

## Characterization of the ArsRS Regulon of *Helicobacter pylori*, Involved in Acid Adaptation†

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**The human gastric pathogen *Helicobacter pylori* is extremely well adapted to the highly acidic conditions encountered in the stomach. The pronounced acid resistance of *H. pylori* relies mainly on the ammonia-producing enzyme urease; however, urease-independent mechanisms are likely to contribute to acid adaptation. Acid-responsive gene regulation is mediated at least in part by the ArsRS two-component system consisting of the essential OmpR-like response regulator ArsR and the nonessential cognate histidine kinase ArsS, whose autophosphorylation is triggered in response to low pH. In this study, by global transcriptional profiling of an ArsS-deficient *H. pylori* mutant grown at pH 5.0, we define the ArsR~P-dependent regulon consisting of 109 genes, including the urease gene cluster, the genes encoding the aliphatic amidases AmiE and AmiF, and the *rocF* gene encoding arginase. We show that ArsR~P controls the acid-induced transcription of *amiE* and *amiF* by binding to extended regions located upstream of the –10 box of the respective promoters. In contrast, transcription of *rocF* is repressed by ArsR~P at neutral, acidic, and mildly alkaline pH via high-affinity binding of the response regulator to a site overlapping the promoter of the *rocF* gene.**

*Helicobacter pylori* is a human pathogen which is associated with gastric diseases like chronic active gastritis, peptic ulceration, adenocarcinoma, and mucosa-associated lymphoid-tissue lymphoma (4, 25, 37). The neutralophilic bacterium, which thrives in the mucus layer covering the gastric epithelium, is extremely well adapted to cope with the fluctuating low-pH conditions encountered in the human stomach and has evolved mechanisms both to survive severe acid shocks and to grow at moderately acidic pH. In accordance with the stomach being its unique habitat, sequencing of the *H. pylori* genome revealed a very restricted repertoire of transcriptional regulators (34); however, it is becoming increasingly evident that a considerable subset of these regulators is involved in the control of the acid response. In three independent studies performing genome-wide transcriptional profiling, between 101 and about 280 genes have been reported to be regulated in response to the exposure of *H. pylori* to low pH (6, 21, 44). Up to now, two transcriptional regulators, the metal-dependent regulators NikR and Fur and a two-component signal transduction system termed ArsRS, have been implicated in the transcriptional regulation of acid-responsive genes (26, 40, 41). The ArsRS system consists of the histidine kinase ArsS, comprising a periplasmic input domain of 111 amino acids and likely to be responsible for low-pH sensing, and the OmpR-like response regulator ArsR. Inactivation of the histidine kinase ArsS renders *H. pylori* unable to colonize mice (24), while the response

regulator ArsR is essential for the in vitro growth of *H. pylori*, suggesting distinct functions for ArsR in its unphosphorylated and phosphorylated states (2, 29).

A major player in the acid resistance of *H. pylori* is the urease system, which is essential for colonization (11, 14, 22, 31, 36). By cleaving urea, the urease enzyme generates ammonia and carbon dioxide, the latter being subsequently converted to HCO<sub>3</sub><sup>–</sup> by a periplasmic carbonic anhydrase. These buffering compounds capture protons that leak into the cytoplasm and periplasm and thereby maintain the cytoplasmic and periplasmic pH of the bacteria near neutrality (18, 33). 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 (28, 43). Transcription of both the *ureAB* operon encoding the enzymatic subunits of urease and the *ureIEFGH* operon encoding the channel protein UreI and accessory proteins which are required for the incorporation of the metal cofactor Ni<sup>2+</sup> into the urease apoenzyme is positively regulated in response to low pH (6, 21, 27). This pH-responsive transcriptional induction is mediated by the ArsRS two-component system via the binding of the phosphorylated response regulator ArsR (ArsR~P) to the promoters of the *ureAB* and *ureIEFGH* operons (27). Several other target genes of the ArsRS two-component system encoding mostly *H. pylori*-specific proteins of unknown function have already been identified (10, 15) and are likely to be involved in urease-independent mechanisms of acid adaptation. Interestingly, the *rocF* gene encoding arginase was also shown to be regulated by ArsRS (15). Arginase produces urea and ornithine by the cleavage of arginine, and it was suggested that this enzyme might contribute to urease-dependent acid resistance when urea is scarce in the surrounding medium (20). Transcription of the *ureAB* genes is also posi-

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tively controlled in response to increasing concentrations of  $\text{Ni}^{2+}$  ions by the pleiotropic, metal-dependent regulator NikR, which is also involved in the regulation of genes involved in  $\text{Ni}^{2+}$  uptake and storage,  $\text{Fe}^{3+}$  uptake and storage, stress responses, motility, and encoding outer membrane proteins (7, 13, 38). The acid stimulon and the NikR regulon defined by transcriptome analyses overlap to some extent, underlining the role of NikR in the process of acid adaptation (6, 7).

Besides that of urease, the expression of other ammonia-producing enzymes was demonstrated to be induced in response to acidic pH (6, 21, 44). Most notably, transcription of the genes *amiE* and *amiF*, which encode aliphatic amidases, was strongly increased upon exposure of *H. pylori* to low pH. Amidase genes which were known so far to be predominantly associated with environmental bacteria are present in several stomach-colonizing *Helicobacter* species, like *H. pylori*, *H. acinonychis*, and *H. felis*, suggesting an important role for the enzymes in nitrogen metabolism and acid resistance for these *Helicobacter* species. However, so far the substrates cleaved by the amidases *in vivo* remain unknown (5, 32). Under iron-replete conditions, transcription of *amiE* has been shown to be repressed by Fur, which binds to the upstream region of *amiE*. To our knowledge, *amiF* transcription is not controlled by Fur (39). However, at low pH the activity of both amidases is conversely affected by the inactivation of Fur or the NikR protein, suggesting a role for both regulators in the acid-responsive regulation of the amidases (6, 40). Interestingly, Fur-deficient mutants are impaired in their acid tolerance, suggesting an important role for Fur in the acid response (3).

The aim of this study was to define the subset of acid-responsive genes which is transcriptionally regulated by the ArsRS two-component system. We show that at pH 5.0, compared to those of the wild type, 109 genes are differentially regulated in a mutant of *H. pylori* G27 lacking the histidine kinase ArsS. Furthermore, we demonstrate that acid-induced transcription of *amiE* and *amiF* is mediated directly by the ArsRS two-component system via binding of ArsR~P to the promoter regions of the amidase genes. Unexpectedly, the *rocF* gene encoding arginase, whose transcription is repressed by ArsR~P, did not show a pH-responsive transcription profile under the applied experimental conditions.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *H. pylori* strains G27 and 26695 are clinical isolates (34, 45). Strain *H. pylori* G27/HP165::km, lacking the histidine kinase ArsS, has been described previously (2). *H. pylori* strain G27/ $\Delta$ arsS $\Delta$ nikR, carrying deletions of both the *arsS* and *nikR* genes, was constructed through allelic replacement by transformation of strain G27/HP165::km with the suicide plasmid pNko::cm. pNko::cm is a derivative of pNko::km (27), in which the kanamycin resistance cassette was replaced by a chloramphenicol resistance cassette of *Campylobacter coli* (42). Chromosomal DNA of the transformants was checked by PCR with primers flanking the integration site for the correct replacement of the *nikR* gene. *H. pylori* strains were grown at 37°C under microaerophilic conditions 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 (BHI) broth containing Dent's or Skirrow's antibiotic supplement and 10% fetal calf serum. When required, blood agar plates or liquid broth were supplemented with kanamycin or chloramphenicol in a final concentration of 20  $\mu\text{g/ml}$ . Acid exposure experiments were performed as follows: bacteria from a liquid culture were harvested at an optical density at 550 nm ( $\text{OD}_{550}$ ) of 0.7 by centrifugation and were resuspended in BHI broth containing fetal calf serum and an antibiotic supplement whose pH had been adjusted to pH 5.0 with hydrochloric acid. Incubation at 37°C under microaerophilic conditions was continued for 1 h. To study gene expression in

bacteria grown under mildly alkaline conditions, cells from a logarithmic culture ( $\text{OD}_{550} = 0.7$ ) were shifted to BHI broth whose pH had been adjusted to pH 8.0 or 8.5 with sodium hydroxide and cultivation was continued for 1 h. *Escherichia coli* DH5 $\alpha$  was grown in Luria-Bertani broth. When necessary, antibiotics were added to the following final concentrations: ampicillin, 100  $\mu\text{g/ml}$ ; kanamycin, 50  $\mu\text{g/ml}$ ; and chloramphenicol, 30  $\mu\text{g/ml}$ .

**RNA isolation.** *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. RNA preparations intended to be used for global transcriptional profiling were further purified using the RNeasy mini kit (QIAGEN) according to the manufacturer's RNA-cleanup protocol. Residual DNA was removed by on-column digestion during RNA purification with QIAGEN RNase-free DNase (QIAGEN). The RNA concentration was quantified by determination of absorbance at 260 and 280 nm, and RNA integrity was checked by visualization on a 1.5% agarose gel.

**Microarray hybridization and data analysis.** Transcriptome analyses were performed using a whole-genome microarray containing 1,649 PCR products generated with specific primer pairs derived from the genome sequences of *H. pylori* 26695 (34) and J99 (1), which were spotted in duplicate. Microarrays were produced as described previously (17). To determine genes which are differentially expressed in the ArsS-deficient mutant G27/HP165::km at pH 5.0, cDNA was prepared from RNA extracted from *H. pylori* G27 and G27/HP165::km after exposure of the bacteria to acidic pH for 1 h. A total of eight RNA samples from two independent preparations of RNA from strains G27 and G27/HP165::km were used for cDNA labeling and hybridization. Equal amounts of RNA (20  $\mu\text{g}$ ) were used to synthesize differentially labeled cDNA (Cy3-dCTP and Cy5-dCTP; Amersham Biosciences) during first-strand reverse transcription reactions with Superscript II reverse transcriptase and 6  $\mu\text{g}$  of random primers (Invitrogen). Dye reversal color swaps were performed as follows: a cDNA sample using Cy3-dCTP and another using Cy5-dCTP were generated, resulting in four labeled cDNAs per color swap. Synthesized cDNAs were purified by using QiaQuick PCR purification columns (QIAGEN) according to the manufacturer's protocol. Cy5-dCTP- and Cy3-dCTP-labeled cDNAs were combined and concentrated by evaporation in a SpeedVac at 45°C. Samples were diluted to 3 $\times$  SSC–0.1% sodium dodecyl sulfate (SDS) and hybridized to the *H. pylori* microarray (50°C, 18 h). The slides were washed for 5 min at room temperature with 0.1% SDS–0.1 $\times$  SSC and then two times with 0.1 $\times$  SSC. After being washed, the slides were scanned, using ScanArray HT, and analyzed by using ScanArray express software (Perkin Elmer). Spots were flagged and eliminated from analysis when the signal-to-background ratio was less than three or in obvious instances of high background or stray fluorescent signals. Median intensities of spots were background corrected, and differences in dye bias were normalized by using the LOWESS algorithm (46). The signal ratios used as measures of differential expression between the red and green channels were obtained from processed signal intensities. The ratios were further analyzed with Microsoft Excel (Microsoft) and SAM software for statistical significance (35). Only those genes which had at least seven unflagged replicates were considered after significance analysis by SAM (Table 1). The parameters used for significance analysis are indicated in the supplemental material (see Table S1 in the supplemental material).

To determine the significance of differential expression, RNA was isolated from the *H. pylori* G27 wild type grown in BHI broth (pH 5.0) and 20  $\mu\text{g}$  of this RNA was labeled with either Cy3-dCTP or Cy5-dCTP. The two cDNA probes generated were hybridized onto the same slide, and the data were analyzed as mentioned above. Signal ratios of <0.5 and >2.0 were analyzed further.

**Primer extension and RNA slot blot analysis.** Primer extension analysis was performed essentially as described previously (26), using 0.5 pMol of  $\gamma$ - $^{32}\text{P}$ -end-labeled oligonucleotides *amiE*-PE, *amiF*-PE, *rocF*-PE, 682-PE, 1104-PE, 1174-PE, 1398-PE, and 1563-PE (Table 2) and 30  $\mu\text{g}$  of RNA. Plasmids which were used as template DNA in the sequencing reactions performed with the aforementioned primers were constructed as follows. EcoRI-BamHI fragments of 328 bp and 517 bp were amplified from chromosomal DNA of *H. pylori* 26695 with primer pairs PamiE-5/PamiE-3 and PamiF-5/PamiF-3, respectively, and were ligated into the cloning vector pSL1180 (Amersham Biosciences) to yield plasmids pSL-P<sub>amiE</sub> and pSL-P<sub>amiF</sub>. Plasmid pSL-P<sub>rocF</sub> was obtained by cloning a 365-bp EcoRI-BamHI fragment that was amplified with primer pair ProcF-5/ProcF-3 into pSL1180 vector DNA. 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 to 100  $\mu\text{g}$ ) was denatured in 1 $\times$  MOPS (morpholinepropane-sulfonic acid) buffer containing 50% formamide and 6% formaldehyde. The

TABLE 1. ArsR~P-activated and -repressed genes

ArsR~P-regulated genes <sup>a</sup>	Category	ORF HP no. <sup>b</sup>	ORF JHP no. <sup>b</sup>	$\Delta arsS/WT^c$ ratio	Gene and function <sup>d</sup>	Genome organization <sup>e</sup>	pH-responsive transcription confirmed by indicated reference <sup>f</sup>	
Activated	Ammonia production	HP0067	JHP0062	0.36	Urease accessory protein, <i>ureH</i>	op HP0071–HP0067	21	
		HP0068	JHP0063	0.22	Urease accessory protein, <i>ureG</i>	op HP0071–HP0067	21	
		HP0069	JHP0064	0.39	Urease accessory protein, <i>ureF</i>	op HP0071–HP0067	21	
		HP0070	JHP0065	0.25	Urease accessory protein, <i>ureE</i>	op HP0071–HP0067	44	
		HP0071	JHP0066	0.21	Urea channel protein, <i>ureI</i>	op HP0071–HP0067	21, 44	
		HP0072	JHP0067	0.24	Urease subunit B, <i>ureB</i>	op HP0073–HP0072	21	
		HP0073	JHP0068	0.20	Urease subunit A, <i>ureA</i>	op HP0073–HP0072	6, 21	
		HP0294	JHP0279	0.06	Aliphatic amidase, <i>amiE</i>	m	6, 21, 44	
		HP1238	JHP1159	0.15	Formamidase, <i>amiF</i>	m	6, 21	
		Amino acid biosynthesis	HP0106	JHP0098	0.27	Cystathionine g-synthase, <i>metB</i>	op HP0107–HP0105 ?	6, 21
			HP0380	JHP1001	0.38	Glutamate dehydrogenase, <i>dghA</i>	m	21, 44
			HP0755	JHP0692	0.43	Molybdopterin biosynthesis protein, <i>moeB</i>	op HP0755–HP0757	6, 21
	Biosynthesis of cofactors, prosthetic groups, and carriers	HP0240	JHP0225	0.34	Octaprenyl-diphosphate synthase, <i>ispB</i>	op HP0243–HP0236 ?		
		HP0306*	JHP0291	0.42	Glutamate-1-semialdehyde 2,1-aminomutase, <i>hemL</i>	op HP0305–HP0308	21, 44	
		HP0824	JHP0763	0.37	Thioredoxin, <i>trxA</i>	op HP0824–HP0825	44	
		HP0825	JHP0764	0.31	Thioredoxin reductase, <i>trxB</i>	op HP0824–HP0825		
	Cell envelope	HP1224	JHP1145	0.39	Uroporphyrinogen III cosynthase, <i>hemD</i>	op HP1223–HP1225		
		HP1191	JHP1116	0.25	LPS heptosyltransferase, <i>rfaF</i>	m	6	
		HP1105	JHP1031	0.47	LPS biosynthesis protein	m		
		HP0078	JHP0073	0.19	Truncated outer membrane protein, <i>omp3</i>	op HP0078–HP0079	6, 44	
		HP0079	JHP0073	0.26	Outer membrane protein, <i>omp3</i>	op HP0078–HP0079	6, 44	
		HP0492	JHP0444	0.30	Predicted lipoprotein	m	6	
	Cellular processes	HP1456	JHP1349	0.48	Membrane-associated lipoprotein	op HP1457–HP1454	44	
		HP1564	JHP1472	0.36	Outer membrane lipoprotein	m		
		HP0389	JHP0992	0.45	Superoxide dismutase, <i>sodB</i>	op HP0389–HP0388		
		HP0390	JHP0991	0.18	Predicted thiol peroxidase	m		
		HP0875	JHP0809	0.43	Catalase, <i>kata</i>	m	44	
		HP1563	JHP1471	0.29	Alkyl hydroperoxide reductase, <i>ahpC</i>	m		
		HP1192	JHP1117	0.06	Secreted protein involved in flagellar motility	m	6, 44	
		HP0243	JHP0228	0.28	Neutrophil-activating protein, <i>napA</i>	op HP0243–HP0236 ?	44	
	DNA metabolism, restriction, and modification	HP1022	JHP0402	0.30	Predicted 5'-3' exonuclease	m	21, 44	
	Energy metabolism	HP0890	JHP0823	0.47	Predicted short-chain oxidoreductase	op HP0890–HP0888		
		HP0924	JHP0858	0.44	4-oxalocrotonate tautomerase, <i>dmpI</i>	m		
		HP0954	JHP0888	0.45	NADPH nitroreductase	op HP0961–HP0953 ?		
		HP1104	JHP1030	0.34	Predicted mannitol dehydrogenase	m	44	
			JHP0585*	0.27	Predicted 3-hydroxyacid dehydrogenase	op JHP0583–JHP0586	21	
	Fatty acid and phospholipid metabolism		JHP1429	0.29	Predicted mannitol dehydrogenase	m		
		HP0891	JHP0824	0.41	Predicted acyl coenzyme A thioesterase	m	44	
	Central intermediary metabolism	HP0757	JHP0649	0.41	Predicted carbon nitrogen hydrolase	op HP0755–HP0757	21	
	Protein fate	HP1332*	JHP1225	0.48	Cochaperone and heat shock protein	op HP1332–HP1330	21	
	Transport and binding proteins	HP0228	JHP0213	0.27	Predicted sulfate permease	m	6, 21	
		HP0889	JHP0822	0.40	Iron (III) dicitrate ABC transporter, permease	op HP0890–HP0888	44	
		HP1339	JHP1258	0.37	Biopolymer transport protein, <i>exbB2</i>	op HP1339–HP1341	21	
		HP1340*	JHP1259	0.47	Biopolymer transport protein, <i>exbD2</i>	op HP1339–HP1341	21	
		HP1341	JHP1260	0.48	Siderophore-mediated iron transport protein, <i>tonB2</i>	op HP1339–HP1341		
HP1331		JHP1251	0.47	Predicted branched-chain amino acid transport protein	op HP1332–HP1330	21		

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TABLE 1—Continued

ArsR~P-regulated genes <sup>a</sup>	Category	ORF HP no. <sup>b</sup>	ORF JHP no. <sup>b</sup>	$\Delta$ arsS/WT <sup>c</sup> ratio	Gene and function <sup>d</sup>	Genome organization <sup>e</sup>	pH-responsive transcription confirmed by indicated reference <sup>f</sup>
		HP1466	JHP1359	0.44	Predicted ABC transport system permease	op HP1466–HP1462 ?	6
	Unknown	HP1427	JHP1320	0.20	Histidine-rich protein, <i>hpn</i>	m	
		HP1432	JHP1321	0.07	Histidine-rich protein	m	6, 21
		HP1098	JHP1024	0.43	Cysteine-rich protein C, <i>hcpC</i>	op HP1100–HP1097 ?	
			JHP1437	0.16	Cysteine-rich protein	m	
		HP1225	JHP1146	0.27	Predicted CrcB integral membrane protein	op HP1223–HP1225	21, 44
	Hypothetical	HP0189	JHP0175	0.47	Hypothetical integral membrane protein	m	
		HP1286	JHP1206	0.48	Conserved hypothetical secreted protein	m	
		HP0081		0.04	Hypothetical protein	m	
		HP0118	JHP0110	0.19	Hypothetical protein	m	6, 21
		HP0120		0.17	Hypothetical protein	m	6
		HP0242*	JHP0227	0.33	Hypothetical protein	op HP0243–HP0236 ?	44
		HP0305	JHP0290	0.24	Hypothetical protein	op HP0305–HP0308 ?	44
		HP0307*	JHP0292	0.48	Hypothetical protein	op HP0305–HP0308 ?	6
		HP0423		0.35	Hypothetical protein	op HP0427–HP0423 ?	6
		HP0425		0.43	Hypothetical protein	op HP0427–HP0423 ?	
		HP0426		0.40	Hypothetical protein	op HP0427–HP0423 ?	6
		HP0641	JHP0584	0.27	Hypothetical protein	op HP640–HP0642	21
		HP0731	JHP0668	0.29	Hypothetical protein	op HP0733–HP0731	
		HP0733	JHP0670	0.47	Hypothetical protein	op HP0733–HP0731	
		HP0963	JHP0897	0.38	Hypothetical protein	op HP0966–HP0962	
		HP0964	JHP0898	0.37	Hypothetical protein	op HP0966–HP0962	
			JHP0899				
		HP1154	JHP1081	0.48	Hypothetical protein	op HP1154–HP1155	21
		HP1187	JHP1113	0.28	Hypothetical protein	m	21, 44
		HP1188		0.12	Hypothetical protein	m	6, 21, 44
		HP1223	JHP1144	0.37	Hypothetical protein	op HP1223–HP1225	
		HP1408	JHP1300	0.20	Hypothetical protein	op HP1408–HP1412 ?	
		HP1412	JHP1307	0.32	Hypothetical protein	op HP1408–HP1412 ?	44
			JHP0959	0.32	Hypothetical protein	m	
Repressed	Cell envelope	HP0229	JHP0214	2.51	Outer membrane protein, <i>omp6</i>	m	6, 21, 44
		HP0722	JHP0659	3.87	Outer membrane protein, <i>omp16</i>	m	6, 21
		HP0725	JHP0662	5.68	Outer membrane protein, <i>omp17</i>	m	6, 21
		HP0788	JHP0725	2.64	Predicted outer membrane protein, <i>hofF</i>	m	
		HP1083	JHP0342	2.79	Predicted outer membrane protein, <i>hofB</i>	m	
	Cellular processes	HP1167	JHP1094	5.33	Predicted outer membrane protein, <i>hofH</i>	m	6, 21
		HP1560*	JHP1468	2.24	Cell division protein, <i>ftsW</i>	m	6
		HP0017	JHP0015	2.22	virB4 homolog involved in natural competence, <i>comB4</i>	op HP0015–HP0017	
		HP1527	JHP1416	3.86	Protein involved in natural competence, <i>comH</i>	m	6, 21
		HP1585	JHP1492	2.09	Flagellar basal-body rod protein		
	DNA metabolism, restriction, and modification	HP1526	JHP1415	2.14	Exodeoxyribonuclease, <i>lexA</i>	m	
	Energy metabolism	HP1398	JHP1428	4.31	Alanine dehydrogenase, <i>ald</i>	m	
		HP1399	JHP1427	3.23	Arginase, <i>rocF</i>	m	
	Protein fate	HP0109*	JHP0101	3.00	Chaperone and heat shock protein 70, <i>dnaK</i>	op HP0111–HP0109	21
		HP0110	JHP0102	2.86	Cochaperone and heat shock protein, <i>grpE</i>	op HP0111–HP0109	21
	Purines, pyrimidines, nucleosides, and nucleotides	HP0382*	JHP0999	2.36	Predicted zinc-metalloprotease	op HP0382–HP0381	21
		HP1178	JHP1104	3.78	Purin nucleoside phosphorylase, <i>deoD</i>	op HP1180–HP1178	21
	Transcription regulation	HP1179	JHP1105	3.86	Phosphopentamutase	op HP1180–HP1178	
		HP0166	JHP0152	2.20	Two-component response regulator, <i>arsR</i>	op HP0166–HP0162	6

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TABLE 1—Continued

RArsR~P-regulated genes <sup>a</sup>	Category	ORF HP no. <sup>b</sup>	ORF JHP no. <sup>b</sup>	$\Delta$ arsS/WT <sup>c</sup> ratio	Gene and function <sup>d</sup>	Genome organization <sup>e</sup>	pH-responsive transcription confirmed by indicated reference <sup>f</sup>
	Transport and binding proteins	HP0686	JHP0626	4.06	Iron(III) dicitrate transport protein, <i>fecAI</i>	m	
		HP1129	JHP1057	2.81	Biopolymer transport protein, <i>exbD1</i>	op HP1137–HP1123 ?	
		HP1130	JHP1058	3.19	Biopolymer transport protein, <i>exbB1</i>	op HP1137–HP1123 ?	
		HP1174	JHP1101	5.65	Glucose/galactose transporter	m	
		HP1180	JHP1106	4.17	Pyrimidine nucleoside transport protein	op HP1180–HP1178	6, 21
	Hypothetical	HP1175	JHP1102	2.12	Hypothetical integral membrane protein	m	6, 21
		HP0681	JHP0622	10.40	Hypothetical protein	op HP0682–HP0681	
		HP0682	JHP0623	16.56	Hypothetical protein	op HP0682–HP0681	
		HP0688*	JHP0628	2.65	Hypothetical protein	op HP0688–HP0689	
		HP0689	JHP0628	2.02	Hypothetical protein	op HP0688–HP0689	
		HP0709	JHP0648	3.97	Hypothetical protein	m	6
		HP0947	JHP0881	3.74	Hypothetical protein	m	
		HP1288*	JHP1208	4.48	Hypothetical protein	op HP1288–HP1289	6
		HP1289	JHP1209	22.77	Hypothetical protein	op HP1288–HP1289	
		HP1322	JHP1242	2.13	Hypothetical protein	m	21

<sup>a</sup> Genes listed are those whose transcription at pH 5.0 differed more than twofold (ratio, >2.0 or <0.5) for the ArsS-deficient mutant *H. pylori* G27/HP165::km compared to that for the G27 wild type, according to microarray analysis.

<sup>b</sup> ORF numbers and the prediction of transcriptional units are based on the genome sequences of *H. pylori* 26695 and J99 (1, 34). Asterisks indicate ORFs which were determined by the SAM algorithm to be not statistically significant (see Table S1 in the supplemental material) but which were retained for data evaluation because they are part of a predicted operon structure with other differentially expressed genes or because their pH-responsive transcription was confirmed in another study.

<sup>c</sup> WT, wild type.

<sup>d</sup> The functional annotation is that used by the PyloriGene database (<http://www.pasteur.fr/english.html>).

<sup>e</sup> m, monocistronically transcribed genes; op, putative transcriptional unit. Question marks indicate that the proposed operon structures cannot be unambiguously deduced from the genome sequences.

<sup>f</sup> Where a reference(s) is given, pH-responsive transcription was confirmed by the studies of Merrell et al. (21), Wen et al. (44), or Bury-Moné et al. (6).

samples were incubated at 65°C for 5 min and cooled on ice before one volume of 20× SSC was added. The denatured samples were filtered through a positively charged nylon membrane (Hybond N<sup>+</sup>, Amersham Biosciences), using a Bio-Dot chamber (Bio-Rad). After UV cross-linking was performed, the nylon mem-

brane was prehybridized for 1 h at 42°C in hybridization buffer (ECL gold hybridization buffer; Amersham Biosciences). The PCR products used as hybridization probes were amplified with primer pairs 229-5/229-3, 725-5/725-3, 686-5/686-3, and 16S-5/16S-3 and were labeled nonradioactively, using the ECL Direct

TABLE 2. Oligonucleotides used in this study

Oligonucleotide or primer	Sequence (5' to 3') <sup>a</sup>	Site <sup>b</sup>	Orientation of strand	Position <sup>c</sup>
PamiE-5	<u>gattttgaattc</u> TATTGTATTAACGCGCTATATGG	EcoRI	+	310783–310805
PamiE-3	attttc <u>ggatcc</u> TCATTCTTAGTGTGGAGTCTAGG	BamHI	–	311089–311111
PamiF-5	caagct <u>gaattc</u> TGGTCATCATGGGAGCAACC	EcoRI	+	1311723–1311742
SamiF-5	ccagcaga <u>attc</u> AGAAAAGTAGCCAGGTCCTAA	EcoRI	+	1311918–1311938
PamiF-3	aatatc <u>ggatcc</u> GCTATTGACAATTGGCACAGG	BamHI	–	1312219–1312239
ProcF-5	atcctt <u>gaattc</u> GATTAGTGCCACATCATCAGG	EcoRI	+	1459647–1459667
ProcF-3	agccat <u>ggatcc</u> GCTTAAAGCCTCTCTCAAACG	BamHI	–	1459992–1460012
amiE-PE	TAGGCATCTTATAATTAATACC	–	–	311068–311092
amiF-PE	AGGAAACTGAATGGCTGCC	–	–	1312203–1312221
rocF-PE	GCTTAAAGCCTCTCTCAAACG	–	–	1459992–1460012
682-PE	ATCCACTCATAACAAATC	–	–	732444–732461
1104-PE	TAGCAAACCTTTAGATTGAAC	–	–	1165334–1165355
1174-PE	GAATAGCGCTGTCAAACCTC	–	–	1241646–1241665
1398-PE	CTCGGGATTCTAAATCCATGC	–	–	1459679–1459699
1563-PE	GGCAGGTGCTTTAAAGTCTGG	–	–	1645245–1645265
229-5	ATGAAAAAACGATTTTACTT	–	–	240139–240159
229-3	TTGATTAAGGTTTTTATTGAA	–	–	239440–239460
725-5	AAGCAAAGCATTCAAACGCC	–	–	780757–780777
725-3	GATGTCTTTAGCGAATTTAGG	–	–	780058–780078
686-5	ATGAAAAGAATTTTACTCTCT	–	–	737126–737146
686-3	GTTGTATCTAAACCTTGCC	–	–	736427–736447
16S-5	GTTAAGAGATCAGCCTATGTCC	–	–	1208855–1208876
16S-3	TGGCAATCAGCGTCAGGTAATG	–	–	1208356–1208377

<sup>a</sup> Sequences in uppercase letters are derived from the genome sequences of *H. pylori* 26695 (34). Sequences introduced for cloning purposes are given in lowercase letters, and restriction recognition sequences are underlined.

<sup>b</sup> Restriction recognition sites.

<sup>c</sup> Nucleotide positions refer to the genome sequence of *H. pylori* 26695 (34).

nucleic acid-labeling system (Amersham Biosciences) 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°C. The membrane was washed two times in prewarmed (42°C) wash solution I (6 M urea, 0.5× SSC, 0.4% SDS) for 20 min at 42°C and two times in wash solution II (2× SSC) at room temperature. For signal detection, the ECL detection system (ECL Direct Nucleic Acid Labeling and Detection system; Amersham Biosciences) and X-ray films (Konica Minolta) were used.

**DNase I footprint analysis.** The recombinant N-terminally His<sub>6</sub>-tagged response regulator ArsR encoded on plasmid pQE-166 was overexpressed in *E. coli* M15[pREP4] (QIAGEN) and was purified by affinity chromatography on Ni<sup>2+</sup>-nitrilotriacetic acid agarose essentially as described previously (2). In vitro phosphorylation of His<sub>6</sub>-ArsR was performed as described by Dietz et al. (10). Plasmids pSL-*P<sub>amiE</sub>*, pSL-*P<sub>amiF</sub>*, and pSL-*P<sub>rocF</sub>* were used for the generation of end-labeled DNA probes for DNase I footprint experiments. pSL-*P<sub>amiF</sub>* was created by ligating a 318-bp EcoRI-BamHI fragment derived from the upstream region of the *amiF* gene, which was amplified with primer pair SamiF-5/PamiF-3 into pSL1180. The promoter DNA fragments were 5'-end labeled with [<sup>γ</sup>-<sup>32</sup>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. (8). 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<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 1 mM dithiothreitol).

**Microarray accession numbers.** The microarray raw data were deposited in the NCBI Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>) and are accessible with the accession number GSE4293.

## RESULTS

**Whole-genome transcriptional profiling of an ArsS-deficient mutant of *H. pylori* G27 exposed to pH 5.0.** Low pH has been demonstrated to be a signal triggering the autophosphorylation of the histidine kinase ArsS and the subsequent phosphorylation of the cognate response regulator ArsR (26). In order to define the complete regulon controlled by ArsR~P, a transcriptome analysis was performed using a whole-genome microarray containing 1,649 PCR products generated with specific primer pairs derived from the genome sequences of *H. pylori* 26695 (34) and J99 (1) and comprising 98% of the coding sequences present in both genomes (17). RNA was extracted from two independent cultures of *H. pylori* G27 and G27/HP165::km, grown to an OD<sub>550</sub> of 0.7, and then exposed to pH 5.0 for one hour. Cy5- and Cy3-labeled cDNA was prepared from these RNAs, and the differentially labeled cDNA pairs derived from the *H. pylori* wild type and *arsS* mutant were hybridized to four independent microarray slides, creating eight sets of hybridization data. A total of 109 genes was identified to be differentially expressed in the *arsS* mutant at pH 5.0 by using a signal ratio cut off of <0.5 and >2.0 (Table 1). Seventy-five genes were positively regulated by ArsR~P, while the transcription of 34 genes was repressed by ArsR~P. In accordance with our previous observation that the promoters of both *ureA*(*P<sub>ureA</sub>*) and *ureI*(*P<sub>ureI</sub>*) are positively regulated by ArsR~P (26, 27), transcription of the complete urease gene cluster was reduced in the *arsS* mutant, as well as transcription of *amiE* and *amiF*, which encode additional ammonia-producing enzymes. Transcription of *rocF*, which encodes arginase, was found to be repressed by ArsR~P. Furthermore, global transcriptional profiling confirmed that ArsR~P acts as a negative autoregulator (10). Transcription of open reading frames (ORFs) HP0165 to HP0162 was reduced in the *arsS* mutant, due to the insertion of the kanamycin resistance cassette into ORF HP0165/HP0164 and its polar effect on expression of the

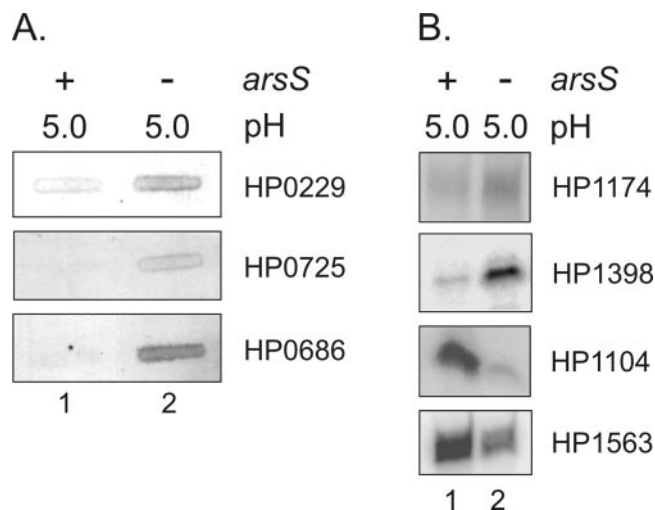


FIG. 1. Analysis of transcription of selected ArsR~P target genes by slot blot Northern hybridization (A) and primer extension (B). Equal amounts of RNA extracted from *H. pylori* G27 (lane 1) and G27/HP165::km (lane 2) exposed to pH 5.0 for 1 h were used in the respective experiments performed with DNA probes or radioactively labeled oligonucleotides specific for the indicated ORFs. RNA slot blot analysis with a labeled probe specific for 16S RNA was performed as a control (not shown). +, presence of gene; -, absence of gene.

downstream genes encoding  $\delta$ -aminolevulinic acid dehydratase (HP0163) and a conserved hypothetical protein of unknown function (HP0162). The ArsR~P-regulated genes were grouped into several categories according to their cellular functions and encode mainly proteins affecting the composition of the cell envelope, transport and binding proteins, detoxifying enzymes, and *H. pylori*-specific proteins of unknown function (Table 1).

To confirm the differential expression of the identified ORFs, seven genes not part of putative operon structures were selected and their transcription was monitored by Northern slot blot analysis or primer extension analysis performed with RNA extracted from the G27 wild type and the isogenic ArsS-deficient mutant exposed to pH 5.0. The selected genes encode two outer membrane proteins (*omp6*, HP0229; *omp17*, HP0725), the ferric citrate receptor *FecA1* (HP0686), a glucose/galactose transporter (HP1174), alanine dehydrogenase (HP1398), mannitol dehydrogenase (HP1104), and alkyl hydroperoxide reductase (HP1563). As shown in Fig. 1 and in agreement with the results from the microarray analysis, in these experiments the transcription of *omp6*, *omp17*, *fecA1*, HP1174, and HP1398 was found to be depressed in the absence of the histidine kinase ArsS, while transcription of HP1104 and HP1563 was reduced in the ArsS-deficient mutant.

**Acid-induced transcription of *amiE* and *amiF* is mediated directly by the binding of ArsR~P to the promoters of the amidase genes.** From the genome organization of *H. pylori* it can be deduced that both *amiE* and *amiF* are monocistronically transcribed. To confirm the ArsR~P-dependent transcription of the amidase genes observed in the transcriptome analyses, primer extension experiments with RNA extracted from *H. pylori* G27 and the isogenic ArsS-deficient mutant grown at neutral pH and exposed to pH 5.0 were performed. The transcriptional start site of *amiE* was mapped to position -44 with respect to the translational start codon (Fig. 2A). The



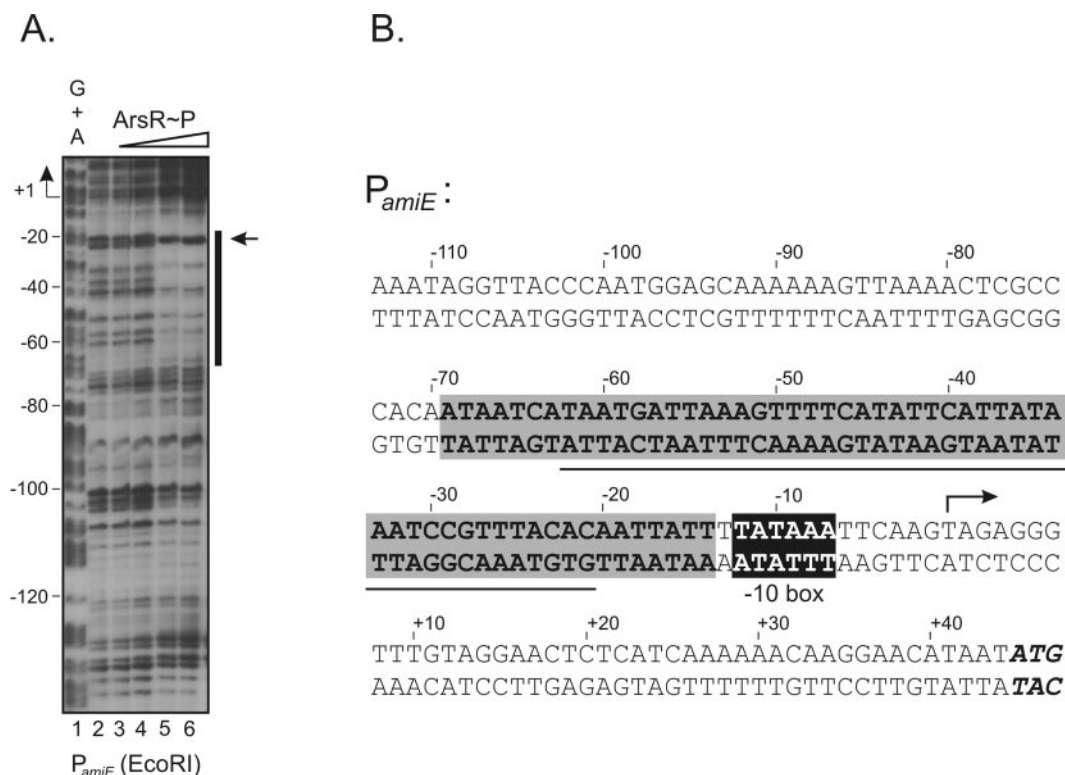


FIG. 3. Binding of ArsR~P to the  $P_{amiE}$  promoter. (A) DNase I footprint experiments were performed on a 328-bp EcoRI-BamHI fragment containing the  $P_{amiE}$  promoter derived from plasmid pSL- $P_{amiE}$ , which was end labeled at the EcoRI terminus by adding increasing amounts of His<sub>6</sub>-ArsR phosphorylated in vitro by acetylphosphate. In lanes 2 to 6, His<sub>6</sub>-ArsR is present in concentrations of 0 (lane 2), 0.37 (lane 3), 0.75 (lane 4), 3.0 (lane 5), and 4.5  $\mu$ M (lane 6). The numbers on the left indicate nucleotide positions with respect to the transcriptional start site, which is marked by an arrow. The solid bar on the right indicates the region of maximum DNase I protection. The arrow on the right indicates a band of hypersensitivity to DNase I digestion. Lane 1 contained a G+A sequence reaction mixture with the DNA probe used as a size marker (19). (B) Schematic representation of the  $P_{amiE}$  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 gray box indicates the region with maximum protection from DNase I digestion by ArsR~P binding to the  $P_{amiE}$  promoter probe labeled at the EcoRI-terminus. The black bar below the sequence indicates the minimum size of the ArsR~P binding site protected from DNase I digestion. Numbers above the sequence indicate the nucleotide position with respect to the transcriptional start site (+1, not shown). The translational start codon of the *amiE* gene is shown in italics.

from position  $-13$  to  $-50$  with respect to the transcriptional start site of *amiF* was protected from DNase I digestion. As already observed for the  $P_{amiE}$  promoter, no binding to the upstream region of *amiF* could be detected when the footprint experiment was performed with unphosphorylated ArsR protein (data not shown). Therefore, we conclude that acid-responsive transcription of the amidase genes is regulated by the ArsRS two-component system via the direct binding of ArsR~P to extended regions overlapping the  $-35$  region of the  $P_{amiE}$  and  $P_{amiF}$  promoters.

**ArsR~P-dependent repression of *rocF* and HP0682 is not responsive to low pH.** Global transcriptional profiling revealed that at pH 5.0 transcription of the arginase gene *rocF* is derepressed in the ArsS-deficient mutant of *H. pylori* G27. However, in none of the previous transcriptome studies analyzing pH-responsive gene regulation in *H. pylori* (6, 21, 44) was expression of *rocF* reported to be repressed at low pH. To further investigate these conflicting observations, transcription of *rocF* was analyzed by primer extension experiments carried out on RNA extracted from *H. pylori* G27 and the isogenic ArsS-deficient mutant grown at neutral pH and exposed to pH 5.0. As shown in Fig. 5A, the 5' end of the *rocF*-specific mRNA

was mapped to position  $-29$  with respect to the translational start site of the *rocF* gene, corresponding to a  $-10$  promoter hexamer of the sequence TAGAAT. An almost equal amount of *rocF*-specific transcript was detected in the G27 wild type irrespective of whether the bacteria were cultivated at pH 7.0 or exposed to pH 5.0. In the *arsS* mutant, transcription of *rocF* was strongly derepressed at both growth conditions; however, an approximately threefold-higher amount of *rocF*-specific transcript was detected at pH 5.0. This result demonstrated that the promoter of the *rocF* gene ( $P_{rocF}$ ) is repressed by ArsR~P; however, growth of *H. pylori* G27 at neutral or acidic pH, supposed to cause alterations of the cellular level of phosphorylated ArsR, did not result in differential transcription of *rocF*. To test whether cultivation of *H. pylori* at mildly basic pH might relieve the ArsR~P-dependent repression of  $P_{rocF}$ , transcription of the *rocF* gene was analyzed by primer extension performed on RNA extracted from strain G27, which was exposed to pH 8.0 or pH 8.5 for 1 h. It has been reported previously that a pH of 8.0 still allows growth of *H. pylori* in the absence of urea (30). No significant change in the amount of *rocF*-specific transcripts was observed under these conditions



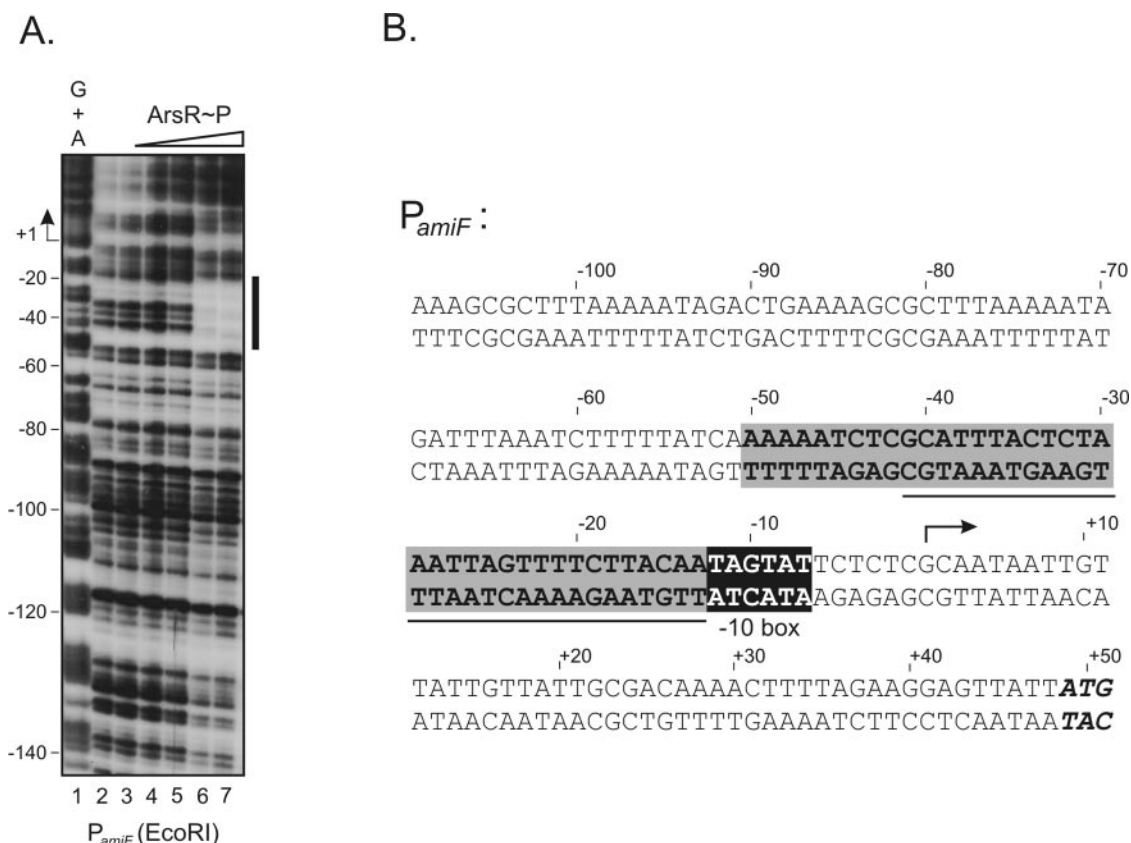


FIG. 4. Binding of ArsR~P to the  $P_{amiF}$  promoter. (A) DNase I footprint experiments were performed on a 318-bp EcoRI-BamHI fragment containing the  $P_{amiF}$  promoter, which was end labeled at the EcoRI terminus by adding increasing amounts of His<sub>6</sub>-ArsR phosphorylated in vitro by acetylphosphate. In lanes 2 to 7, His<sub>6</sub>-ArsR is present in concentrations of 0 (lane 2), 0.37 (lane 3), 0.75 (lane 4), 1.5 (lane 5), 3.0 (lane 6), and 4.5  $\mu$ M (lane 7). The numbers on the left indicate nucleotide positions with respect to the transcriptional start site, which is marked by an arrow. The solid bar on the right indicates the maximum region of DNase I protection. Lane 1 contained a G+A sequence reaction mixture with the DNA probe used as a size marker (19). (B) Schematic representation of the  $P_{amiF}$  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 gray box indicates the region with maximum protection from DNase I digestion by response regulator binding to the  $P_{amiF}$  promoter probe labeled at the EcoRI terminus. The black bar below the sequence indicates the minimum size of the ArsR~P binding site protected from DNase I digestion. Numbers above the sequence indicate the nucleotide position with respect to the transcriptional start site (+1, not shown). The translational start codon of the *amiF* gene is given in italics.

compared to that of strain G27 grown at neutral pH (data not shown).

In our transcriptome analysis, the paralogous ORFs HP0682 and HP1289, which encode *H. pylori*-specific proteins of unknown function, showed the highest ratio of ArsR~P-dependent repression. Both genes are organized in bicistronic transcriptional units with the ORFs HP0681 and HP1288, respectively, and had been previously shown to be negatively regulated by ArsR~P (15). However, as is the case with *rocF*, pH-dependent repression of these genes has not been noted in previous studies (6, 21, 44). The transcriptional start site of ORF HP0682 was mapped by Forsyth et al. (15) to position -6 with respect to the annotated translational start codon, corresponding to a TATAAA -10-box hexamer. When the transcription of ORF HP0682 was compared in the G27 wild-type strain and the ArsS-deficient mutant grown at neutral pH or exposed to pH 5.0, respectively, the same expression profile as observed for the *rocF* gene was detected (Fig. 5B). Similarly, no significant change in the amount of HP0682-specific transcript was detected when the

bacteria were shifted from pH 7.0 to pH 8.0 or pH 8.5 (data not shown). Therefore, we conclude that the transcription of a subset of genes which are clearly regulated by ArsR~P is not responsive to pH changes in a range from pH 5.0 to 8.5.

**ArsR~P binds to a high-affinity binding site overlapping the  $P_{rocF}$  promoter.** To investigate whether transcription of *rocF* is regulated directly by ArsR~P, DNase I footprint experiments were carried out on a 365-bp DNA probe comprising the upstream region of the *rocF* gene (Fig. 6). In the presence of 0.37 to 0.75  $\mu$ M ArsR which was phosphorylated in vitro with acetylphosphate, a region protected from DNase I digestion, ranging from position -6 to -67 with respect to the transcriptional start site and overlapping the -10 promoter box, became visible. When the binding reactions were performed with unphosphorylated ArsR protein, footprinting of this region could be observed at a protein concentration of 4.5  $\mu$ M (data not shown). Therefore, the results of the footprint experiments demonstrate that transcription of the *rocF* gene is repressed by ArsR~P, which binds with high affinity to a region overlapping the -10 box of the  $P_{rocF}$  promoter.

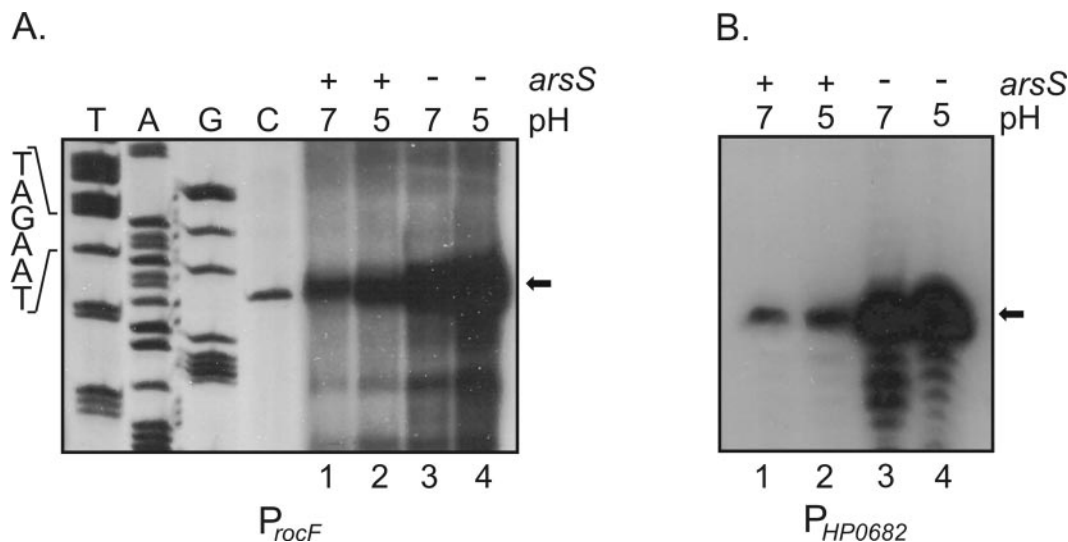


FIG. 5. Analysis of transcription of *rocF* and ORF HP0682 in *H. pylori* G27 and G27/HP165::km. (A) Primer extension experiments using the radiolabeled oligonucleotide rocF-PE were performed on RNAs extracted from the wild-type strain G27 grown at pH 7.0 and pH 5.0 (lanes 1 and 2) and the ArsS-deficient mutant G27/HP165::km grown at neutral pH and pH 5.0 (lanes 3 and 4). The elongated primer products are indicated by an arrow on the right. The sequences of the  $-10$  element of the  $P_{rocF}$  promoter is given on the left. The sequencing ladder (lanes T, A, G, C) was obtained by annealing primer rocF-PE to plasmid pSL- $P_{rocF}$ . (B) Primer extension experiments using the radiolabeled oligonucleotide 682-PE were performed on RNAs extracted from strains G27 (lanes 1 and 2) and G27/HP165::km (lanes 3 and 4) grown at pH 7.0 (lanes 1 and 3) and exposed to pH 5.0 for 1 h (lanes 2 and 4). The cDNAs specific for ORF *hp682* are indicated by an arrow on the right. +, presence of gene; -, absence of gene.

## DISCUSSION

*H. pylori* is a highly specialized bacterium which, to our knowledge, colonizes the human stomach as its unique habitat and, therefore, is endowed with highly efficient acid acclimation mechanisms distinguishing it from other neutralophilic bacteria. Due to the continuous alteration between starvation and digestive phases following food intake, *H. pylori* is likely to encounter considerable pH fluctuations within its niche, posing the necessity of efficient modulation of the pH-adaptive response. Three independent global transcriptome studies have been performed so far in order to completely define the pH-responsive stimulon comprising the genes involved in acid acclimation. However, there was only a rather low overlap among the data raised in the individual studies, reflected by a total of 429 genes reported to be differentially expressed upon acid exposure added from the three individual data sets comprising between 101 and 279 genes (6, 21, 44). This discrepancy might be explained by differences in the experimental setups, including the particular *H. pylori* strains analyzed. The ArsRS two-component system has been identified previously as an important regulator of the acid response, mediating the low-pH induction of urease expression upon phosphorylation of the response regulator ArsR (26, 27). Therefore, we intended to characterize the acid-responsive regulon controlled by ArsR~P by transcriptional profiling of *H. pylori* G27 and an isogenic mutant lacking the histidine kinase gene *arsS*. The level of transcription of 109 genes differed more than twofold between the two strains, with 75 genes being positively regulated by ArsR~P and 34 genes being repressed by ArsR~P. Acid-responsive regulation of 64 of these genes (48 induced, 16 repressed) has previously been reported on the basis of global transcriptome analysis (6, 21, 44). From the 109 genes sup-

posed to be regulated by ArsR~P, 45 are predicted to be monocistronically transcribed (pH regulation of 25 of these genes was confirmed in the previous array studies) according to the genome sequences of *H. pylori* 26695 and J99 (1, 34), while the remaining genes are members of 33 predicted transcriptional units. In 10 of these transcriptional units, transcription of all the members of the respective operon was found to be altered in the ArsS-deficient mutant, and in 5 predicted operons comprising three or more genes, all but one of their members were found to be differentially regulated. Most convincingly, in accordance with our previous observation that the promoters located upstream of *ureAB* and *ureIEFGH* are controlled by ArsR~P (26, 27), in our microarray analysis transcription of the complete urease gene cluster was found to be altered, which was not observed in previous global transcriptome studies (6, 21, 44). Differential expression of 11 genes identified in the microarray analysis was confirmed by Northern and primer extension analysis. pH-responsive or ArsR~P-dependent regulation of four of these genes had not been reported so far. The highest ratio of transcriptional induction by ArsR~P showed ORFs HP0081 (25-fold) and HP0079 (17-fold), encoding a hypothetical protein and a secreted protein supposed to be involved in flagellar motility, respectively, and the amidase gene *amiE* (16-fold) and ORF HP1432 (15-fold), encoding a histidine-rich protein predicted to be involved in nickel binding, *amiF* and ORF HP1427, encoding another histidine-rich protein, exhibited an approximately threefold-lower transcriptional induction than their respective paralogous genes. The highest ratio of ArsR~P-dependent repression was observed with ORFs HP0682 and HP1289 (16- and 22-fold, respectively), encoding hypothetical proteins of unknown function (see below). Induction of amidase expression in response

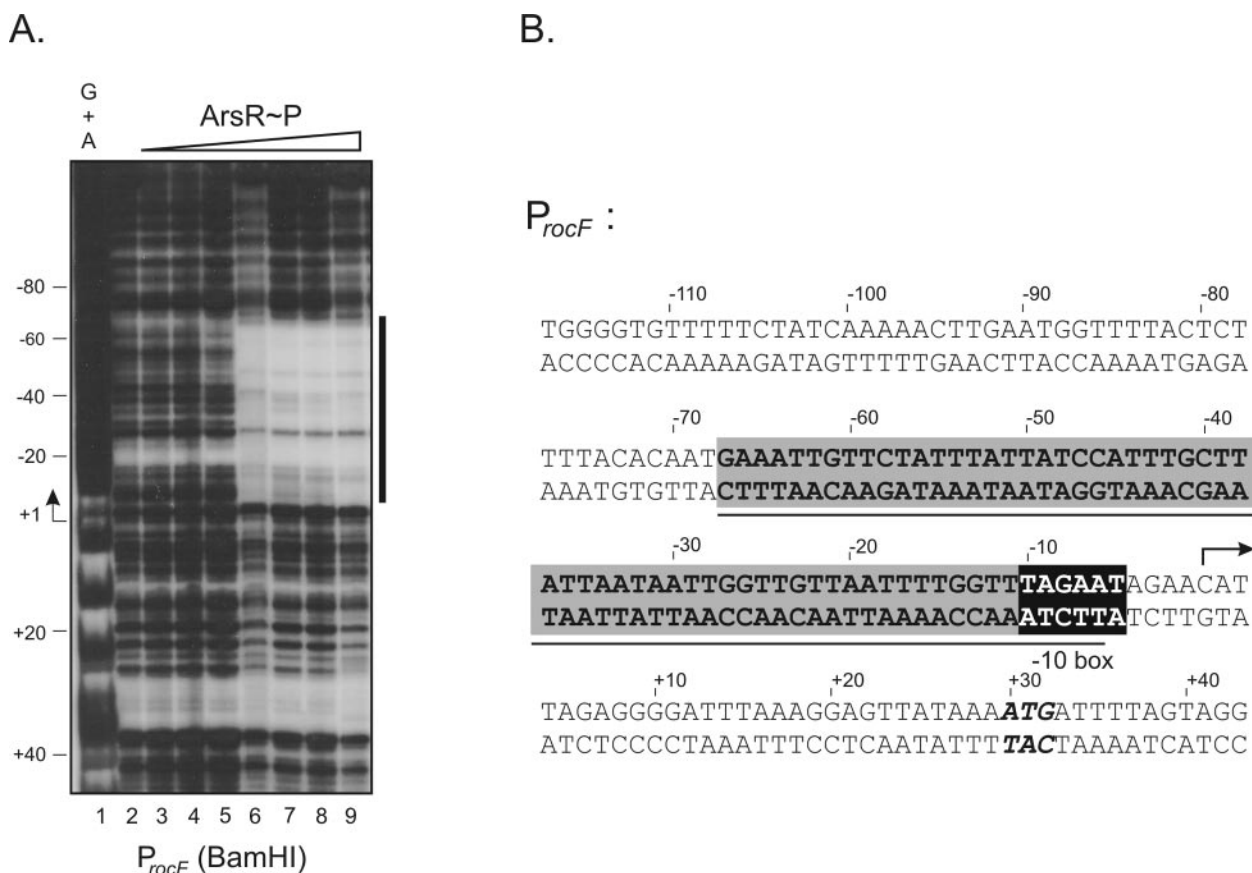


FIG. 6. Binding of ArsR~P to the  $P_{rocF}$  promoter. (A) DNase I footprint experiments were carried out on the  $P_{rocF}$  promoter fragment labeled at the BamHI terminus. In lanes 2 to 9, His<sub>6</sub>-ArsR was added in concentrations of 0 (lane 2), 0.09 (lane 3), 0.18 (lane 4), 0.37 (lane 5), 0.75 (lane 6), 1.5 (lane 7), 3.0 (lane 8) and 4.5  $\mu$ M (lane 9). The numbers on the left indicate nucleotide positions with respect to the transcriptional start site, which is marked by an arrow. The solid bar on the right indicates the region of DNase I protection. Lane 1 contained a G+A sequence reaction mixture with the DNA probe used as a size marker (19). (B) Schematic representation of the  $P_{rocF}$  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 gray box indicates the region protected from DNase I digestion by response regulator binding to the  $P_{rocF}$  promoter probe. The black bar below the sequence highlights the complete ArsR~P binding site, which covers the -10 promoter element. Numbers above the sequence indicate the nucleotide position with respect to the transcriptional start site (+1, not shown). The translational start codon of the *rocF* gene is given in italics.

to low pH has been observed previously (6, 21, 40, 44) and suggests an important role for these ammonia-producing enzymes in the acid adaptation of *H. pylori*. However, the amidase genes are not essential for colonization in the mouse model of *H. pylori* infection (5). Here we demonstrate that expression of the three major ammonia-producing pathways—urease, AmiE, and AmiF—is under the control of the ArsRS two-component system. Interestingly, besides the nickel-binding proteins HP1432 and HP1427, expression of a number of detoxifying enzymes (thiol peroxidase, alkyl hydroperoxide reductase, catalase, and superoxide dismutase) was induced by ArsR~P. This might reflect protection against the toxic effects caused by high intracellular concentrations of the bivalent metal ions Ni<sup>2+</sup> and Fe<sup>2+</sup>, whose solubility and therefore bioavailability increase under acidic conditions. In particular, Fe<sup>2+</sup> is known to be involved in the triggering of radical reactions whose products need to be detoxified. In this context, it should be emphasized that low pH was shown to induce the expression of NikR, which is a repressor of *fur* transcription (9, 40). As a consequence, the amount of Fur protein present under acidic con-

ditions might be too low to allow efficient repression of the iron uptake systems, increasing the necessity of protection against the toxic effects of Fe<sup>2+</sup>. ArsR~P is also involved in changing the composition of the outer membrane in response to low pH by regulating the expression of several outer membrane proteins and of enzymes involved in lipopolysaccharide (LPS) biosynthesis. Although an intriguing speculation, the pI of these outer membrane proteins and therefore their buffering capacity seem not to be correlated with their predominant expression at neutral or acidic pH. Changes in LPS composition upon exposure of *H. pylori* to acidic conditions are known to occur (23) and might involve ORFs HP1191 and HP1105, regulated by ArsR~P. A number of transport-associated proteins were also found to be both positively and negatively regulated by ArsR~P, possibly reflecting adaptation to changes in the supply of nutrients, which might be associated with the fluctuations in gastric pH. However, about one-third of the genes regulated by ArsR~P encode hypothetical proteins whose roles in the acid acclimation of *H. pylori* remain to be elucidated.

As observed previously, the ArsR~P regulon and the Ni<sup>2+</sup>-



responsive NikR regulon overlap to some extent and have several genes involved in nickel homeostasis in common, such as the urease genes and HP1432 and HP1427, whose products are involved in nickel storage (7, 38). No significant effect of ArsR~P on the transcription of *nikR* was observed, indicating that the acid-induced increase in NikR expression (40) is mediated by an additional pH-responsive regulatory system. In the transcriptome studies performed by Merrell et al. (21), Wen et al. (44), and Bury-Moné et al. (6), four genes (HP0624, HP1050, HP1440, and HP1457) were consistently found to be differentially expressed at low pH versus neutral pH, but no influence of ArsR~P on their transcription was detected. Therefore, it is likely that such systems are present in *H. pylori* despite its general paucity of transcriptional regulators. Interestingly, of the 29 genes in common between our study and the transcriptome analysis performed by Bury-Moné et al. (6), 20 were reported to have lost their pH-responsive transcription profile in an *H. pylori* double mutant deficient in both NikR and Fur, suggesting a prominent role for these proteins as coregulators in the control of pH-responsive gene expression. The genes aberrantly transcribed in the *nikR fur* mutant at pH 5.0 included *ureA*, *amiE*, *omp16*, *omp17*, and *arsR* itself, while pH-responsive transcription of HP1432, *amiF* and *omp3* was retained in the double mutant (6). Gancz et al. (16) reported recently that several members of the ArsR~P regulon were aberrantly transcribed at pH 5.0 in a Fur-deficient mutant that includes *amiE*, HP1432, and *arsR* (16). However, we could not detect any significant effect on *ureA* transcription in a NikR-deficient mutant grown in standard broth at pH 7.0 and pH 5.0 (27), and no influence of Fur on pH-responsive transcription of the urease genes was observed in the study of Gancz et al. (16). Furthermore, no indications for metal-responsive regulation of *arsR* by Fur and NikR were obtained in recent microarray analyses (7, 12). These partially conflicting data point out the complex interplay between ArsR and the metal-dependent regulators, which must be dissected in future studies. Note also that we and Bury-Moné et al. (6), in accordance with the mapping of an ArsR~P binding site downstream of the promoter of the *arsR* gene (10), detected repression of *arsR* transcription at pH 5.0, while an increase in *arsR* transcription was reported in the transcriptome study of Wen et al. (44).

The finding that ArsR~P footprinted the  $P_{amiE}$  and  $P_{amiF}$  promoters clearly demonstrated that the pH-responsive regulation of the amidase genes is mediated directly by the ArsRS two-component system (Fig. 3 and 4). While in the case of *amiE* we detected a basal level of ArsR~P-independent transcription, as was previously observed for the *ureA* and *ureI* genes (26, 27), transcription from the  $P_{amiF}$  promoter absolutely required ArsR~P, since no *amiF*-specific transcript could be detected in the ArsS-deficient mutant, irrespective of the pH of the growth medium (Fig. 2). In the *arsS* mutant, we found a slight increase in *amiE* transcription at low pH, which might be caused by a repressor cascade affecting the  $P_{amiE}$  promoter, which involves the metal-dependent regulators NikR and Fur (39, 40). Although no evidence for a role of NikR in transcriptional regulation of the *amiF* gene has been obtained (6, 7) and our data suggest a role for ArsR~P as the prominent regulator of pH-responsive transcription of the amidase genes, the pH-responsive induction of the enzymatic activity of both AmiF and AmiE is clearly reduced in a *nikR* mutant (6, 40). This

might suggest that a gene product of the NikR regulon contributes to the enzymatic activity of the amidases or is involved in providing their cellular substrates, which are unknown so far.

The regions protected from DNase I digestion by the binding of ArsR~P to the  $P_{amiE}$  and  $P_{amiF}$  promoters spanned 56 bp and 38 bp, respectively, and overlapped with the -35 promoter elements. Extended binding regions for ArsR~P have also been found in the  $P_{ureA}$  and  $P_{ureI}$  promoters (27). These observations might suggest that two consecutive ArsR~P dimers are binding to the promoters of the respective genes or that the active response regulator forms a tetrameric protein complex. As noticed for the  $P_{amiE}$  and  $P_{amiF}$  promoters in this study, the binding regions of ArsR~P mapped in the  $P_{ureA}$  and  $P_{ureI}$  promoters were not recognized by the unphosphorylated ArsR protein in DNase I footprint experiments (27). This similarity in promoter characteristics might be correlated with similar kinetics of pH-responsive transcription of the amidase and *ureA* and *ureI* genes. According to the microarray study of Merrell et al. (21), these genes reached their maximum level of transcription 90 min after the shift to low pH. In contrast, other ArsR~P-regulated genes whose promoters harbor short binding sites for ArsR~P (10) were maximally transcribed 15 min after the pH shift (21).

Most surprisingly, in this study we identified the *rocF* gene and ORF HP0682 as members of the ArsR~P regulon which are not responsive to pH changes in a range of 5.0 to 8.5. As ArsR~P footprinted the  $P_{rocF}$  promoter (Fig. 6), it is likely that ArsS-dependent repression of the *rocF* gene is mediated by ArsR~P and not by an alternative transcriptional regulator interacting with ArsS. Interestingly, a region of 62 bp overlapping the -10 box of the  $P_{rocF}$  promoter exhibited the highest affinity for ArsR~P noted so far in DNase I footprint experiments. While this high-affinity binding site interacted with ArsR~P present in concentrations of 0.37 or 0.75  $\mu$ M, depending on the particular experiment, binding to  $P_{ureA}$ ,  $P_{ureI}$ ,  $P_{amiE}$ ,  $P_{amiF}$ , and the promoters of the ArsR~P regulated ORFs HP1408 and HP0119 required ArsR~P in concentrations of 1.5  $\mu$ M and above (10, 27). Since from the comparison of the transcription of ArsR~P-dependent genes in the G27 wild type and the ArsS-deficient mutant at pH 7.0 it is clear that at neutral pH ArsS autophosphorylates to some extent (10, 15), we hypothesize that the low cellular concentration of ArsR~P already present at pH 7.0 is sufficient to repress the *rocF* gene. Increasing the pH of the surrounding medium up to pH 8.5 did not result in derepression of *rocF*. In the ArsS-deficient mutant, we observed an approximately threefold increase in *rocF* transcription at pH 5.0 compared to that at pH 7.0, suggesting pH-responsive positive regulation of *rocF* by an unknown mechanism which comes into play when the ArsR~P-dependent repression of *rocF* is relieved. In this context, note that Wen et al. (44) observed a 1.7-fold increase in *rocF* transcription when *H. pylori* 26695 was exposed to pH 5.5. So far, the biological significance of this complex mode of regulation of *rocF* remains obscure. In addition to ORFs HP1288 and HP1289 being paralogs of HP0682 and HP0681, several genes, including HP0081, and HP1398, showed a rather high ratio of differential expression in the ArsS-deficient mutant at pH 5.0 but were not detected in the transcriptome studies investigating pH-responsive gene regulation (6, 21, 44). Therefore, for the moment it cannot be ruled out that these genes also belong to the class of



ArsR~P-dependent genes which do not exhibit a pH-responsive transcription profile.

In conclusion, we have shown that the major ammonia-producing pathways which are central to the acid resistance of *H. pylori* are regulated in response to low pH by the ArsRS two-component system. Several members of the ArsR~P regulon defined here are under the additional control of the metal-dependent regulators NikR and Fur, indicating a complex regulatory interplay in the pH-responsive control of transcription. This complexity is further underlined by the identification of ArsR~P-dependent genes whose transcription is not responsive to low pH.

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