Ni^{2+} ions (35), and it was demonstrated that nickel-responsive regulation is mediated by the NikR protein (10, 36), which is a global autoregulator controlling the expression of nickel-activated and -repressed genes, including genes involved in nickel and ferric iron uptake and storage, metal metabolism, motility, and stress responses as well as genes encoding outer membrane proteins (10). Interestingly, NikR represses transcription of the *fur* gene, encoding the iron-responsive repressor protein Fur, which is involved in acid resistance (7, 10, 38). Recently, the binding sites of NikR in the promoters of the *fur* and *ureA* genes have been mapped (13). Global transcriptome analysis revealed that transcription of *ureAB*, *ureIEFGH*, and several genes encoding components of alternative pathways for the production of ammonia is induced by acidic pH (9, 23, 42). Since low pH is thought to increase the solubility and therefore the intracellular availability of Ni^{2+} ions, it was speculated that NikR might act as a master regulator of acid adaptation by directly mediating acid-induced transcription of *ureAB* and by controlling the transcription of other pH-regulated genes via a regulatory cascade involving the Fur repressor (39). However, it was clearly demonstrated that acid induction of transcription of *ureA* requires the HP166-HP165 two-component system (27), which is composed of the essential OmpR-like response regulator HP166 and the nonessential histidine kinase HP165. Since HP166-HP165 was shown to control the transcription of several *H. pylori*-specific genes in response to acidic pH (4, 15, 19, 27), the two-component system has recently been renamed ArsRS (*a*cid *r*esponsive *s*ignaling) (27).

In this study we demonstrate that the ArsRS two-component system directly controls acid-induced transcription from the *ureA* (P_{ureA}) and *ureI* (P_{ureI}) promoters. Through footprint analyses with the phosphorylated ArsR response regulator (ArsR∼P), we identify binding sites of ArsR∼P at the P_{ureA} and P_{ureI} promoters. Furthermore, we provide evidence that pH-responsive regulation and nickel-responsive regulation of the P_{ureA} promoter are mediated by independent mechanisms involving the ArsR response regulator and the NikR protein.

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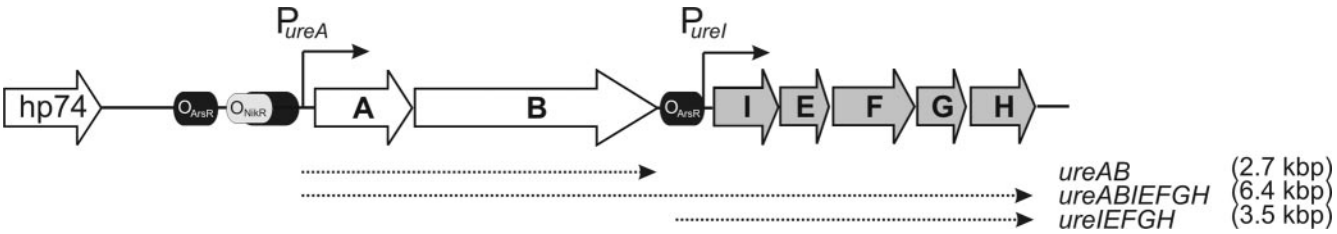

FIG. 1. Schematic representation of the urease operon. The urease genes and the upstream ORF HP0074 (hp74) encoding a lipoprotein signal peptidase (32) are shown, and the directions of transcription of these genes are indicated by arrows. The P_{ureA} and P_{ureI} promoters are marked by thin arrows. The gray oval upstream of P_{ureA} indicates the operator of the NikR regulator mapped by Delany et al. (13). The black ovals indicate the binding sites of the response regulator ArsR. Dotted arrows represent the transcripts whose synthesis is directed by the P_{ureA} and P_{ureI} promoters, respectively, and which are further processed by endonucleolytic cleavage (1). The sizes of these transcripts are indicated on the right. The figure is not drawn to scale.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* G27 and 26695 are clinical isolates (33, 43). *H. pylori* strain G27/HP165::km has been described previously (4). *H. pylori* strains were grown at 37°C under microaerophilic conditions (Oxoid) on Columbia agar plates containing 5% horse blood, 0.2% cyclodextrin, and Dent's or Skirrow's antibiotic supplement. Liquid cultures were grown in brain heart infusion or brucella broth containing Dent's or Skirrow's antibiotic supplement and 10% fetal calf serum (FCS). When required, blood agar plates or liquid broth was supplemented with chloramphenicol or kanamycin at a final concentration of 20 μg/ml. Acid exposure experiments were performed as follows. Bacteria from a liquid culture were harvested at an optical density at 590 nm of 0.8 by centrifugation and were resuspended in brain heart infusion broth containing FCS and antibiotic supplement with the pH adjusted to 5.0 with hydrochloric acid. Incubation at 37°C under microaerophilic conditions was continued for 1 hour. To study the Ni^{2+}-responsive induction of *ureA* transcription, *H. pylori* was grown in brucella broth containing Dent's or Skirrow's antibiotic supplement, 10% FCS, and 100 μM $NiCl_2$ to an optical density at 590 nm of 0.8. *Escherichia coli* DH5α was grown in Luria-Bertani broth. When necessary, antibiotics were added to the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 30 μg/ml.

Construction of *H. pylori* strains with deletions within the P_{ureA} promoter. Two constructs were generated, pSLΔPureA and pSLparΔPureA, to replace regions within the P_{ureA} promoter ranging from position +7 to −176 or −88 to −176, respectively, with a chloramphenicol resistance cassette. Numbering is with respect to the transcriptional start site of the *ureA* gene (32). To construct suicide plasmid pSLΔPureA, a 520-bp EcoRI-BamHI fragment, comprising 407 bp of the 3′ region of open reading frame (ORF) HP0074 and 124 bp of the intergenic region between HP0074 and *ureA*, and a 483-bp PstI-SacI fragment, comprising 50 bp of the upstream region of *ureA* and 433 bp of the 5′ region of *ureA*, were PCR amplified from chromosomal DNA of *H. pylori* 26695 with primer pairs hp74-5/hp74-3 and ureA-5/ureA-3 (Table 1), respectively, and were subsequently cloned into pSL1180 vector DNA (Amersham-Pharmacia). The resulting plasmid was linearized by digestion with BamHI and PstI, and a chloramphenicol resistance cassette from *Campylobacter coli* (40) was inserted in a way that the transcriptional direction of the *cat* gene is divergent from *ureA*, yielding plasmid pSLΔPureA. pSLparΔPureA is a derivative of pSLΔPureA in which the 483-bp PstI-SacI fragment was replaced by a 577-bp PstI-SacI fragment comprising 144 bp of the upstream region of *ureA* and 433 bp of the 5′ region of *ureA*, which was amplified with primer pair ureA-5p/ureA-3. Natural transformation of the *H. pylori* strains G27 and G27/HP165::km with the suicide plasmid pSLΔPureA and transformation of G27 with pSLparΔPureA were performed as described previously (3). Chromosomal DNA of the resulting chloramphenicol-resistant transformants was checked for the correct allelic replacement event by PCR with primers flanking the integration site. This resulted in the generation of strains G27/ΔP_{ureA}, G27/Δ*arsS*ΔP_{ureA}, and G27/parΔP_{ureA}, respectively.

TABLE 1. Oligonucleotides used in this study

Name	Sequence (5′ to 3′)[a]	Site[b]	Strand	Position[c]
ureAPE	CATAGTGGAGCATCAAC		+	77911–77927
ureIPE	ACATATAACAATACAAGTCC		+	75309–75328
Aprom-5	gcataggaattcTAGAGCACTACCTTGACATGG	EcoRI	−	78262–78242
Aprom-3	cattttggatccATCAACTTGTCTAACTCTTTTGG	BamHI	+	77922–77944
ureAHyp-5	CAUCAUCAUGATGAAACTCACCCCAAAAGA		−	77957–77937
ureAHyp-3	CUACUACUATTACTCCTTAATTGTTTTTAC		+	77240–77260
Iprom-5	agaagaggatccGCTTGAAAGACAAGTGTTGCC	BamHI	−	75713–75693
Iprom-3	aaaacagaattcACATATAACAATACAAGTCC	EcoRI	+	75309–75328
ureI-5	ctcttaggatccGAAGTAACTTCTAAACCAGCC	BamHI	−	75586–75566
ureI-3	ttttacgaattcATAGAAACTAGTCAAATGGTGGCG	EcoRI	+	75107–75129
hp74-5	ttaatggagctcGGATCAAGCGATTAAATACGC	SacI	−	78710–78690
hp74-3	taattgctgcagAAAATTGGAGTGATAATGGTGG	PstI	+	78190–78211
ureA-5	tacatcggatccTGATTTCGTTATGTCTTCAAGG	BamHI	−	78006–77985
ureA-5p	tagcgcggatccGATATAACACTAATTCATTTTAAATA	BamHI	−	78100–78075
ureA-3	ttcaaggaattcAAGTGTGAGCCGATTTGAACC	EcoRI	+	77523–77543
NKTB-F	attcagggatccCGGTTGTCTAATTCGTC	BamHI	+	1398691–1398707
NKTB-R	cggatgaattcCTCCTTGTCTATGATAAAAC	EcoRI	−	1399093–1399074
Nko-F	attcagggatccGACTAAGGCGTCTAGCTTTG	BamHI	−	1398360–1398341
Nko-R	attcagccatggACTAGCCCTAATCGCGCTAG	NcoI	+	1397831–1397850
16SHyp-5	GCTAAGAGATCAGCCTATGTCC		−	1208876–1208855
16SHyp-3	TGGCAATCAGCGTCAGGTAATG		+	1208356–1208377

[a] Sequences in uppercase letters are derived from the genome sequences of *H. pylori* 26695 (33). Sequences introduced for cloning purposes are given in lowercase letters, and restriction recognition sequences are underlined.
[b] Restriction recognition site.
[c] Nucleotide positions refer to the genome sequence of *H. pylori* 26695 (33).

RNA isolation, primer extension, and RNA slot blot analysis. *H. pylori* RNA was isolated from bacteria grown to the logarithmic phase in liquid broth by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Primer extension analysis was performed essentially as described previously (27), using 0.5 pmol of γ-^{32}P-end-labeled oligonucleotides ureAPE and ureIPE. Plasmid pSL-ureI, which was used as template DNA in the sequencing reactions performed with primer ureIPE, contains a 480-bp BamHI-EcoRI fragment comprising the promoter region and part of the 5′ region of the *ureI* gene, which was PCR amplified with primers ureI-5/ureI-3. Primer extension experiments were performed three times with independently prepared RNAs. Quantification of the signals from the primer extension products was performed using a Typhoon 9200 variable-mode imager (Amersham Biosciences) and ImageMaster TotalLab software (Amersham Biosciences). RNA slot blot analysis was performed as follows. RNA (20 μg) was denatured in 1× MOPS (morpholinepropanesulfonic acid) buffer containing 50% formamide and 6% formaldehyde. The samples were incubated at 65°C for 5 min and cooled on ice before addition of 1 volume of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The denatured samples were filtered through a positively charged nylon membrane (Hybond N$^+$; Amersham), using a Bio-Dot chamber (Bio-Rad). After UV cross-linking, the nylon membrane was prehybridized for 1 h at 42°C in hybridization buffer (ECL gold hybridization buffer; Amersham). The PCR products used as hybridization probes were nonradioactively labeled using the ECL direct nucleic acid labeling system (Amersham) according to the manufacturer's instructions. The labeled probes were added to the hybridization solution, and hybridization was performed for 12 to 16 h at 42°. The membrane was washed two times in prewarmed (42°C) wash solution I (6 M urea, 0.5× SSC, 0.4% sodium dodecyl sulfate) for 20 min at 42°C and two times in wash solution II (250 ml 2× SSC) at room temperature. For signal detection, the ECL detection system (ECL direct nucleic acid labeling and detection system; Amersham) and X-ray films (Konica Minolta) were used.

DNase I footprint analysis. The recombinant N-terminally His_6-tagged response regulator ArsR encoded on plasmid pQE-166 was overexpressed in *E. coli* M15 and was purified by affinity chromatography on Ni^{2+}-nitrilotriacetic acid agarose essentially as described previously (4). In vitro phosphorylation of His_6-ArsR was performed as described by Dietz et al. (15). Plasmids pSL-ureAProm and pSL-ureIProm, used for the generation of end-labeled DNA probes for DNase I footprint experiments, were constructed by cloning a 339-bp EcoRI-BamHI fragment derived from the upstream region of *ureA* and a 414-bp BamHI-EcoRI fragment derived from the upstream region of *ureI*, respectively, into pSL1180. The cloned DNA fragments were PCR amplified from chromosomal DNA of *H. pylori* 26695 with primer pairs Aprom-5/Aprom-3 and Iprom-5/Iprom-3, respectively. The promoter DNA fragments were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase at one extremity and gel purified, and approximately 100,000 cpm of each probe was used for footprint experiments, which were performed essentially as described by Delany et al. (12). The binding reactions were performed for 20 min at room temperature in 50 μl binding buffer (50 mM Tris-HCl, pH 7.9, 40 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM dithiothreitol).

Construction of *H. pylori* G27/*nikR*::km. G27/*nikR*::km was constructed through allelic replacement by transformation of the G27 wild-type strain with plasmid pNko::Km, which was generated as follows. An upstream flanking region consisting of a 406-bp BamHI-EcoRI fragment amplified from chromosomal DNA of *H. pylori* G27 with primers NKTB-F/NKTB-R, including the *nikR-exbB* intergenic region and 87 bp and 58 bp of the 5′ regions of the respective genes, was cloned into pGemT (Promega), generating the pNKTB plasmid. A downstream flanking region was then amplified from G27 chromosomal DNA with primers Nko-F/Nko-R, generating a 529-bp NcoI-BamHI fragment, including 30 bp of the 3′ end of the *nikR* gene, and cloned into the pNKTB plasmid. A kanamycin cassette of *C. coli* (21) was then cloned between the flanking regions, generating the pNko:Km suicide plasmid. Chromosomal DNA of the transformants was checked by PCR with primers external and internal to the replacement site to verify the desired double-crossover event. A transformant with the correct double-crossover PCR profile was analyzed by Western blotting with a polyclonal mouse antiserum raised against the purified recombinant NikR protein (13), which confirmed the lack of expression of the NikR protein.

RESULTS

Acid-induced transcription from the P_{ureI} promoter requires the ArsRS two-component system. We have reported recently that the increase of *ureAB* transcription observed at acidic pH is largely dependent on the presence of the histidine kinase ArsS (27). To test whether acid-induced transcription of *ureI* and its downstream genes, which was observed in the global transcriptome studies performed by Merrell et al. (23) and Wen et al. (42), is also controlled by the ArsRS two-component system, we investigated the expression of *ureI* in the *H. pylori* G27 wild-type strain and the isogenic *arsS* deletion mutant G27/HP165::km which had been exposed to pH 5.0 for 1 hour. As shown in Fig. 2A, two major elongated primer products could be detected when a primer extension experiment with a *ureI*-specific primer was performed on RNA which was extracted from *H. pylori* G27. The shorter product (band 1) is derived from a transcript starting at position −65 with respect to the translational start codon of *ureI*. The nucleotide sequence upstream revealed a −10 promoter element (TATGAT). This is in agreement with the P_{ureI} promoter proposed by Akada et al. (1). The longer product (band 2) corresponds to a *ureI*-specific transcript starting at position −119 with respect to the start codon of *ureI*. This putative transcriptional start site is not preceded by a reasonable −10 promoter element. Both *ureI*-specific transcripts were synthesized to a greater extent when *H. pylori* G27 was cultivated at low pH, indicating acid induction, which was estimated as approximately 2.5-fold for the shorter transcript. However, acid exposure did not increase the synthesis of these transcripts in G27/HP165::km, indicating a role of the ArsRS two-component system in the pH regulation of *ureIEFGH*, as was observed for *ureAB*.

To investigate whether band 2 corresponds to a transcript whose synthesis is driven by an alternative promoter of *ureIEFGH* or represents a specific cleavage product of a readthrough transcript directed from the P_{ureA} promoter, the *H. pylori* strains G27/ΔP_{ureA} and G27/Δ*arsS*ΔP_{ureA} were constructed. In these strains, which are derived from the G27 wild-type strain and the isogenic *arsS* deletion mutant G27/HP165::km, respectively, the intergenic region between *ureA* and the upstream ORF HP0074 comprising the P_{ureA} promoter was largely replaced by a chloramphenicol resistance cassette which is transcribed in the opposite direction to *ureA*. Integration of the chloramphenicol cassette resulted in the deletion of the region ranging from position +7 to −176 with respect to the transcriptional start site of *ureA* (32). When primer extension experiments with a *ureI*-specific oligonucleotide were performed on RNAs extracted from G27/ΔP_{ureA} and G27/Δ*arsS*ΔP_{ureA}, product 2 could no longer be detected, indicating that the corresponding mRNA is dependent on P_{ureA} and may represent a processing product of the full-length *ureABIEFGH* readthrough transcript (Fig. 2B). In strain G27/ΔP_{ureA}, acid-induced transcription from P_{ureI} was estimated to be increased about fivefold compared to that in the G27 wild-type strain grown at pH 5.0, while pH-dependent transcription of *ureI* was not observed in strain G27/Δ*arsS*ΔP_{ureA}, demonstrating again that the histidine kinase ArsS is required for the pH regulation of P_{ureI}. In conclusion, the ArsRS two-component system controls acid-induced transcription from the P_{ureI} promoter as well as from the P_{ureA} promoter (27).

ArsR binds to the P_{ureA} and P_{ureI} promoters. To investigate whether the ArsRS two-component system participates directly or indirectly in the pH-dependent regulation of the urease genes, DNase I footprint experiments with the purified response regulator protein ArsR were performed. Figure 3

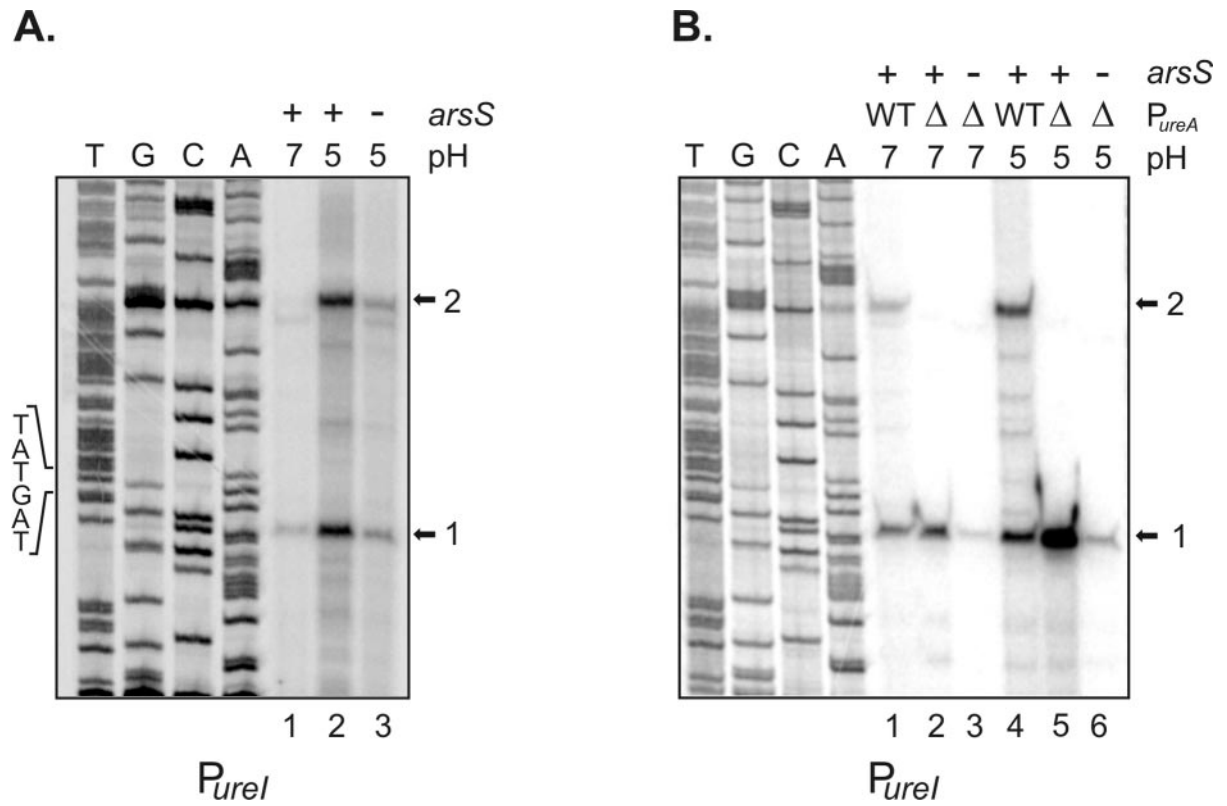

FIG. 2. Analysis of transcription from the P_{ureI} promoter in *H. pylori* strains grown at neutral pH and exposed to pH 5.0. A. Primer extension experiments using the radiolabeled oligonucleotide ureIPE were performed on equal amounts of RNAs extracted from *H. pylori* G27 grown at neutral pH (lane 1) and from strains G27 and G27/HP165::km which were exposed to pH 5.0 for 60 min (lanes 2 and 3, respectively). The elongated primer products 1 and 2 are indicated by arrows. The sequence of the −10 element of the P_{ureI} promoter is given on the left. The sequencing ladders (lanes T, G, C, and A) were obtained by annealing primer ureIPE to plasmid pSL-ureI. B. Primer extension experiments with oligonucleotide ureIPE were performed on equal amounts of RNAs extracted from *H. pylori* G27 (lanes 1 and 4), G27/ΔP_{ureA} (lanes 2 and 5), and G27/Δ*arsS*ΔP_{ureA} (lanes 3 and 6) grown at neutral pH or exposed to pH 5.0 for 60 min. WT, wild type.

shows the results of footprint experiments carried out with a 339-bp radioactively labeled P_{ureA} promoter probe. In the presence of 1.5 μM ArsR which was phosphorylated in vitro with acetylphosphate (ArsR∼P), a region of 54 bp spanning from position −21 to −74 with respect to the transcriptional start site of *ureA* was protected from DNase I digestion. At higher concentrations of ArsR∼P (4.5 μM), a second protected region appeared, spanning from position −105 to −139, and bands with enhanced DNase I sensitivity centered around position −91 became visible. When the same experiments were performed with unphosphorylated ArsR, no binding of the response regulator to the P_{ureA} promoter probe could be detected (data not shown). Therefore, ArsR binds to the P_{ureA} promoter at two distinct sites exclusively in its phosphorylated state.

Footprint analysis with ArsR∼P on a 414-bp DNA probe containing the P_{ureI} promoter was also performed, and the results are shown in Fig. 4. When 3.0 μM in vitro-phosphorylated ArsR∼P was added to the probe, a region spanning from position −3 to −50 with respect to the transcriptional start site of *ureI* was protected from DNase I digestion. At higher protein concentrations (>6.0 μM), the protected region extended up to position +9. Again no protection was observed when unphosphorylated ArsR was included in the reaction mixture (data not shown). Therefore, ArsR∼P binds directly to the P_{ureI} promoter, which was clearly shown to be positively regulated by ArsS in a pH-dependent manner (Fig. 2).

From the results of the footprint experiments, we conclude that pH regulation of the urease genes is mediated by the ArsRS two-component system through direct binding of ArsR∼P to the P_{ureA} and P_{ureI} promoter regions.

Deletion of the upstream binding site of ArsR in the P_{ureA} promoter abrogates pH regulation of *ureA* transcription. In the promoters of pH-responsive target genes of ArsR which have been characterized so far, the binding site of ArsR consists of a 26-bp sequence motif located at a distance of 9 bp upstream of the −10 promoter element (15). In the footprint experiments shown in Fig. 3, we have identified two ArsR binding sites within the P_{ureA} promoter overlapping and upstream of the core promoter element, respectively. To confirm the role of the distal upstream binding site of ArsR∼P in acid regulation of P_{ureA}, we generated a mutant strain (G27/parΔP_{ureA}) harboring a deletion in the P_{ureA} promoter ranging from position −89 to −176 and analyzed transcription of *ureA* in this strain, which retains the downstream ArsR binding site as well as the recently mapped NikR operator of P_{ureA} (13) (Fig. 3B). As shown in Fig. 5, no increase in transcription of *ureA* was observed when the mutant G27/parΔP_{ureA} was ex-

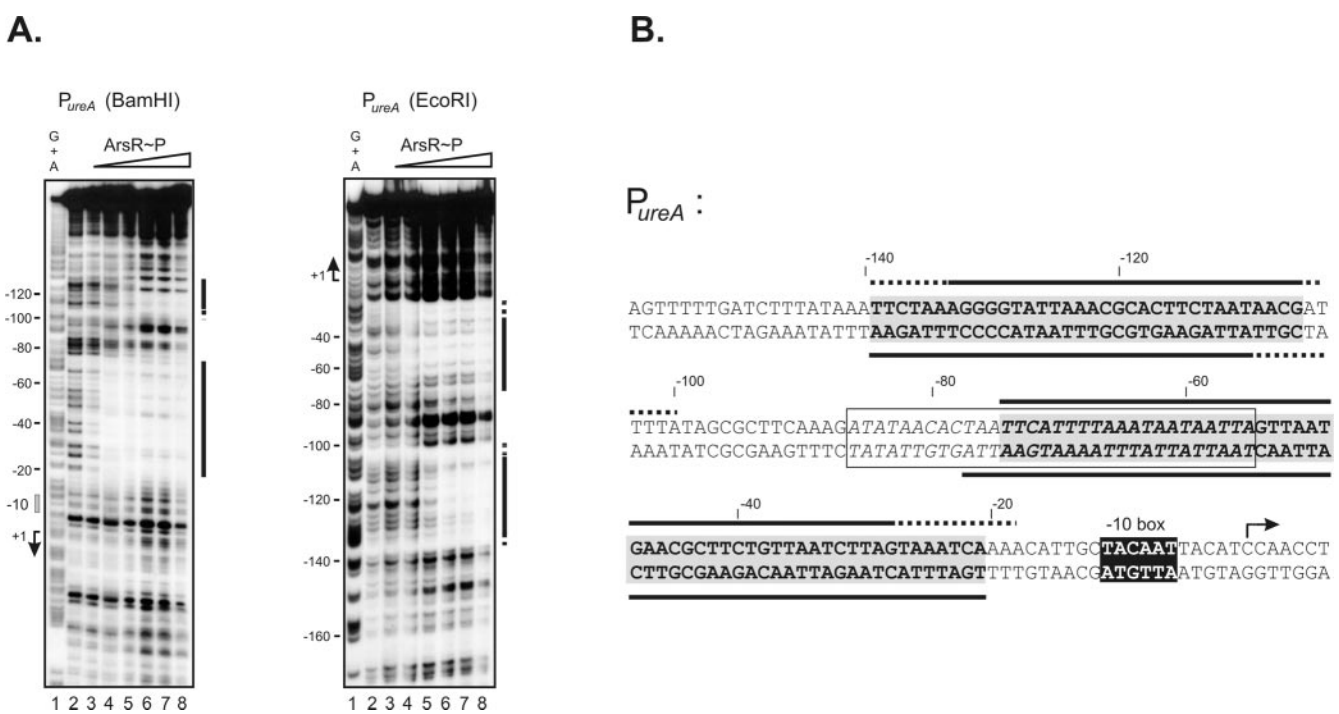

FIG. 3. Binding of ArsR~P to the P_{ureA} promoter. A. DNase I footprint experiments were performed on a 339-bp EcoRI-BamHI fragment containing the P_{ureA} promoter which was end labeled at the BamHI and EcoRI termini, respectively, by adding increasing amounts of His_6-ArsR phosphorylated in vitro by acetylphosphate. In lanes 2 to 8, His_6-ArsR is present at concentrations of 0, 0.75, 1.5, 3.0, 4.5, 6.0, and 7.5 μM, respectively. The numbers on the left indicate nucleotide positions with respect to the transcriptional start site, which is marked by an arrow. The open bar indicates the position of the −10 promoter element. The solid and broken bars on the right indicate the minimum and maximum regions of DNase I protection, respectively. Lane 1 contained a G+A sequence reaction mixture with the DNA probe used as a size marker (22). B. Schematic representation of the P_{ureA} promoter. The −10 promoter element is highlighted by black shading, and the transcriptional start site is indicated by an arrow above the double-stranded sequence. Black bars below and above the sequence indicate the minimum (solid lines) and maximum (dashed lines) regions protected from DNase I digestion by the binding of ArsR~P to the P_{ureA} promoter probe labeled at the BamHI and EcoRI termini, respectively. The overlapping regions which were clearly protected on both probes are highlighted by gray shading. The NikR binding site mapped by Delany et al. (13) is boxed, and the respective sequence motif is in italics. Numbers above the sequence indicate the nucleotide position with respect to the transcriptional start site (+1).

posed to pH 5. This demonstrates that removal of the upstream distal ArsR binding site abrogates acid induction of P_{ureA}, verifying the role of the in vitro-identified ArsR binding sites. Furthermore, as the NikR operator is retained in this promoter mutant, regulation of transcription in response to acidity appears to be dependent only on the ArsRS two-component system.

Acid-induced transcription of *ureA* is independent of the metal-responsive regulator NikR. To investigate the possible role of NikR in acid response regulation of P_{ureA}, hypothesized by van Vliet and coworkers (39), we constructed a *nikR* deletion mutant of *H. pylori* G27 and analyzed the pH- and metal-dependent regulation of the urease genes. The growth of the parent strain G27 in liquid culture was unaffected by the presence of Ni^{2+} at concentrations ranging from 100 to 500 μM. In contrast, strain G27/*nikR*::km showed reduced growth in the presence of 100 μM Ni^{2+}, and no growth was observed when the broth was supplemented with Ni^{2+} at concentrations of 250 μM or higher. As expected, in strain G27/*nikR*::km expression of urease was no longer induced in response to supplementation of the culture broth with 100 μM Ni^{2+} (Fig. 6A), as had been observed with similar mutants of *H. pylori* 26695 and SS1 (10, 36). Importantly, an increase of transcription was observed in G27/*nikR*::km exposed to pH 5.0 (Fig. 6B, lane 4 versus lane 2), which was comparable to the increase observed in the wild-type strain (lane 3 versus lane 1). This demonstrates that acid-induced transcription of P_{ureA} is independent of NikR. To delineate the role of ArsRS in nickel-dependent regulation of *ureA*, we investigated transcription of *ureA* in the G27 wild-type strain and G27/HP165::km when the strains were cultivated in the presence of 100 μM Ni^{2+}. As shown in Fig. 6C, Ni^{2+} induction could be detected in the *arsS* deletion mutant. From these results, we conclude that pH- and Ni^{2+}-dependent transcription of *ureA* represent different and independent regulatory mechanisms mediated by the ArsRS two-component system and the NikR protein, respectively.

DISCUSSION

Due to the rather high basal level of urease expression at pH 7.0, resulting in urease concentrations approaching 10% of the total cellular protein content (2), it was initially assumed that transcription of the urease genes is constitutive. However, it is well established now that transcription of *ureABIEFGH* is positively regulated in response to acidic pH (9, 23, 27, 42) and that transcription of *ureAB* is induced in the presence of ele-

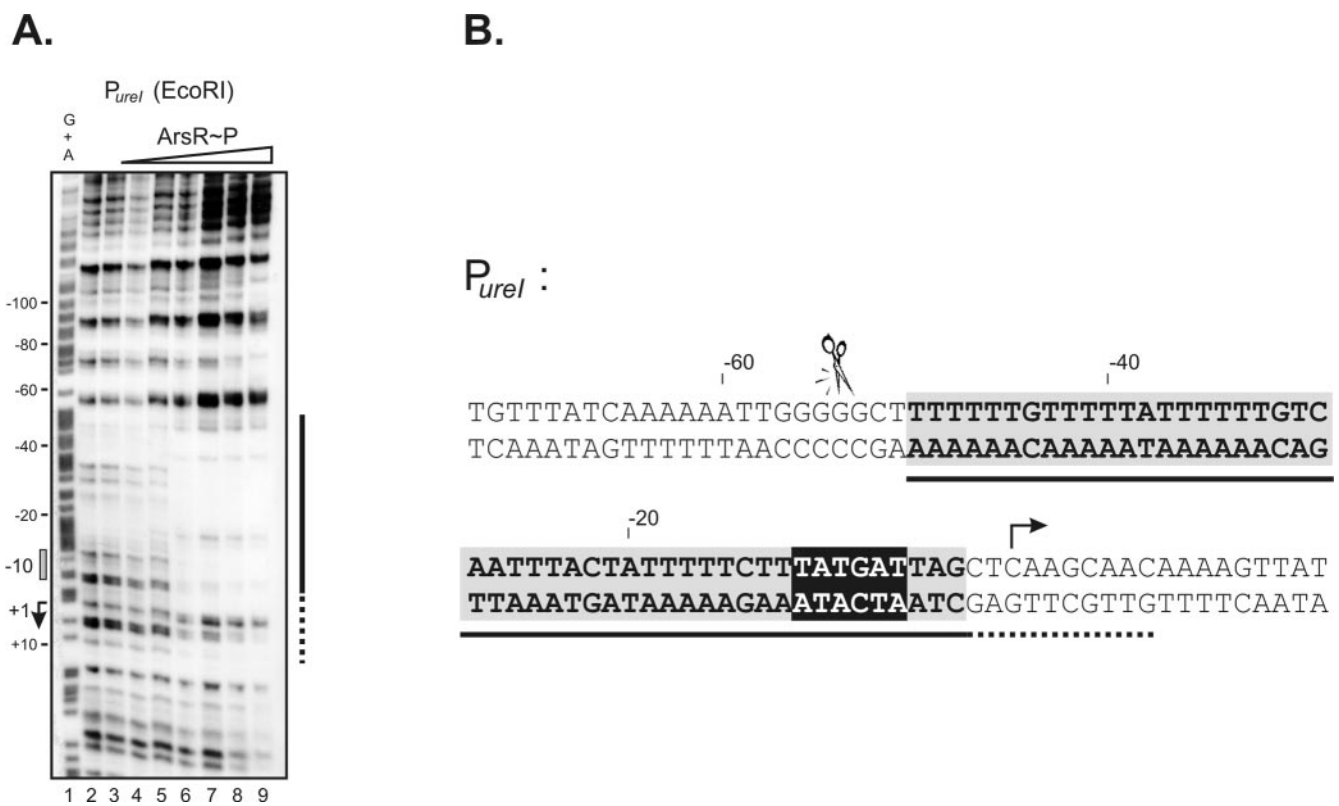

FIG. 4. Binding of ArsR~P to the P$_{ureI}$ promoter. A. A DNase I footprint experiment was performed on a 414-bp BamHI-EcoRI fragment containing the P$_{ureI}$ promoter which was end labeled at the EcoRI terminus. In lanes 2 to 9, His_6-ArsR which was phosphorylated in vitro by acetylphosphate is present at concentrations of 0, 0.37, 0.75, 1.5, 3.0, 4.5, 6.0, and 7.5 μM, respectively. The numbers on the left indicate nucleotide positions with respect to the transcriptional start site, which is marked by an arrow. The open bar indicates the position of the −10 promoter element. The solid and broken bars on the right indicate the minimum and maximum regions of DNase I protection, respectively. Lane 1 contained a G+A sequence reaction mixture with the DNA probe used as a size marker (22). B. Schematic representation of the P$_{ureI}$ promoter. The −10 promoter element is highlighted by black shading, and the transcriptional start site is indicated by an arrow above the double-stranded sequence. The black bars below the sequence indicate the minimum (solid lines) and maximum (dashed lines) regions protected from DNase I digestion by the binding of ArsR~P to the P$_{ureI}$ promoter probe. The minimum region of protection is also highlighted by gray shading. Numbers above the sequence indicate the nucleotide position with respect to the transcriptional start site (+1). The scissors above the sequence indicate the 5′ end of the *ureI*-specific transcript observed in the primer extension experiments whose synthesis is directed by the P$_{ureA}$ promoter.

vated concentrations of Ni^{2+} ions (35). As recently reported for the pH-dependent regulation of *ureAB* (27), we show here that acid-induced transcription of *ureIEFGH* is mediated by the ArsRS two-component system. The *ureIEFGH* operon is transcribed by the P$_{ureI}$ promoter located in the short intergenic region between *ureB* and *ureI* (1) (Fig. 2). However, we reproducibly detected significant amounts of an additional *ureI*-specific transcript whose synthesis is directed by the P$_{ureA}$ promoter (Fig, 2). Therefore, two promoters, P$_{ureA}$ and P$_{ureI}$, control the expression of the *ureIEFGH* genes at the transcriptional level, and both are controlled by ArsRS in response to acidic pH. The presence of a *ureABIEFGH* readthrough transcript which is mostly endonucleolytically cleaved to give rise to a *ureABI* mRNA of 3.4 kbp was already observed by Akada et al. (1). Those authors also detected three short *ureI*-specific mRNAs ranging in size from 0.8 to 1.1 kbp which were expected to be derived from a *ureIEFGH* transcript by endonucleolytic cleavage within the region encoding UreE. Since the 5′ end of the *ureI*-specific mRNA detected in our primer extension experiments is located only 54 bp upstream of the transcriptional initiation site of the *ureI* gene, it is likely that this transcript represents one of the mRNA molecules observed in the Northern hybridizations performed by Akada et al. (1). Therefore, we hypothesize that an additional endonucleolytic cleavage site involved in the processing of the *ureABIEFGH* mRNA is located in the intergenic region between *ureB* and *ureI*.

DNase I footprint analysis demonstrated that the ArsRS two-component system mediates pH-dependent regulation of P$_{ureA}$ and P$_{ureI}$, directly through binding of the phosphorylated response regulator ArsR~P to the respective promoter regions (Fig. 3 and 4). At neutral pH the basal level of transcription of P$_{ureI}$, as in the case of P$_{ureA}$ (27), is largely unaffected in the absence of ArsS and, consequently, ArsR~P (data not shown). Furthermore, removal of the distal upstream ArsR binding site of P$_{ureA}$ had no effect on the basal level of transcription at neutral pH. Consequently, the ArsRS two-component system is required for activation of transcription only under acidic conditions. Acid-induced transcription from P$_{ureI}$ is more pronounced when the P$_{ureA}$ promoter is deleted (Fig. 2B). Currently this observation remains unexplained, but it might reflect a titration effect caused by the competition of response

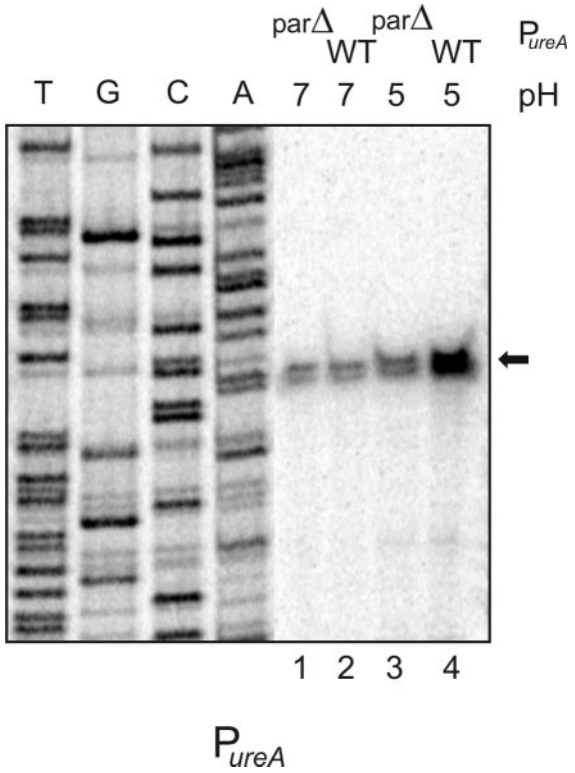

FIG. 5. Analysis of *ureA* expression in *H. pylori* strains G27 and G27/parΔP_{ureA} by primer extension analysis. Primer extension experiments using the radiolabeled oligonucleotide ureAPE were performed on equal amounts of RNAs extracted from *H. pylori* G27 grown at neutral pH (lane 2) and pH 5.0 (lane 4) and from strain G27/parΔP_{ureA} grown at neutral pH (lane 1) and at pH 5.0 (lane 3). The arrow on the right indicates the position of the cDNA corresponding to the *ureA*-specific transcript. The sequencing ladders (lanes T, G, C, and A) were obtained by annealing primer ureAPE to plasmid pSLparΔPureA.

regulator binding sites of different affinity for a limited pool of ArsR~P molecules, or it might be due to changes in DNA topology of the *ureI* promoter region favoring transcription from P_{ureI} which are caused by the deletion of the intergenic region between *ureA* and ORF HP0074.

The binding sites mapped for ArsR in the P_{ureA} and P_{ureI} promoters show certain peculiarities with respect to ArsR binding sites which were identified in the promoters of other pH-regulated genes. In the promoters of ORFs HP01408 and HP0119, the binding sites of ArsR consist of a well-conserved 26-bp motif located at a distance of 9 bp upstream of the −10 promoter element (15). Furthermore, a 20-bp ArsR binding site mapping downstream of the P_{arsR} promoter, where ArsR~P is likely to act as a repressor, showed some conservation of this motif (15). In both the P_{ureA} and the P_{ureI} promoters, extended ArsR binding sites were mapped which do not exhibit clear similarity either with the 26-bp ArsR binding motif identified previously or with each other. Nonetheless, deletion of the upstream binding site of ArsR within P_{ureA} abrogated pH-dependent regulation of the *ureAB* genes, indicating a role for this binding site in the acid response activation of transcription in vivo. While the ArsR binding site in other positively pH-regulated promoters overlaps the −35 promoter region, the binding site mapped in the P_{ureI} promoter also covers the −10 promoter element. Despite this atypical position of the binding site, ArsR clearly acts as an activator of transcription of *ureI* (Fig. 2B), suggesting that ArsR~P is repositioned by the binding of RNA polymerase. The observation that the binding site of a transcriptional activator overlaps with the −10 promoter element in in vitro DNA binding experiments is not unprecedented, since, for example, Kinnear et al. (20) reported a similar pattern for the binding of the response regulator BvgA to the promoter of the pertactin gene. Interestingly, no binding of the unphosphorylated ArsR protein to the P_{ureA} and P_{ureI} promoters could be observed in the footprint experiments, while it bound to the P_{1408} and P_{119}

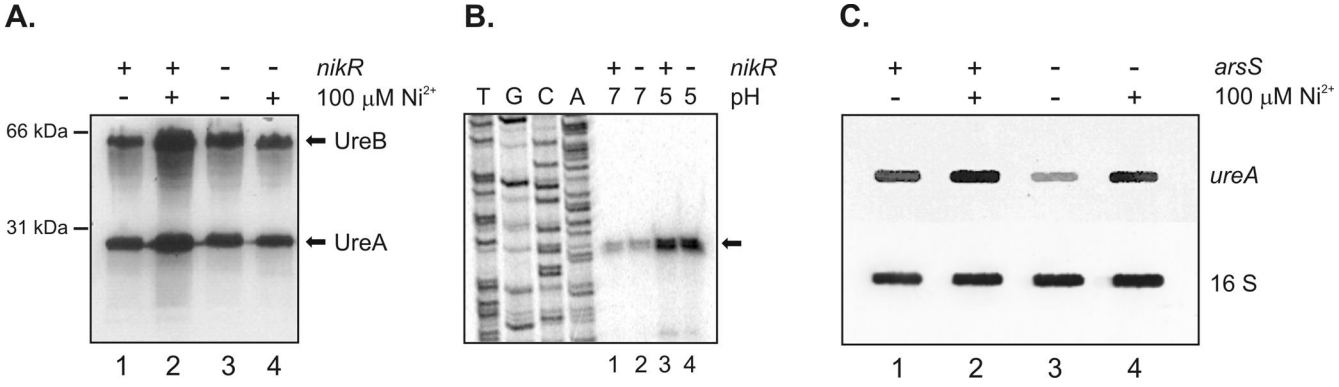

FIG. 6. Analysis of the expression of urease in *H. pylori* strains G27, G27/*nikR*::km and G27/HP165::km. A. Immunoblot analysis of equal amounts of whole-cell protein prepared from *H. pylori* G27 grown in brucella broth (lane 1) or brucella broth supplemented with 100 μM $NiCl_2$ (lane 2) and from G27/*nikR*::km grown in brucella broth (lane 3) or brucella broth supplemented with 100 μM $NiCl_2$ (lane 4) with a polyclonal antiserum directed against *H. pylori* urease. The UreB and UreA proteins are indicated on the right, and the positions of molecular mass markers are given on the left. B. Primer extension analysis using the radiolabeled oligonucleotide ureAPE was performed on equal amounts of RNAs extracted from *H. pylori* G27 grown at neutral pH (lane 1) and exposed to pH 5.0 (lane 3) and from G27/*nikR*::km grown at neutral pH (lane 2) and exposed to pH 5.0 (lane 4). The arrow on the right indicates the position of the cDNA corresponding to the *ureA*-specific transcript. C. Slot blot Northern hybridization performed with RNAs extracted from *H. pylori* G27 grown in brucella broth (lane 1) or brucella broth supplemented with 100 μM $NiCl_2$ (lane 2) and from G27/HP165::km grown in brucella broth (lane 3) or brucella broth supplemented with 100 μM $NiCl_2$ (lane 4). Hybridization was performed with *ureA*- and 16S rRNA-specific probes as indicated on the right.

promoters at elevated concentrations (>4.5 mM) (15), suggesting a lower affinity of the urease promoters for ArsR. This difference in affinity might be reflected in a different kinetics of acid induction, since Merrell et al. (23) observed by global transcriptome analysis that transcription from the P_{119} promoter is strongly induced after 30 min of acid exposure and declines at later time points, while the amount of *ureAB* transcript is maximal 90 min after the shift to low pH.

In addition to pH-dependent regulation, transcription of *ureAB* is also positively regulated in response to increasing concentrations of Ni^{2+} ions (35), while Ni^{2+}-dependent regulation of *ureIEFGH* has not been reported. The transcriptional response of *ureAB* to Ni^{2+} is mediated by the NikR protein. In contrast to the orthologous repressor protein from *E. coli*, NikR from *H. pylori* has been reported to be a pleiotropic regulator (10, 14, 35). Recently, a high-affinity binding site of NikR was mapped in the P_{ureA} promoter, and it partially overlaps with the downstream binding site of ArsR identified in this study (13). van Vliet and colleagues inferred that NikR might act as a master regulator of acid adaptation, responding to the increased bioavailability of Ni^{2+} ions under acidic conditions (39). This was based on the observations that (i) the ferric uptake regulator protein Fur is involved in urease-independent mechanisms of acid resistance in *H. pylori*, including expression of the amidase AmiE (7, 17, 37); (ii) the Fur and NikR regulons partially overlap, with NikR acting as a repressor of Fur transcription (10, 13, 38); and (iii) urease transcription is positively controlled by NikR (36). We show here that acid induction of *ureAB* transcription is independent of the NikR protein and relies instead on the ArsS two-component pH sensor. In fact, the *nikR* deletion mutant showed a pH-responsive *ureA* transcription profile identical to that of the G27 wild-type strain (Fig. 6B), while deletion of *arsS* abrogates pH regulation of *ureA* and *ureI* almost completely (27). This implication is also corroborated by the observation that the acid-induced increase in urease activity does not differ significantly between *H. pylori* 26695 and an isogenic *nikR* deletion mutant (10, 38). Although pH-induced transcription of *ureA* was identical in the wild type and the *nikR* mutant, the slight increase in *ureA* transcription observed when the *arsS* mutant was exposed to pH 5.0 (27) might be attributed to the NikR protein being activated by the higher availability of Ni^{2+} at low pH, since in the *arsS* knockout strain NikR does not have to compete with ArsR~P for binding to the P_{ureA} promoter. Otherwise, an additional transcriptional activator is likely to interact with the P_{ureA} promoter at low pH. Ni^{2+}-dependent transcription of *ureA* at pH 7.0 was virtually unaltered in the *arsS* deletion mutant (Fig. 6C), demonstrating that ArsRS is not involved in the basal expression of NikR and supporting the conclusion that pH-dependent transcription and Ni^{2+}-dependent transcription of *ureA* are mediated by independent regulatory mechanisms involving transcriptional activators competing for the interaction with partially overlapping binding sites and responding to different environmental stimuli.

van Vliet et al. reported acid-induced transcription of the *nikR* gene (38), which was, however, not detected in transcriptome analyses studying the global pH response (9, 23, 42). Therefore, it is tempting to speculate that pH control of *nikR* transcription might be mediated by the ArsRS two-component system, which is currently being tested in our laboratory. It is well established now that the acid-adaptive response of *H. pylori* is controlled in an intricate way involving at least the transcriptional regulators ArsR, NikR, and Fur (9, 27, 38). However, based on the observation that an *H. pylori* mutant lacking the *arsS* gene is completely unable to establish an infection in the mouse stomach (25), while mutants deficient in either NikR, Fur, or both metal-dependent regulators are still able to colonize mice (albeit with a clearly reduced efficiency compared to the wild-type strain) (9), it can be hypothesized that the ArsRS two-component system is the prominent player in the control of acid resistance genes during infection.

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