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Involvement of the HP0165-HP0166 Two-Component System in Expression of Some Acidic-pH-Upregulated Genes of *Helicobacter pylori*†

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About 200 genes of the gastric pathogen *Helicobacter pylori* increase expression at medium pHs of 6.2, 5.5, and 4.5, an increase that is abolished or much reduced by the buffering action of urease. Genes up-regulated by a low pH include the two-component system HP0165-HP0166, suggesting a role in the regulation of some of the pH-sensitive genes. To identify targets of HP0165-HP0166, the promoter regions of genes up-regulated by a low pH were grouped based on sequence similarity. Probes for promoter sequences representing each group were subjected to electrophoretic mobility shift assays (EMSA) with recombinant HP0166-His$_6$ or a mutated response regulator, HP0166-D52N-His$_6$, that can specifically determine the role of phosphorylation of HP0166 in binding (including a control EMSA with in-vitro-phosphorylated HP0166-His$_6$). Nineteen of 45 promoter-regulatory regions were found to interact with HP0166-His$_6$. Seven promoters for genes encoding α-carbonic anhydrase, *omp11*, *fecD*, *lpp20*, *hypA*, and two with unknown function (*p*HP1397-1396 and *p*HP0654-0675) were clustered in gene group A, which may respond to changes in the periplasmic pH at a constant cytoplasmic pH and showed phosphorylation-dependent binding in EMSA with HP0166-D52N-His$_6$. Twelve promoters were clustered in groups B and C whose up-regulation likely also depends on a reduction of the cytoplasmic pH at a medium pH of 5.5 or 4.5. Most of the target promoters in groups B and C showed phosphorylation-dependent binding with HP0166-D52N-His$_6$, but promoters for *ompR* (p*HP0166-0162*), p*HP0682-0681*, and p*HP1288-1289* showed phosphorylation-independent binding. These findings, combined with DNase I footprinting, suggest that HP0165-0166 is an acid-responsive signaling system affecting the expression of pH-sensitive genes. Regulation of these genes responds either to a decrease in the periplasmic pH alone (HP0165 dependent) or also to a decrease in the cytoplasmic pH (HP0165 independent).

Helicobacter pylori is the only neutralophilic bacterium that colonizes the human stomach. Infection by this pathogen is primarily responsible for peptic ulcer disease and mucosa-associated lymphoid tissue maltoma and is associated with an increased incidence of gastric cancer (9, 16, 27, 28). The organism is unique among bacterial pathogens in that it can colonize the acidic environment of the human stomach. To be able to inhabit the stomach, *H. pylori* has developed a number of acid resistance and what we have termed acid acclimation mechanisms. Acid acclimation is defined as the ability of *H. pylori* to maintain a nearly neutral periplasmic pH and a high enough cytoplasmic pH in the presence of an acidic environment that allows *H. pylori* to colonize its gastric niche. This term, acid acclimation, distinguishes the response of *H. pylori* to acid from the more common acid resistance and tolerance mechanisms described in a variety of neutralophiles such as *Vibrio cholerae* or pathogenic strains of *Escherichia coli* that survive exposure to high acidity during transit through the gastric lumen but cannot inhabit the stomach (15).

Both acid resistance and acclimation require the function of a number of proteins whose expression is increased in response to the acidic environment via a signal transduction system.

Among the genes essential for acid acclimation and gastric colonization are those of the urease gene cluster (11, 13, 25, 42). This cluster consists of the genes encoding the urease apoprotein, *ureA* and *ureB* (32, 37, 38); a pH-gated urea transporter, *ureI* (31, 36, 44); and four Ni^+ insertion genes, *ureE*, *ureF*, *ureG*, and *ureH* (43). In addition, the periplasmic α-carbonic anhydrase is also important for acid survival in vitro (22) and, along with the cytoplasmic β-carbonic anhydrase, is required for infection of the mouse stomach (G. E. Ball, S. Bury-Mone, H. De Reuse, A. Labigne, G. L. Mendz, and J.-M. Thiberge, 105th Gen. Meet. Am. Soc. Microbiol., abstr. B-142, 2005). The fact that these pH homeostatic genes are essential for animal models of stomach colonization shows that a low pH is indeed present in the ecological niche of *H. pylori*.

The limited environment of the human stomach is the unique habitat of *H. pylori* and explains the observation that this organism has a very limited number of regulatory genes (6, 41). Containing only three histidine kinases and five response regulators involved in transcriptional regulation (1, 41), *H. pylori* is one of the organisms with the smallest number of two-component systems whose genome sequences have been characterized, although only a few target genes, which are regulated by two-component systems in

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H. pylori, have been identified (10, 14, 29, 30). All of the specific environmental stimuli sensed by these two-component systems have not been identified, but given gastric acidity with a median diurnal pH of 1.4 and a pH of ~1.0 in the absence of food and a pH of ~5.0 during the digestive phase (3, 7, 33, 34), the environmental pH is likely to be the major challenge encountered, and indeed *H. pylori* requires acid to survive in the presence of urea (8). *H. pylori* is found adhering to the gastric surface and in the gastric mucus (17, 19). Although the pH of the surface of the gastric epithelium, which is the niche colonized by *H. pylori*, is thought to be less acidic than the gastric lumen, it appears that after the initial colonization phase, which requires transit of the stomach lumen, *H. pylori* still must encounter an acidic pH in its habitat as a consequence of changes in the luminal pH. Hence, there are likely to be times when *H. pylori* is exposed to a highly acidic pH environment and it is thought to colonize a niche in the stomach at a specific pH (20).

In a search for other genes involved in acid acclimation, recent microarray analyses (24, 45) have demonstrated that the expression of a large number of genes (~200) is up-regulated in response to an acidic pH. Among these are the genes for one of the two-component systems, HP0165-HP0166 (45), and several genes belonging to the regulon controlled by HP0165-HP0166 were shown to be acid regulated (24). The expression of several genes is altered when the medium pH is lowered to 6.2. The periplasmic domain of HP0165 contains several histidines which, with a pK_a of ~6.0, would change protonation state as the periplasm fell from pH 7.4 to 6.2 or below. Histidine is the only amino acid with a pK_a in this range, and protonation of one or more of the seven probable exoplasmic histidines as the periplasmic pH falls may be responsible for activation of the HP0165 signaling with this small change in the periplasmic pH. Recently, evidence has been presented that increased transcription at an acidic pH from the promoters controlling the expression of open reading frames (ORFs) *HP0119* and *HP1432*, which belong to the HP0165-HP0166 regulon, is strictly dependent on the presence of HP0165 (29). Another study has also suggested that the HP0165-HP0166 two-component system directly controls acid-induced transcription from the *ureA* and *ureI* promoters (30). Only a few target genes regulated by the HP0165-HP0166 two-component system in *H. pylori* have been identified (10, 14, 29, 30). However, the studies described above imply that the HP0165-HP0166 two-component system can contribute to regulation of some of the ~200 genes that are upregulated by acid.

The purpose of the present study was to identify some of the target genes for the two-component system HP0165-HP0166 present in the group of acidic-pH-regulated genes discovered by microarray profiling by electrophoretic mobility shift assay (EMSA) and DNase I footprinting. In addition, the requirement for phosphorylation of HP0166 was investigated with a mutated response regulator (HP0166-D52N-His_6) in which the aspartate phosphorylation site is removed. Here we distinguish between the target genes that depend on the histidine kinase activity of HP0165 (and thence phosphorylation of HP0166) and those genes that, although regulated by HP0166, are less dependent on the kinase activity of HP0165. This distinction may depend on regulation only by acidification of the periplasm or by additional acidification of the cytoplasm, both being normalized by intrabacterial urease activity.

MATERIALS AND METHODS

Construction of HP0166-His_6 expression plasmid. The DNA fragment coding for response regulator HP0166 was amplified by PCR with genomic DNA from *H. pylori* strain 26695 as the template and primers HP0166-5′P (174431; 5′-CAC CATGATAGAAGTTTTAATGATAGAAG-3′) and HP0166-3′P (173775; 5′-ATATCAGTATTCTAATTTATAACCAATCC-3′). The 5′ primer HP0166-5′P (174431) contained a four-nucleotide sequence (CACC, underlined) immediately 5′ to the start codon (ATG) to facilitate directional cloning into the pET100/D-TOPO vector (Invitrogen, Carlsbad, CA), which allows expression of recombinant HP0166 with an N-terminal tag containing the Xpress epitope and a six-His tag that enables further purification. The nucleotide sequences of the cloned PCR products were verified by sequencing of both strands.

Overproduction and purification of HP0166-His_6. HP0166-His_6 recombinant protein was expressed in *E. coli* BL21 Star(DE3) containing plasmid pET100/D-TOPO-HP0166. Bacteria were grown in 50 ml of LB broth at 37°C to an optical density at 600 nm of 0.5. Subsequently, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM, and incubation was continued for 2 h. Cells were harvested and suspended in 8 ml of native binding buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM Tris-HCl, pH 8.0) with 10 mM imidazole and 1 mg/ml lysozyme. After incubation on ice for 30 min, the bacteria were further disrupted by sonication. Cell debris was pelleted by centrifugation, and 8 ml of the supernatant was added to the purification column containing 1.5 ml of 50% Ni-nitrilotriacetic acid resin (Invitrogen, Carlsbad, CA) and incubated for 3 h at room temperature on an orbital shaker. The column was washed four times with 8 ml of native wash buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM Tris-HCl, pH 8.0, 20 mM imidazole). His-tagged proteins were eluted with 8 ml of native elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM Tris-HCl, pH 8.0, 250 mM imidazole). An aliquot (10 μl) from each eluted fraction was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the fractions with purified proteins were concentrated and desalted with Amicon Ultra-4 centrifugal filter devices (Millipore, Bedford, MA).

Mutagenesis and HP0166-D52N-His_6 purification. A mutated expression construct for HP0166-D52N-His_6 was generated on the construct pET100/D-TOPO-HP0166 with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Sense and antisense oligonucleotides were designed to contain the mutation that replaces the single nucleotide T with A in codon 52 (GAT) of the wild-type *HP0166* gene sequence coding for aspartic acid, which results in a mutated codon (GAA) coding for asparagine. The entire HP0166-coding region of the mutant construct was sequenced from both directions to ensure that it contained only the desired mutation. The mutated construct was expressed in *E. coli* BL21 Star(DE3), and HP0166-D52N-His_6 was purified as described above.

In vitro phosphorylation of HP0166-His_6. A sample of recombinant HP0166-His_6 was phosphorylated in vitro in a buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ 25 mM KCl, 1 mM dithiothreitol, and 10 mM carbamoyl phosphate over a period of 60 min at 25°C.

Probe preparation by PCR. To prepare the probes for EMSA, we performed PCR by using *H. pylori* strain 26695 chromosomal DNA as a template and the primer pairs (Table 1) that will generate DNA fragments corresponding to the promoter-regulatory regions of the selected genes. The PCR products with correct size were gel purified with a MinElute gel extraction kit (QIAGEN, Valencia, CA).

EMSAs. DNA fragments corresponding to the promoter-regulatory regions of different genes were generated by PCR and 5′ end labeled radioactively by using T4 polynucleotide kinase (Promega, Madison, WI) with [γ-^{33}P]ATP (50 μCi). Binding of HP0166-His_6 to DNA was carried out in a 10-μl reaction mixture containing 10^4 cpm of ^{33}P-labeled DNA, 1 μg of poly(dI-dC) (Sigma, St. Louis, MO), 25 mM $NaPO_4$ (pH 7), 150 mM NaCl, 0.1 mM $MgSO_4$, and 1 mM dithiothreitol. The DNA binding reaction was initiated by the addition of HP0166-His_6 (or HP0166-D52N-His_6), and the mixture was incubated at room temperature for 20 min. Radiolabeled, PCR-generated probes corresponding to the promoter-regulatory regions for the low-pH-up-regulated genes were incubated with increasing amounts of purified proteins. Cold competitor chase experiments with a 50-fold excess of unlabeled probe as a specific competitor were used to demonstrate the specificity of HP0166 binding. Samples were then loaded directly onto a 6% DNA retardation polyacrylamide gel (Invitrogen, Carlsbad, CA). Electrophoresis was carried out for 1 h at room temperature (14 V/cm), and the gels were then dried and analyzed by autoradiography. Each EMSA experiment was repeated at least three times.

DNase I footprinting. DNA fragments used for DNase I footprinting were prepared by PCRs with the *Pfu* polymerase (Stratagene, La Jolla, CA) and 20 pmol of each primer, one of which was previously labeled with T4 polynucleotide kinase and [γ-^{33}P]ATP. Labeled PCR products were purified with the QIAquick PCR

TABLE 1. Oligonucleotide primers used for promoter fragment preparation by PCR

Primer	Sequence (5′→3′)[a]	Position[b]	Primer	Sequence (5′→3′)[a]	Position[b]
p*HP0082_5′*	TCTCTATTTCTTTTTGCCTCTCCTT	−155 to −120	p*HP0875-0874_5′*	CAACAACTCTCCTTAATCTAA	−321 to −301
p*HP0082_3′*	CATTTGCCCTTTTAAAACGA	−3 to −22	p*HP0875-0874_3′*	TTCCTTTTATGATCTTAAATC	−6 to −26
p*HP0181-0185_5′*	GGCTAAATTTTGAAACCTTG	−257 to −238	p*HP0019-0020_5′*	TTTCAAAACCCCCACCAAAGGAA	−107 to −85
p*HP0181-0185_3′*	CGCATAAAGATAGTCATAGC	−58 to −77	p*HP0019-0020_3′*	GACTTCCCCTTAATTTAGGT	−1 to −20
p*HP0194-0196_5′*	AAACTGCTCCTAAAAACTCA	−212 to −193	p*HP0305-0308_5′*	TTTTCTGTATCCAAGGTTAG	−241 to −222
p*HP0194-0196_3′*	GATCCTTATTTTATAGGTGG	−6 to −25	p*HP0305-0308_3′*	TCCTTTAATGTGGTCTTATC	−9 to −28
p*HP0243-0235_5′*	TTTGGATAACTATCCCCCTT	−205 to −186	p*HP0166-0162_5′*	AAAACGATCAAAGGAATTGT	−235 to −216
p*HP0243-0235_3′*	AAAATTGTTTTAGGCTTTTATTTTC	−16 to −40	p*HP0166-0162_3′*	CAATTAACTCCTTCAATGAT	−5 to −24
p*HP0294_5′*	CTATAAAAACAGAGCGGCTA	−192 to −173	p*HP0711-0713_5′*	GCGTGAAATAAATTGATAAA	−189 to −160
p*HP0294_3′*	TACAAACCCTCTACTTGAAT	−31 to −50	p*HP0711-0713_3′*	ACTTTCCTTTTTATACTATT	−2 to −21
p*HP0399-0391_5′*	TTTTCTTTAAAAAAACCGCTAATTG	−106 to −81	p*HP0025_5′*	TTTACTAACCGCATGCAAAC	−509 to −480
p*HP0399-0391_3′*	GCCTTACTATAAAATAAATATAAAA	−5 to −29	p*HP0025_3′*	CCGCAAGCTTTCGTTAATATAGTAT	−79 to −103
p*HP0437_5′*	AACTCCTTATCTTTATCTTTTAT	−65 to −43	p*HP0051-0047_5′*	AGCATTGAAAAATTAAGGGAGC	−434 to −413
p*HP0437_3′*	CTTACTTCTGTTGGTTGAAATAT	+54 to +32	p*HP0051-0047_3′*	CGTTGAAAAATCATCGGTTT	−29 to −48
p*HP0472_5′*	GAACTCCCTTTGATTAAATG	−224 to −205	p*HP1559-1558_5′*	TTTTGGTTTTTGAATAGGCGTTCTC	−193 to −169
p*HP0472_3′*	CCTTCTTGTTGAATTAAAGTTA	−7 to −28	p*HP1559-1558_3′*	CCTTTTTCAAACCCTTGCAA	−10 to −29
p*HP0601_5′*	ATTATAGCCCATTTTCATGC	−134 to −115	p*HP0193_5′*	ACGATCCTTATTTTATAGGTGG	−211 to −189
p*HP0601_3′*	TGTTGTAACTCCTTGTTATAAA	−1 to −22	p*HP0193_3′*	CAAAATTAACCTAAAATCGC	−7 to −26
p*HP0682-0681_5′*	TTTGTTTGAATAATGAAAAA	−153 to −134	p*HP0876_5′*	AATCTTCGTAATATGACACTAAGCC	−300 to −277
p*HP0682-0681_3′*	TTGATAAGAATATTTATACC	−2 to −21	p*HP0876_3′*	CCAACAACTCTCCTTAATCT	−1 to −20
p*HP0723_5′*	TAACCCTTTTTAAATTTATTTTAA	−128 to −105	p*HP0588-0595_5′*	AAGGCATGCCTTTACAAATGGA	−474 to −453
p*HP0723_3′*	CTGCCCCTTCAAACAAAATA	−4 to −23	p*HP0588-0595_3′*	TGCATTTAAAGCCCTAGCAA	−24 to −43
p*HP0813-0826_5′*	GATTTTCCTTTCATCAAACT	−132 to −113	p*HP0692-0694_5′*	TTCTATTAAAACCCTGAAACTAGGC	−279 to −255
p*HP0813-0826_3′*	ACTCCAAACTCCCTTCAAAC	−1 to −20	p*HP0692-0694_3′*	CACCCTAAACGGTTGGATTTTATTT	−54 to −78
p*HP0871-0866_5′*	GATAATCGTCCTTATTGTGG	−351 to −332	p*HP1067-1074_5′*	TTACTTGTAAAATCCTTGTCT	−290 to −270
p*HP0871-0866_3′*	GCAAACCCTCTTGTTATTTT	−33 to −52	p*HP1067-1074_3′*	CTTCTCCTTTTAAGATTGCA	−5 to −24
p*HP0896_5′*	CCAATCCTTTAAACCACACC	−193 to −174	p*HP1399_5′*	GTTAATCCTTTAAATAATAGTGTC	−201 to −177
p*HP0896_3′*	TTTTCTCCTTGAAATTAAAA	−1 to −21	p*HP1399_3′*	AAATCCCCTCTAATGTTCTA	−3 to −34
p*HP0990_5′*	CGGTTGGGTAATTCACTCCA	−93 to −74	p*HP1582-1584_5′*	TTTTTATTGGATTTAGTATT	−126 to −107
p*HP0990_3′*	TTCTCCCCACGCTCTACCAC	−1 to −20	p*HP1582-1584_3′*	ACTATCCTTTATTTTATAAT	−2 to −21
p*HP1186_5′*	TTAAATCTTAAGGGGTTGAA	−271 to −252	p*HP0890-0888_5′*	TGTTTCTCCTTAATTAAAATT	−217 to −97
p*HP1186_3′*	TAATTCTAGCGCCTAATCCA	−46 to −65	p*HP0890-0888_3′*	ACGCACACTCCAACCCAATC	−3 to −23
p*HP1326-1329_5′*	GCTAATCCTTTGAAATTTGA	−174 to −155	p*HP0520_5′*	GATCCTTTTACAATTTGAGC	−171 to −152
p*HP1326-1329_3′*	CGTTTTGACCTTTACATTAAG	−1 to −21	p*HP0520_3′*	GCATTACCTCCATACATAAT	−2 to −21
p*HP1397-1396_5′*	CGAGCTTTAAAAAGAGCATG	−112 to −93	p*HP0546-0539_5′*	AATGCAACGCTAAAGATGGT	−533 to −513
p*HP1397-1396_3′*	ACCCCTTATATTGTTTAGGA	−5 to −24	p*HP0546-0539_3′*	GCGTTTCCTTTCAAATTGAA	−1 to −20
p*HP1399_5′*	GTTAATCCTTTAAATAATAGTGTC	−201 to −78	p*HP1457-1454_5′*	GGTATCATAAAGGGCGTTAT	−367 to −348
p*HP1399_3′*	AAATCCCCTCTAATGTTCTA	−14 to −33	p*HP1457-1454_3′*	TGATAGAAACTCCCTATTCA	−3 to −22
p*HP1408-1412_5′*	GACAGAGTTGTTGTAGTTTTAC	−637 to −616	p*HP0654-0675_5′*	AGTATCTCCTTTGTGTTGGTT	−296 to −276
p*HP1408-1412_3′*	TAGTGGTATTCAATCAATGG	−13 to −33	p*HP0654-0675_3′*	GTTAAATTGCCCTTTCGTTT	−2 to −21
p*HP0071-0067_5′*	TATTTTTTAGGAGCAACGCT	−192 to −173	p*HP1440_5′*	AAACCTTTGCGATAAGCTTT	−120 to −101
p*HP0071-0067_3′*	GCCTTTTCCTTCCAAACAAA	−2 to −21	p*HP1440_3′*	TATCCAACTTACCGCTTAAA	−1 to −20
p*HP1288-1289_5′*	ACACTCCTAAAATCCAATTC	−519 to −500	p*HP0252-0259_5′*	AAATAAAATTAAGCGAGCAT	−160 to −141
p*HP1288-1289_3′*	CCACAACCCTATTAAAAGCA	−19 to −38	p*HP0252-0259_3′*	AAAAATATCCTTTTTGCTTT	−1 to −20
p*HP0524-0530_5′*	GAAGCTCCTTTCAAGAATTA	−365 to −346	p*HP0786-0788_5′*	TATTCAAACCTTAAAACAATC	−147 to −127
p*HP0524-0530_3′*	GCGACAGCTTTATTGTTTAA	−1 to −20	p*HP0786-0788_3′*	TAGTAATAACCTTATTAAGA	−3 to −22
p*HP0485_5′*	TCCTCTTCCTTACTTTACCT	−282 to −266	p*HP1241-1240_3′*	GGTTTTCTCCTTTAATTTTG	−119 to −100
p*HP0485_3′*	TCTTGATCCTTATTATTATTTT	−1 to −22	p*HP1241-1240_3′*	GGGTATTCCTTTTTGTTGTT	−1 to −20

[a] The nucleotide sequences are derived from the genome sequences of *H. pylori* 26696.
[b] The nucleotide positions are the positions in relation to the translational start codon of the first ORF in each operon.

purification kit (QIAGEN, Valencia, CA). HP0166 binding to DNA was performed as described above with the addition of bovine serum albumin (0.1 μg). The footprinting reactions were performed in a 50-μl volume, and concentrations of $MgCl_2$ and $CaCl_2$ were adjusted to 1 mM and 0.5 mM, respectively, before the addition of 0.06 U of DNase I (Invitrogen, Carlsbad, CA). After incubation for 1 min at room temperature, the reaction was stopped by adding 3 volumes of stop buffer (20 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.8% SDS, 0.1 μg/μl sonicated calf thymus DNA), followed by proteinase K (25 μg/μl) digestion at 50°C for 1 h. DNA fragments were ethanol precipitated, and an equivalent number of counts per minute (5×10^4) from each reaction mixture was loaded onto 6% polyacrylamide–7 M urea sequencing gels. Sequencing reactions with a Sequenase version 2.0 PCR product sequencing kit (USB, Cleveland, OH) were carried out on the appropriate PCR products and the same ^{33}P-labeled primers that were used for labeling of the probes and loaded alongside the DNase I footprinting reaction mixtures. Gels were dried and analyzed by autoradiography.

Microarray analysis of *H. pylori* under acidic conditions. The microarray method was used previously (45) to survey the general transcriptional activity of the whole genome in *H. pylori* maintained at neutral pH (pH 7.4) compared to a gradually decreasing external pH (pH 6.2, pH 5.5, and pH 4.5) in the absence and presence of 5 mM urea to mimic conditions in the natural environment of the organism. The bacterial strains and growth conditions and the methods used for microarray preparation, hybridization, array quantification, and data processing, as well as measurement of intrabacterial pH, have been previously described (45). To identify patterns of low-pH-regulated gene expression, we performed an average-linking hierarchical cluster analysis on those genes that were up-regulated more than twofold by the medium pH (pH_{out}) at 6.2, 5.5, and 4.5. Cluster and Treeview (12) were used to select, group, and visualize genes whose expression varied across the samples. We extracted tables (rows of genes, columns of individual microarray hybridizations) of normalized fluorescence ratios from the database. The subsets of genes that are significantly regulated by a low pH (those ratios with values showing a change of more than or equal to twofold and having a 95% confidence interval as determined by the *t* test were considered significantly regulated genes) were selected from the 1,534 ORFs on the array. Before clustering and display, the logarithm of the measured fluorescence ratios for each gene was centered by subtracting the arithmetic mean of all ratios measured for that gene. Then a hierarchical clustering algorithm was applied to the genes under each experimental condition with the Pearson correlation coefficient as the measure of similarity and average linkage clustering (12).

RESULTS

Identification of promoter sequences for acid-regulated genes. In order to identify the promoter sequences, each of the ~200

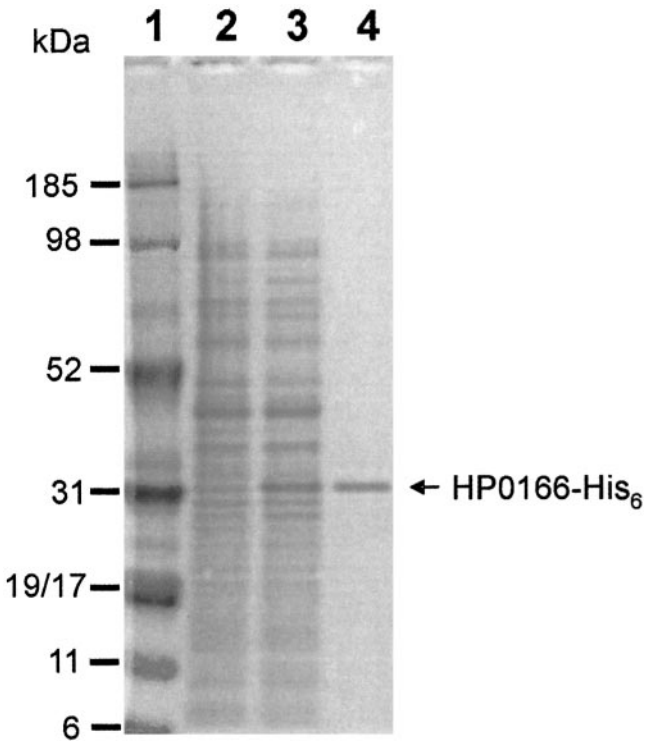

FIG. 1. Overproduction and purification of recombinant HP0166-His_6. SDS-PAGE analysis of crude extracts from *E. coli* BL21 Star(DE3) carrying pET100/D-TOPO-HP0166 without IPTG induction (lane 2) and with 2 h of IPTG induction (lane 3) and purified HP0166-His_6 protein after Ni-nitrilotriacetic acid affinity chromatography (lane 4). Molecular weight standards were loaded in lane 1.

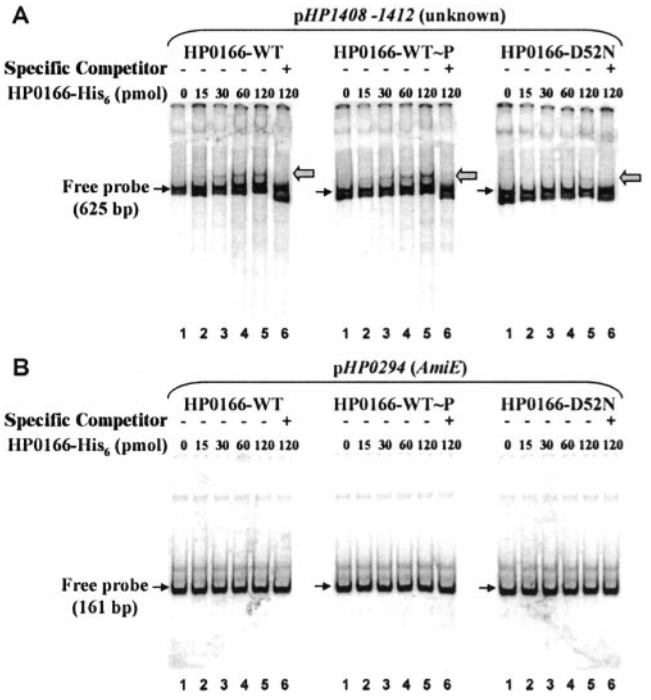

FIG. 2. Control experiments for EMSA with HP0166-His_6 purified from *E. coli* (HP0166-WT) (left parts), HP0166-His_6 subsequently phosphorylated in vitro by carbamylphosphate (HP0166-WT~P) (center parts), and a mutated response regulator (HP0166-D52N) that is not able to be phosphorylated at the aspartate phosphorylation site (right parts). A previously identified HP0166 target promoter region for p*HP1408-1412* (10) was used as a positive control (A). A 161-bp probe for p*HP0294* (*amiE*) that did not bind to HP0166-His_6 protein under the same experimental conditions served as a negative (nonspecific) control (B). PCR products for promoters were labeled with [γ-^{33}P]ATP. Labeled probes were incubated in the absence (lanes 1) or presence of increasing amounts of response regulator protein (15, 30, 60, and 120 pmol, lanes 2 to 5, respectively) or in the presence of both the protein (120 pmol) and a 50-fold excess of unlabeled probe as a specific competitor (lanes 6). The open and line arrows indicate the shifted bands and free probes, respectively.

genes that were up-regulated in a urea-sensitive manner in response to a low pH, as profiled in our microarray database (45), was analyzed with the complete genome sequences from *H. pylori* strain ATCC 26695, and 134 operons were identified for these acid-regulated genes. The likely promoter-regulatory sequences for these operons were identified with prokaryotic promoter prediction tools in the neural network (http://searchlauncher.bcm.tmc.edu/seq-search/gene-search.html). The 134 promoter-regulatory sequences were organized into 18 groups based on their sequence similarity with the sequence alignment tools from the Vector NTI Suite (InforMax, Inc.) (Fig. S1 and S2 in the supplemental material). One or two promoter sequences were randomly picked from each group and amplified to prepare the probes for the EMSA. The sizes of the PCR products ranged from 102 bp (p*HP0391-0399* from group 2) to 625 bp (p*HP1408-1412* from group 10), as shown in Fig. S3 in the supplemental material.

Purification of recombinant HP0166. To identify the target genes for two-component system HP0166-0165 among the low-pH-regulated genes, the HP0166 response regulator protein was overproduced and purified. The HP0166 coding sequence was cloned in the pET100/D-TOPO vector (Invitrogen, Carlsbad, CA), generating an N-terminal translational fusion with six histidine residues under the control of a T7 bacteriophage promoter. Analysis by SDS-PAGE of crude extracts from *E. coli* cells containing pET100/D-TOPO-HP0166 induced with 1 mM IPTG for 2 h showed an overproduced band of the expected apparent molecular mass (approximately 31 kDa), which was absent in the control extract from the cells carrying the same construct without IPTG induction (Fig. 1, lanes 2 and 3). SDS-PAGE analysis of the purified, His_6-tagged HP0166 protein revealed a purity of greater than 90% (Fig. 1, lane 4) with a yield of approximately 7.2 mg of purified protein per liter of culture.

To identify whether the interactions between HP0166 and its target promoters depend on the HP0166 aspartate phosphorylation thought to be triggered by the histidine kinase HP0165, a mutant derivative of ORF *HP0166* which resulted in a change in the probable phosphate-accepting aspartic acid residue at position 52 to asparagine was constructed. The mutated protein (HP0166-D52N) was expressed in *E. coli* as an N-terminal His_6 fusion, purified by affinity chromatography to the same level as the wild type (data not shown), and subsequently used for EMSA.

HP0166 binds specifically to the promoter-regulatory regions of acidic-pH-up-regulated genes. The purified recombinant response regulator HP0166-His_6 was used in the EMSA with DNA fragments corresponding to the promoter-regula-

TABLE 2. Promoter-regulatory probes with no direct binding with HP0166-His_6 in EMSA[a]

Promoter	GenBank accession no.	Description[b] of gene(s) under control
p*HP0082*	HP0082	Methyl-accepting chemotaxis transducer (*tlpC*)
p*HP0181-0185*	HP0182	Lysyl-tRNA synthetase (*lysS*)
	HP0183	Serine hydroxymenthyltransferase (*glyA*)
p*HP0243-0235*	HP0243	Neutrophil-activating protein (*napA*)
p*HP0294*	HP0294	Aliphatic amidase (*amiE*)
p*HP0437*	HP0437	IS*605* transposase (*tnpA*)
p*HP0601*	HP0601	Flagellin A (*flaA*)
p*HP0723*	HP0723	L-Asparaginase II (*ansB*)
p*HP0813-0826*	HP0824	Thioredoxin (*trxA*)
p*HP0896*	HP0896	Outer membrane protein (*omp19*)
p*HP0990*	HP0990	Unknown function
p*HP1187*	HP1187	Unknown function
p*HP0524-0530*	HP0528	Cag pathogenicity island protein (*cag8*)
p*HP0019-0020*	HP0020	Carboxynorspermidine decarboxylase (*nspC*)
p*HP0305-0308*	HP0306	Glutamate-1-semialdehyde-2,1-aminomutase (*hemL*)
p*HP0051-0047*	HP0047	Hydrogenase expression/formation protein (*hypE*)
p*HP1559-1558*	HP1559	Flagellar basal-body rod protein (*flgB*)
p*HP0193*	HP0193	Fumarate reductase, cytochrome b subunit (*frdC*)
p*HP0876*	HP0876	Iron-regulated outer membrane protein (*frpB*)
p*HP0692-0694*	HP0692	3-Oxoadipate coenzyme A-transferase subunit B (*yxjE*)
p*HP1067-1074*	HP1067	Chemotaxis protein (*cheY*)
p*HP1582-1584*	HP1582	Pyridoxal phosphate biosynthetic protein J (*pdxJ*)
	HP1583	Pyridoxal phosphate biosynthetic protein A (*pdxA*)
p*HP0520*	HP0520	Cag pathogenicity island protein (*cag1*)
p*HP0546-0539*	HP0540	Cag pathogenicity island protein (*cag19*)
	HP0541	Cag pathogenicity island protein (*cag20*)
	HP0542	Cag pathogenicity island protein (*cag21*)
	HP0543	Cag pathogenicity island protein (*cag22*)
p*HP0252-0259*	HP0259	Exonuclease VII, large subunit (*xseA*)
p*HP0786-0788*	HP0786	Preprotein translocase subunit (*secA*)
p*HP1241-1240*	HP1240	Conserved hypothetical protein

[a] A total of 45 probes representing promoter-regulatory regions for low-pH upregulated genes were investigated by EMSA, and 26 of them showed no direct binding with HP0166-His_6 in EMSA (including both the ones without a gel shift and the ones with a nonspecific shift indicated by unlabeled-competitor chase experiments).
[b] Gene names and designations of the loci in strain 26696 (41) are indicated.

tory regions of randomly selected representatives from each group of the low-pH-up-regulated genes that had been profiled in our microarray database (45). The selected promoter sequences from each group were 5′ end labeled with [γ-^{33}P]ATP and analyzed by EMSA after incubation with HP0166-His_6. Additionally, a previously identified HP0166 target promoter region, p*HP1408-1412* (10), was used as a positive control to validate our EMSA experiments. As shown in Fig. 2A, a 625-bp probe was 5′ end labeled by [γ-^{33}P]ATP and incubated with increasing amounts of HP0166-His_6. A gel shift band was observed when 30 pmol of HP0166-His_6 was added to the reaction mixture (Fig. 2A, HP0166-WT, lane 3), and the intensity of the shifted band became stronger with increasing amounts of HP0166-His_6 protein up to 60 and 120 pmol (Fig. 2A, HP0166-WT, lanes 4 and 5). The addition of 50-fold excess amounts of unlabeled DNA fragments corresponding to the promoter regions effectively prevented DNA binding of the corresponding radiolabeled probes (Fig. 2A, HP0166-WT, lane 6), showing the specificity of the shift. To investigate the level of phosphorylation in a preparation of *H. pylori* HP0166-His_6 isolated from *E. coli* (HP0166-WT), EMSA experiments were also performed with HP0166-His_6 that was subsequently phosphorylated in vitro by carbamylphosphate (HP0166-WT~P) and a mutated response regulator (HP0166-D52N) that is not able to be phosphorylated at the aspartate phosphorylation site, with the same promoter probe. A similar gel mobility shift pattern was detected with in vitro-phosphorylated HP0166-His_6 (Fig. 2A, HP0166-WT~P), whereas no gel shift was detected until 120 pmol of HP0166-D52N was added (Fig. 2A, HP0166-D52N). These data show that the preparation of *H. pylori* HP0166-His_6 isolated from *E. coli* is functionally phosphorylated and that using mutant HP0166-D52N is a better strategy to define the requirement of HP0166 phosphorylation for binding to its target promoters. A 161-bp probe for p*HP0294* (*amiE*) that did not bind to HP0166-His_6 protein under the same experimental condition is shown in Fig. 2B to serve as a negative (nonspecific) control. About 26 probes for the promoter-regulatory regions of acidic-pH-up-regulated genes which were examined by EMSA showed negative results similar to those for p*HP0294* (*amiE*) (Table 2), suggesting that these promoters are not regulated by HP0166.

The initial EMSA results indicated that nine promoter-regulatory sequences, representing 8 of the 18 groups, interacted with the response regulator HP0166. Further EMSA studies on the promoter-regulatory sequences in some of those positive groups identified 10 additional HP0166 target genes that were up-regulated by a low pH. These initial EMSA results suggest that a significant number (19 probes) of the pH-regulated genes are regulated by HP0166.

HP0166 target genes that may be HP0165 dependent and respond directly to periplasmic pH. When we compared the patterns of low-pH-regulated gene expression by cluster analysis (45), seven HP0166 target promoters for genes including *fecD* (p*HP0890-0888*), *lpp20* (p*HP1457-1454*), *hypA* (p*HP0871-*

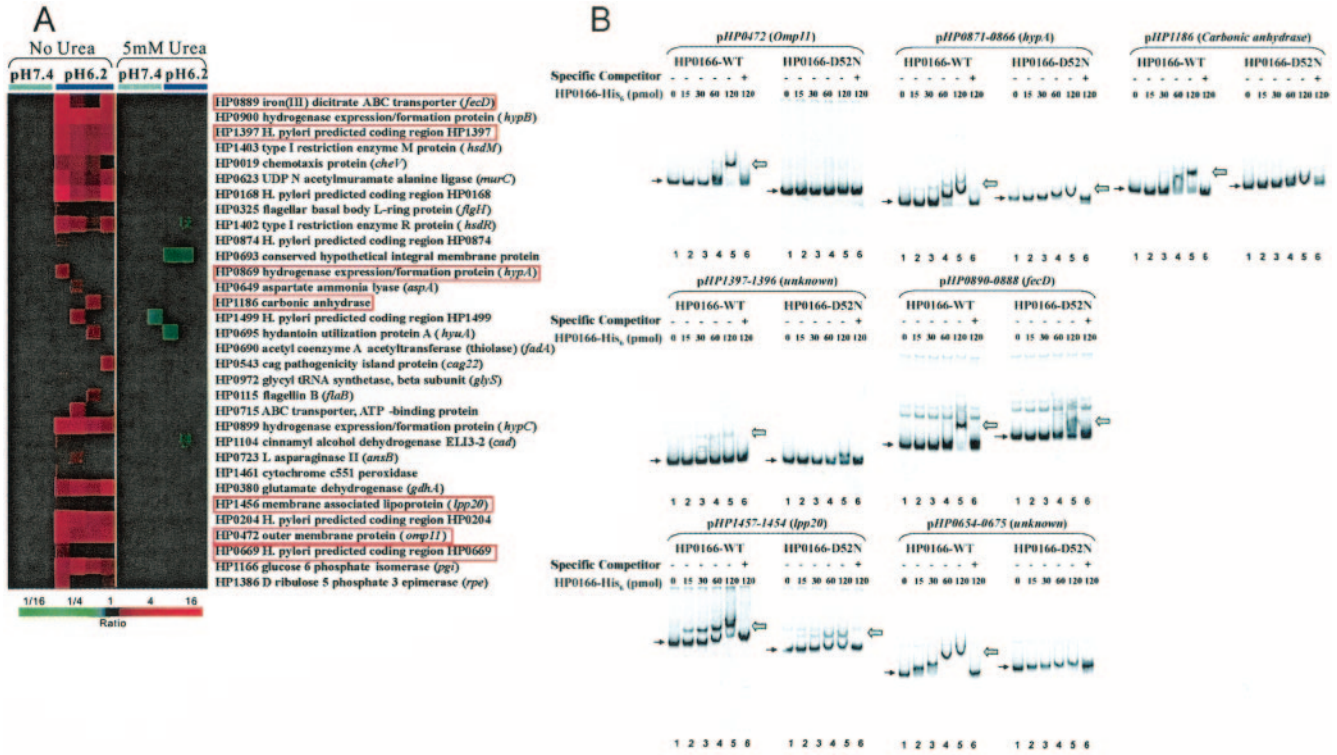

FIG. 3. HP0166 target promoters in group A. HP0166 target promoters in group A may be HP0165 dependent and responsive to the periplasmic pH. (A) Enlargement of the gene cluster segment assigned to group A from the cluster analysis of gene expression patterns showing microarray experiments at a medium pH of 6.2 versus a medium pH of 7.4 with and without urea. The presence of urea on this group of genes up-regulated at a medium pH of 6.2 abolishes their up-regulation. The HP0166 target genes identified by EMSA are highlighted with rectangles. The medium pH for each individual microarray experiment is indicated. The color scale used to represent the expression ratios is shown at the bottom. (B) HP0166 target promoters in group A identified by EMSA. PCR products for promoters were labeled with [γ-^{33}P]ATP. Labeled probes were incubated in the absence (lane 1) or presence of increasing amounts of wild-type HP0166-His$_6$ (HP0166-WT) or mutated protein HP0166-D52N (15, 30, 60, and 120 pmol, lanes 2 to 5, respectively) or in the presence of both HP0166-His$_6$ (120 pmol) and a 50-fold excess of unlabeled probe as a specific competitor (lane 6). The open and line arrows indicate the shifted bands and free probes, respectively.

0866), that for carbonic anhydrase (p*HP1186*), and *omp11* (p*HP0472*) and two promoters for genes with unknown function (p*HP1397-1396* and p*HP0654-0675*) were found to fall into the group of genes here classified as group A (cluster B in the previous study [45]) (Fig. 3). These genes of group A were found to be up-regulated at a medium pH of 6.2, but their up-regulation was ablated by the addition of urea. Urea addition at this pH has been shown to normalize the periplasmic pH (38), while the intrabacterial pH is relatively unchanged with or without urea (45) (Fig. 3A). The low-pH-up-regulated genes in group A are illustrated in Fig. 3A. HP0166 binding to these seven promoter-regulatory regions was detected with wild-type HP0166 (HP0166-WT in Fig. 3B) by EMSA.

To define the requirement of HP0166 phosphorylation for binding to its target promoters found in group A, a mutated response regulator (HP0166-D52N) which replaced the aspartic acid residue at position 52 with asparagine was also used for EMSA (HP0166-D52N in Fig. 3B). As expected, with the mutated protein HP0166-D52N, the target promoters in group A showed either no binding for *omp11* (p*HP0472*), carbonic anhydrase (p*HP1186*), and two promoters for the genes with unknown function (*pHP1397-1396* and *pHP0654-0675*) or much-reduced binding affinities for *fecD* (*pHP0890-0888*), *lpp20* (p*HP1457-1454*), and *hypA* (*pHP0871-0866*). This demonstrates that the phosphorylated form of HP0166 is required to efficiently bind the target promoters in group A, the periplasmic-pH-sensitive group of genes found on the microarray.

HP0166 target genes that may be HP0165 independent and respond to changes in the cytoplasmic pH. Eight HP0166 target promoters (identified with HP0166-WT by EMSA as shown in Fig. 4B) for the gene *ureI* (p*HP0071-0067*), the gene for a catalase-like protein (p*HP0485*), the gene *rocF* (p*HP1399*), the gene for catalase (p*HP0875-0874*), the gene for histidine kinase/response regulator OmpR (p*HP0166-0162*), the gene for a ferredoxin-like protein (p*HP0588-0595*), and two promoters for genes with unknown function (p*HP0682-0681* and p*HP1326-1329*) were found to fall into a group of genes, group B (cluster D in the previous study [45]). These genes were up-regulated at a medium pH of 5.5 in the absence but not in the presence of urea (Fig. 4). The genes of group B are illustrated in Fig. 4A. At this medium pH, the cytoplasmic pH (pH_{in}) falls to 6.5 in the absence of urea and then is increased to 6.9 with urea addition (45).

Additionally, four HP0166 target promoters (identified with HP0166-WT in the EMSA shown in Fig. 5B), for the genes *tpi* (p*HP0194-0196*) and *cheV* (p*HP0399-0391*) and for the genes with unknown function (p*HP0711-0713* and p*HP1288-1289*), were found to fall into group C of genes (cluster E in the previous study [45]) that were up-regulated at a medium pH of

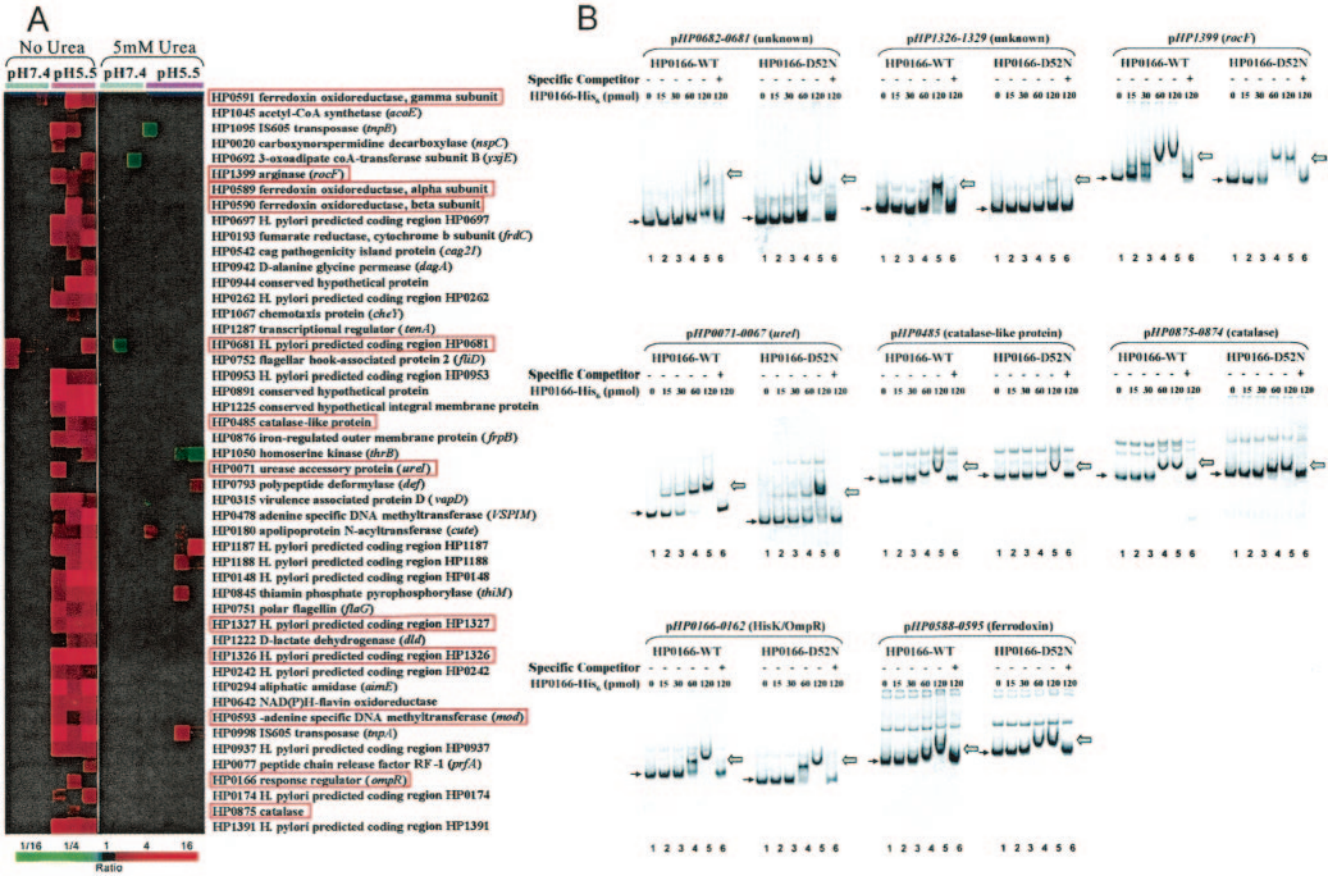

FIG. 4. HP0166 target promoters in group B. HP0166 target promoters in group B may be HP0165 independent and responsive to a cytoplasmic pH lower than pH 6.5. (A) Enlargement of the gene cluster segment assigned to group B on the basis of the cluster analysis of gene expression patterns in microarray experiments at a medium pH of 5.5 versus a medium pH of 7.4 with and without urea. In this group, the presence of urea also abolished up-regulation of these genes at a fixed medium pH of 5.5. The HP0166 target genes identified by EMSA are highlighted with rectangles. The medium pH for each individual microarray experiment is indicated. The color scale used to represent the expression ratios is shown on the bottom. (B) HP0166 target promoters in group B identified by EMSA. PCR products for promoters were labeled with [γ-^{33}P]ATP. Labeled probes were incubated in the absence (lane 1) or presence of increasing amounts of wild-type HP0166-His$_6$ (HP0166-WT) or mutated protein HP0166-D52N (15, 30, 60, and 120 pmol, lanes 2 to 5, respectively) or in the presence of both HP0166-His$_6$ (120 pmol) and a 50-fold excess of unlabeled probe as a specific competitor (lane 6). The open and line arrows indicate the shifted bands and free probes, respectively.

4.5, and the up-regulation was abolished in the presence of urea (Fig. 5). The cytoplasmic pH (pH_{in}) falls further at this medium pH to 5.3 in the absence of urea and rises with the addition of urea (45). The low-pH-up-regulated genes in group C are illustrated in Fig. 5A.

While not excluding the possibility that the genes in these groups are also responsive to the periplasmic pH, the gene expression patterns in these two groups in relation to the periplasmic pH (pH_{out}) and cytoplasmic pH (pH_{in}) indicate that some of these genes may also be responsive to a cytoplasmic pH that has decreased to 6.5 and 5.3, respectively, as shown by direct measurement of the cytoplasmic pH (45). With the mutated protein HP0166-D52N, most of the target promoters in groups B and C showed reduced binding affinities (Fig. 4B and 5B, with HP0166-D52N), indicating that their effective interaction with HP0166 requires the phosphorylation of the HP0166 response regulator. However, the target promoters for the gene with unknown function (p*HP0682-0681*) and histidine kinase/response regulator OmpR (p*HP0166-0162*) from group B, as well as a target promoter for the gene with unknown function (p*HP1288-1289*) from group C, showed almost no effect on their binding to the mutated response regulator HP0166-D52N (Fig. 4B and 5B), demonstrating that the phosphorylation of response regulator HP0166 is not required for binding to these particular target promoters. Interestingly, the target promoter for *ureI* (p*HP0071-0067*) in group B showed a reduced affinity of binding to HP0166-D52N, compared with binding to HP0166-WT (Fig. 4B), but it still retained reasonably high affinity for the mutated response regulator HP0166-D52N by showing a gel shift with the addition of the minimal amount of protein of 15 pmol. Hence, for this particular gene, binding of HP0166 may also not be fully dependent on phosphorylation.

DNase I footprinting reveals a discrepancy in the binding sites of HP0166 with different promoter-regulatory regions. To confirm the results from EMSA and to determine the location and sequence of the HP0166 binding site, DNase I footprinting assays were performed with DNA fragments carrying promoter-regulatory regions for *HP0871-0866* (*hypA*) and *HP1186* (carbonic anhydrase). As shown in Fig. 6, when the DNA fragments (225 bp) carrying the *HP0871-0866* (*hypA*) promoter

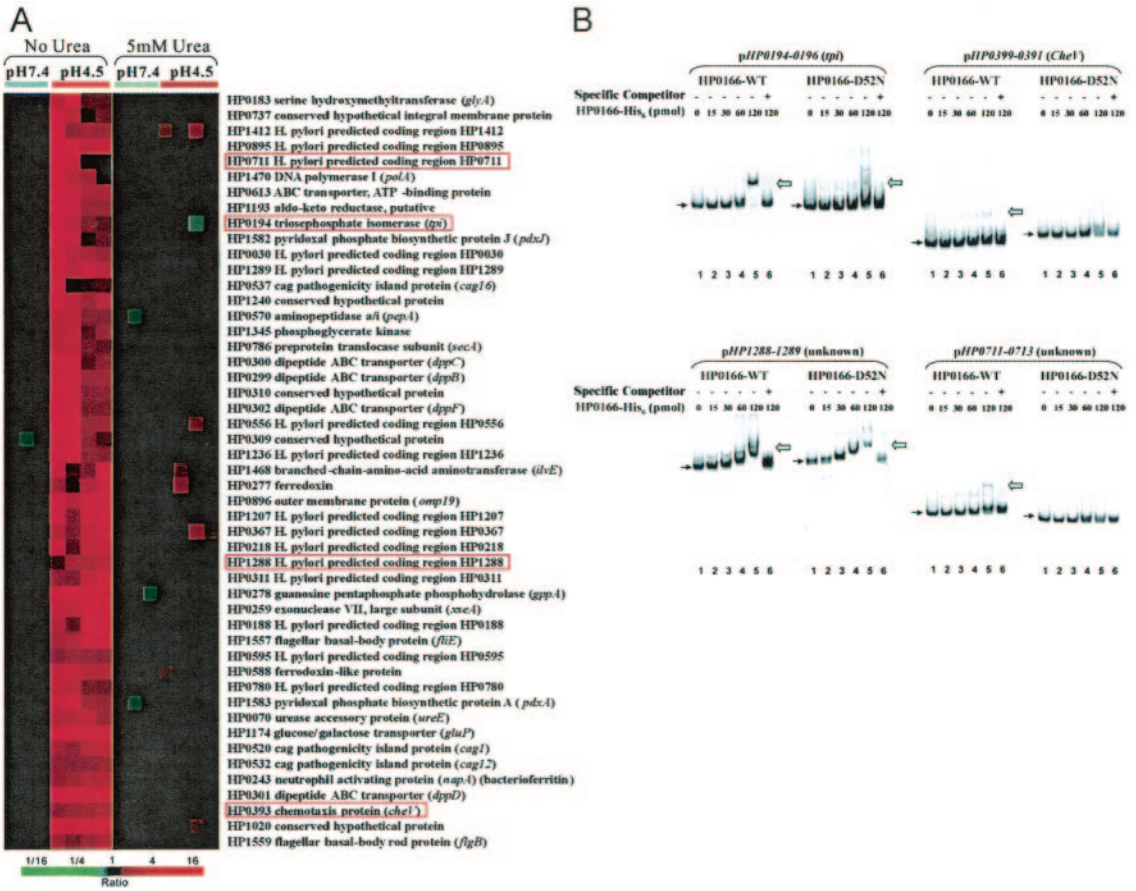

FIG. 5. HP0166 target promoters in group C. HP0166 target promoters in group C may be HP0165 independent and responsive to a cytoplasmic pH lower than pH 5.3. (A) Enlargement of the gene cluster assigned to group C from the cluster analysis of gene expression patterns showing microarray experiments at a medium pH of 4.5 versus a medium pH of 7.4 with and without urea. In this group also, the presence of urea abolished up-regulation at pH 4.5. The HP0166 target genes identified by EMSA are highlighted with rectangles. The fixed medium pH for each individual microarray experiment is indicated. The color scale used to represent the expression ratios is shown at the bottom. (B) HP0166 target promoters in group C identified by EMSA. PCR products for promoters were labeled with [γ-^{33}P]ATP. Labeled probes were incubated in the absence (lane 1) or presence of increasing amounts of wild-type HP0166-His$_6$ (HP0166-WT) or mutated protein HP0166-D52N (15, 30, 60, and 120 pmol, lanes 2 to 5, respectively) or in the presence of both HP0166-His$_6$ (120 pmol) and a 50-fold excess of unlabeled probe as a specific competitor (lane 6). The open and line arrows indicate the shifted bands and free probes, respectively.

region were end labeled on the nontemplate strand, the protected region extended from −86 to −111 in relation to the translational start codon at the lower HP0166-His$_6$ concentration (60 pmol) and from −62 to −111 and −141 to −165 at a higher concentration (120 pmol) (Fig. 6A). These results suggest the presence of at least two binding sites with different affinities for HP0166 in the *HP0871-0866* (*hypA*) promoter regions, in agreement with the multiple complexes observed in EMSAs (Fig. 3B).

The extent of the protected region of the *HP1186* (carbonic anhydrase) promoter also varied depending on the HP0166-His$_6$ concentrations. When the 225-bp promoter fragment of *HP1186* (carbonic anhydrase) DNA was end labeled on the nontemplate strand, HP0166 protected a region extending from position −106 to position −128 at a lower concentration (60 pmol) and from −95 to −144 at a higher concentration (120 pmol) (Fig. 6B).

These data indicate that HP0166 binds directly to the promoter-regulatory regions for *HP0871-0866* (*hypA*) and *HP1186* (carbonic anhydrase), and the binding sites are located between positions −111 and −62 (and likely between −165 and −141) of the *HP0866-0871* (*hypA*) promoter region (Fig. 6C) and between positions −144 and −95 of the *HP1186* (carbonic anhydrase) promoter region (Fig. 6D).

A nucleotide sequence comparison of the *HP1186* (carbonic anhydrase) and *HP0871-0866* (*hypA*) protected regions (Fig. 6E) did not reveal a direct or inverted repeat sequence. However, a conserved, AT-rich heptanucleotide sequence (AATG/CATT) was found around the center of the protected regions for *HP1186* (carbonic anhydrase) and *HP0866-0871* (*hypA*).

DISCUSSION

The unique ability of the neutralophile *H. pylori* to colonize the human stomach depends on a series of responses developed by this organism, especially those termed acid acclimation. The acid acclimation response is in contrast to the acid resistance or tolerance response possessed by other neutralo-

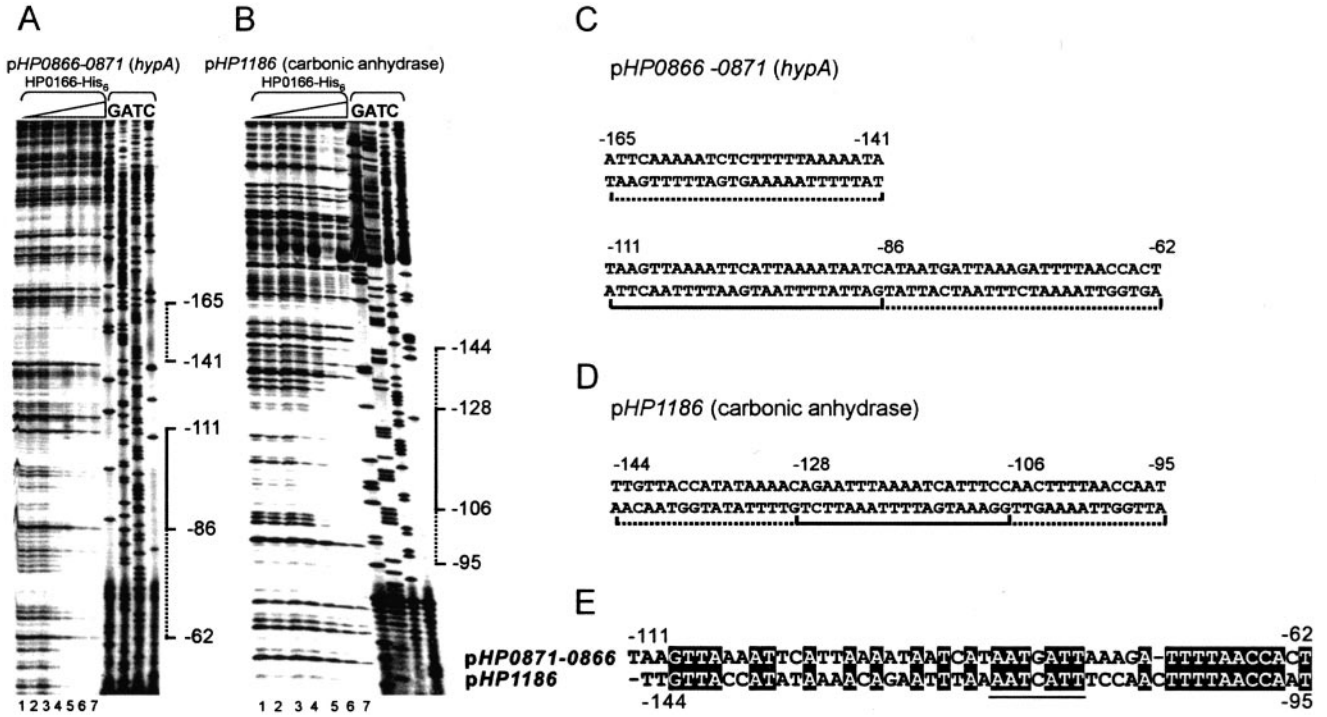

FIG. 6. HP0166 binding sites for the *HP0871-0866* (*hypA*) and *HP1186* (carbonic anhydrase) promoter regions. DNase I footprinting analysis of HP0166 binding to the *HP0871-0866* (*hypA*) and *HP1186* (carbonic anhydrase) promoter regions was carried out with purified HP0166-His_6. Each lane contains 0.5 pmol of the γ-^{33}P-end-labeled nontemplate strand of *HP0871-0866* (−34, −351) (A) or the nontemplate strand of *HP1186* (−47, −271) (B). Fragments were incubated with increasing amounts of purified HP0166-His_6: lane 1, no protein; lane 2, 15 pmol; lane 3, 30 pmol; lane 4, 60 pmol; lane 5, 120 pmol; lane 6, 240 pmol; lane 7, 480 pmol. Sequencing reaction mixtures of appropriate DNA fragments were loaded next to lane 7. Regions protected by HP0166 are indicated by vertical solid lines (at a lower HP0166 concentration) and dotted lines (at a higher HP0166 concentration). The double-stranded sequences of regions protected from DNase I digestion by the binding of HP0166 for *HP0871-0866* (C) and *HP1186* (D) are shown. (E) Alignment of the protected regions for the *HP0871-0866* (*hypA*) and *HP1186* (carbonic anhydrase) promoter regions. The conserved, AT-rich sequence (AATG/CATT) found between protected regions for *HP0871-0866* and *HP1186* is underlined. Positions relative to the translational start codon are indicated.

philes that can transit through the acidic gastric lumen but cannot colonize the stomach. These acid resistance mechanisms of other neutralophiles maintain the cytoplasmic pH between 4.0 and 5.0 in acid, allowing survival but not growth. Many of these acid resistance pathways involve amino acid decarboxylases that result in uptake of a cytoplasmic proton with decarboxylation and then are coupled to an exchanger exporting the amine product in exchange for the amino acid, resulting in cytoplasmic pH regulation between 4.0 and 5.0 by proton export (15, 21). Other acid regulation responses include proton extrusion by the F_1 F_0 ATPase (15), which may also be functional in this sense in *H. pylori*, in contrast to the amino acid decarboxylase system, which is absent in the genome of the organism.

Critical to acid acclimation is the ability of *H. pylori* to maintain an inner membrane potential and cytoplasmic pH at levels compatible with growth in the relatively acidic environment colonized by the organism at the surface of the antral or fundic gastric mucosa. When the gastric luminal pH falls below 3.0, the surface of the stomach becomes acidic, as has been demonstrated severally by microelectrode or fluorescent-probe measurements (3, 34). Given that the mean diurnal pH in the lumen of the human stomach is 1.4, *H. pylori* is likely exposed to an acidic environment for much of the time at its site of infection.

Several genes of *H. pylori* have been shown to be up-regulated or even down-regulated as a result of progressive exposure to acidic medium pHs of 6.2, 5.5, and 4.5 (45). In this particular microarray study, the effect of the presence of urea at physiological levels was also investigated since urease activity is essential for survival of *H. pylori* in acidic medium and for gastric colonization (2, 11, 42). The presence of UreI, a pH-gated urea channel, accelerates urea access at an acidic medium pH to the intrabacterial urease compartment and thence generation of NH_3, which can neutralize entering protons and CO_2, which, along with both cytoplasmic and periplasmic carbonic anhydrase, generates buffering HCO_3^- (22).

In our microarray studies, *H. pylori* genes regulated by medium acidity were identified by comparison of genes expressed at pHs 6.2, 5.5, and 4.5 to expression at pH 7.4 in the absence or presence of urea (45). In the course of these investigations with clustering analysis, it was observed that the expression patterns in the genes in response to acidity could be separated into groups that were up-regulated at pH 6.2 (group A), at pH 5.5 (group B), and at pH 4.5 (group C), whose up-regulation was eliminated in the presence of urea. From these observa-

tions, it was inferred that the genes in group A responded largely to changes in the periplasmic pH whereas the genes of groups B and C might also respond to changes in the cytoplasmic pH (45).

Among the genes identified as those up-regulated by medium acidity were *HP0165* and *HP0166*, two genes of one of the four two-component systems expressed by this organism. It seemed possible that this two-component system might be responsible for regulation of some of the acid-responsive genes. The gene for response regulator HP0166 has been shown to be an essential gene for *H. pylori* cell growth, while the cognate histidine kinase HP0165 is not essential under in vitro culture conditions (4), suggesting that the HP0165-independent (or unphosphorylated) response regulator HP0166 is necessary for transcription of target genes essential for in vitro growth, while an additional set of genes is activated or repressed under environmental conditions which trigger the histidine kinase activity of the sensor protein HP0165 and accordingly lead to the phosphorylation of protein HP0166. It is not clear whether there is any other kinase that may lead to the phosphorylation of HP0166. However, a recent study (35) has demonstrated that the essential but unknown growth functions of response regulator HP0166 can be provided by a mutated derivative carrying a D52N substitution at the site of aspartate phosphorylation, suggesting that the transcriptional control of the essential genes by the response regulator HP0166 is independent of its aspartate phosphorylation. Therefore, more target genes remain to be identified and the promoters of the two subsets of target genes should contain response regulator binding sites with different affinities (high-affinity binding sites which are recognized by the unphosphorylated protein and lower-affinity binding sites whose binding requires phosphorylation of HP0166) (4). The results described here, that a significant number of promoters for pH-regulated genes showed direct binding with response regulator HP0166, strongly support the hypothesis that the HP0165-HP0166 two-component system is one system responsible for the regulation of many genes in *H. pylori* in responding to an acidic environment.

Seven promoters in group A for the gene *fecD* (*pHP0890-0888*), the gene *lpp20* (*pHP1457-1454*), the gene *hypA* (*pHP0871-0866*), the gene for carbonic anhydrase (*pHP1186*), and the gene *omp11* (*pHP0472*) and two promoters for the genes with unknown function (*pHP1397-1396* and *pHP0654-0675*) were found to be able to directly bind to the response regulator HP0166 (Fig. 3), suggesting that these HP0166 target genes are responsive to the periplasmic pH and therefore dependent on the sensor protein HP0165 located on the inner membrane. The EMSA results obtained with HP0166-D52N confirmed that the phosphorylated form of HP0166 is required to bind the target promoters in this group of genes, presumably dependent on phosphorylation of HP0165 and transfer of the phosphate to HP0166.

Among these acid-responsive HP0166 target genes found in this group was α-carbonic anhydrase (HP1186), which has been demonstrated to be an important periplasmic buffering protein that catalyzes the conversion of CO_2, produced by urease, to HCO_3^- (22). Another acid-responsive HP0166 target gene found in this group, *hypA* (*HP0871-0866*), is considered to have a role in the uptake of nickel, the cation essential for activation of the urease apoprotein UreA/UreB (26), although it is part of the hydrogenase assembly complex (5). It is of interest that the genes investigated in this group were all dependent on the phosphorylation site on HP0166, perhaps due to the phosphorylation of HP0165 and phosphate transfer to HP0166. Probably the cognate transmembrane sensor histidine kinase HP0165 monitors environmental changes, namely, in the periplasmic pH, by an input domain exposed to the periplasm which contains seven histidines with a pK_a in the range of pH 6.0 and, upon detecting a fall in pH, likely undergoes autophosphorylation of a well-conserved histidine residue in the transmitter domain located on the cytoplasmic side of the membrane (40). Therefore, these HP0166 target promoters in group A may be HP0165 dependent and directly responsive to a slight decrease in the periplasmic pH.

The expression patterns of the genes responding to pH 5.5 in group B (Fig. 4A) and to pH 4.5 in group C (Fig. 5A) indicate that these genes change their expression levels in response to a lowering of the cytoplasmic pH (pHs 6.5 and 5.3, respectively) in addition to the periplasmic pH (45). Eight and four acid-responsive HP0166 target genes were found in groups B and C, respectively (Fig. 4 and 5). Assuming that the periplasmic domain of the sensor histidine kinase HP0165 only detects the environmental stimulus in the periplasm, some of the target genes regulated by the response regulator HP0166 in groups B and C may be HP0165 independent. The EMSA studies with HP0166-D52N (Fig. 4B and 5B) showed that the target promoters for the genes with unknown function (p*HP0682-0681*) and histidine kinase/response regulator OmpR (p*HP0166-0162*) from group B, as well as a target promoter for the gene with unknown function (p*HP1288-1289*) from group C, are not dependent on the presence of the phosphorylation site for their interactions with HP0166. The other target promoters in groups B and C may require HP0166 phosphorylation for maximal binding.

Our finding that the regulation by the response regulator HP0166 of the genes in response to a fall in the cytoplasmic pH may be independent of the histidine kinase HP0165 is consistent with the observation that the response regulator HP0166 is essential for *H. pylori* growth while the cognate histidine kinase HP0165 is dispensable under in vitro culture conditions (4). This may suggest that there are different types of input into the response regulator HP0166 in response to the cytoplasmic pH.

The promoter for *HP0067-0071* (*ureI*) in group B was found to be able to directly bind to HP0166, and this is in agreement with the recent report by Pflock et al. (30). The gene product encoded by *ureI*, UreI, the proton-gated urea entry channel that allows buffering of the periplasm of the organism by urease activity, is essential for survival and growth in acidic medium (36, 44), as well as for colonization of mouse or gerbil stomachs (25, 39). These observations show the absolute necessity for expression of this protein and also show that the organism colonizes the stomach in regions of acidic pH.

Another pH homeostatic gene in group B, *HP1399* (*rocF*), was found to be an HP0166 target gene in our EMSA experiments. The gene product of *HP1399* (*rocF*), arginase, is an enzyme of the *H. pylori* urea cycle and synthesizes urea and ornithine from the catabolism of arginine. Arginase has been found to be crucial for acid protection in vitro in the absence of urea but is not essential for in vivo colonization of mice or for urease activity (23). Presumably this is because it produces an intrabacterial source of urea that is bypassed in vivo by an adequate supply of gastric juice urea and may be required only when the gastric juice urea falls below threshold levels.

The promoter for *HP0166-0162*, which governs transcription of the HP0165-0166 two-component system, also showed direct binding to the response regulator HP0166 in an HP0165-independent manner (Fig. 4), which is in agreement with the finding that the response regulator HP0166 may negatively autoregulate expression of the HP0165-0166 two-component system (10).

In our EMSA and DNase I footprinting experiments, the HP0166-His_6 protein isolated from *E. coli* was used without going through in vitro phosphorylation, since our control experiment (Fig. 2) has shown that the response regulator HP0166 purified from *E. coli* is already adequately phosphorylated. This is consistent with a recent study (18) that showed that the response regulator Spo0A from *Bacillus subtilis* is efficiently phosphorylated in *E. coli*. The comparison with the EMSA results obtained with the nonphosphorylatable mutated response regulator HP0166-D52N (35) also shows that it is likely that our preparation of wild-type HP0166-His_6 was at least partially phosphorylated in *E. coli*. The use of the mutant HP0166-D52N allowed a distinction to be made between genes that have an absolute requirement for regulation by phosphorylation of HP0166 and those that are less dependent on the state of phosphorylation of HP0166.

However, while the strategy used in this study is able to quickly identify some of the acid-responsive target promoters of HP0166, it is not efficient enough to identify the complete regulons of the HP0165-0166 two-component system as an acid-responsive signaling system, especially those possibly essential genes that are HP0165 independent. Studies with an HP0165 knockout strain exposed to a low pH will be helpful in finding those HP0166 target genes independent of the function of the sensor protein HP0165.

The binding sites for the response regulator HP0166 were identified in the *HP0871-0866* (*hypA*) and *HP1186* (carbonic anhydrase) promoter-regulatory regions by DNase I footprinting analysis. Although no direct or inverted repeat sequences were present in the sequence motif identified, a conserved, AT-rich heptanucleotide sequence (AATC/GATT) was found between the promoter-regulatory regions of *HP1186* (carbonic anhydrase) and *HP0866-0871* (*hypA*), which fell into group A (45), suggesting their possible role in responding to the periplasmic pH in an HP0165-dependent manner. A similar conserved, AT-rich heptanucleotide sequence (ATTNAAT) was also found in the HP0166 binding sites for the *HP1408* and *HP0199* promoter regions (10), which have been demonstrated to be positively regulated by the phosphorylated response regulator in an HP0165-dependent manner (10).

In this study, we have demonstrated by EMSA and DNase I footprinting analysis that a significant number of low-pH-responding genes are directly regulated by the response regulator HP0166, suggesting that the HP0165-0166 two-component system is part of the acid-responsive signaling system in *H. pylori*, which may regulate gene expression in response to either a decrease in the periplasmic pH alone or also to a decrease in the cytoplasmic pH which may be HP0165 independent. The expression of genes contained in the urease operon and carbonic anhydrase have been shown to be part of the repertoire used for gastric acid acclimation by the organism. The other genes identified here as interacting with HP0166 may also play a role yet to be established for colonization of the stomach.

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