Delineation of the pH-Responsive Regulon Controlled by the *Helicobacter pylori* ArsRS Two-Component System

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ABSTRACT *Helicobacter pylori* encounters a wide range of pH within the human stomach. In a comparison of *H. pylori* cultured *in vitro* under neutral or acidic conditions, about 15% of genes are differentially expressed, and corresponding changes are detectable for many of the encoded proteins. The ArsRS two-component system (TCS), comprised of the sensor kinase ArsS and its cognate response regulator ArsR, has an important role in mediating pH-responsive changes in *H. pylori* gene expression. In this study, we sought to delineate the pH-responsive ArsRS regulon and further define the role of ArsR in pH-responsive gene expression. We compared *H. pylori* strains containing an intact ArsRS system with an *arsS* null mutant or strains containing site-specific mutations of a conserved aspartate residue (D52) in ArsR, which is phosphorylated in response to signals relayed by the cognate sensor kinase ArsS. We identified 178 genes that were pH-responsive in strains containing an intact ArsRS system but not in Δ *arsS* or *arsR* mutants. These constituents of the pH-responsive ArsRS regulon include genes involved in acid acclimatization (*ureAB*, amidases), oxidative stress responses (*katA*, *sodB*), transcriptional regulation related to iron or nickel homeostasis (*fur*, *nikR*), and genes encoding outer membrane proteins (including *sabA*, *alpA*, *alpB*, *hopD* [*labA*], and *horA*). When comparing *H. pylori* strains containing an intact ArsRS TCS with *arsRS* mutants, each cultured at neutral pH, relatively few genes are differentially expressed. Collectively, these data suggest that ArsRS-mediated gene regulation has an important role in *H. pylori* adaptation to changing pH conditions.

KEYWORDS gene regulation, two-component signal transduction systems, RNA-seq, signal transduction, two-component regulatory systems

The Gram-negative bacterium *Helicobacter pylori* persistently colonizes the stomach in about half of the human population (1–4). Although the majority of individuals colonized with *H. pylori* do not experience adverse consequences, *H. pylori* has an important role in the pathogenesis of gastric cancer and peptic ulcer disease (5–7).

H. pylori encounters considerable pH fluctuation *in vivo*. Within a normal human stomach, the intraluminal pH is very acidic (pH 2) under fasting conditions (8–11), and the pH increases when food is consumed. The pH within the gastric mucus layer, where *H. pylori* is localized, varies from about pH 4 near the luminal surface to nearly neutral pH at the surface of gastric epithelial cells (12–14). In contrast to the acidic pH of a normal human stomach, atrophic gastritis (a premalignant condition that sometimes

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develops in response to *H. pylori*) is associated with a loss of normal gastric acidification. When *H. pylori* exits the stomach (for example, during transmission to new hosts), the bacteria encounter neutral pH conditions in the mouth or mild acidic conditions in the colon.

The capacity of *H. pylori* to tolerate large variations in pH is likely dependent on its capacity to sense pH variations and respond by altering gene expression. Transcriptional profiling studies have shown that there are substantial differences in *H. pylori* gene expression at neutral pH compared to gene expression when the bacteria are exposed to acidic pH (15–21). For example, transcription of ammonia-producing enzymes (urease and amidases) is increased when the bacteria are exposed to low pH conditions (15–17, 22). These enzymes contribute to acid tolerance by buffering and maintaining the cytoplasmic pH of *H. pylori* (23, 24). Similarly, *H. pylori* exposure to low pH results in alterations in the bacterial proteome (25–27).

Several previous studies reported the identification of *H. pylori* genes that are pH-responsive, but there has been limited agreement in the genes identified (15–21, 28). This is probably attributable to multiple factors, including variations in the low pH conditions and the use of different *H. pylori* strains in individual studies. In addition, most previous studies examining the effect of low pH on *H. pylori* gene expression were conducted using microarray methodology (15, 17–19, 21, 28), a technique that is limited by potential problems related to hybridization specificity.

Bacteria commonly alter gene expression in response to environmental changes through the actions of two-component signal transduction systems (TCSs), which are comprised of a sensor kinase and a cognate response regulator (29–32). Transduction of the environmental signal from the sensor kinase to the response regulator occurs through phosphorylation of a conserved aspartate residue in the activator domain of the response regulator. In *H. pylori*, the ArsRS TCS has been implicated in pH sensing and mediating pH-responsive changes in expression of genes involved in acid acclimatization and pH homeostasis (for example, genes encoding urease and amidases) (16, 21, 27, 33–37). In previous studies, target genes controlled by the ArsRS TCS have been identified by analyzing pH-responsive gene transcription in wild-type strains compared with that in Δ arsS mutant strains, using a variety of approaches, including array-based methods (33, 34, 38, 39), quantitative real-time PCR (RT-qPCR), electrophoretic mobility shift assays, proteomic analysis, or Western blotting (16, 27, 35, 40–43). In contrast to Δ arsS mutant strains, which have been analyzed in multiple studies, Δ arsR mutant strains have not been studied because such strains are reported to be nonviable (44). In contrast, strains harboring a D52N substitution mutation at the ArsR phosphorylation site are viable (36, 44). It has been proposed that the ArsRS regulon is comprised of genes with essential functions that are regulated by ArsR in its unphosphorylated form, as well as pH-responsive genes that are regulated by ArsR in its phosphorylated form (36, 45–48).

In the present study, we report an RNA-seq analysis of *H. pylori* transcriptional responses to low pH and we delineate the role of the ArsRS TCS in regulating *H. pylori* gene expression in response to low pH. In contrast to previous studies that relied on array-based methods and use of Δ arsS mutants, we analyzed multiple ArsRS mutant strains, including a Δ arsS null mutant and strains harboring mutations of the phospho-accepting aspartate residue in ArsR. Specifically, we analyzed a strain with an ArsR-D52N mutation (predicted to be a nonphosphorylatable form of ArsR) (36, 44) and a strain with an ArsR-D52E mutation. Previous studies have reported that some response regulators harboring the latter mutation can act as phosphomimetics (49), eliminating the requirement of phosphorylation. We report the identification of 178 genes that are pH-responsive in strains containing an intact ArsRS system but not in Δ arsS or arsR-D52 mutants. These results provide a robust definition of the pH-responsive ArsRS regulon and extend our understanding of its role in regulating pH-responsive gene expression in *H. pylori*.

FIG 1 *H. pylori* transcriptional alterations in response to acidic pH. Seed cultures of *H. pylori* strain 26695 grown overnight in BB-Chol medium (pH 7.0) were inoculated into BB-Chol medium (pH 5.3 or pH 7.0) and were cultured for either 1 h or 6 h. RNA was isolated from these cultures and transcript levels were quantified by RNA-seq as described in Materials and Methods. For each gene, the transcript level at pH 5.3 was compared to the transcript level at pH 7.0. (A) 1 h time point. (B) 6 h time point. The y axis shows the statistical significance of differences in transcript abundance when comparing bacteria grown at pH 5.3 and pH 7.0 (negative $\log_{10} P$ values; a higher value indicates greater significance), and the x axis shows the magnitude of differences ($\log_2$ fold change values). FC, fold change. Positive x axis values represent upregulation of gene expression in response to low pH and negative x axis values represent downregulation in response to low pH. Vertical dotted lines correspond to 2.5-fold changes when comparing cultures grown at pH 5.3 with cultures grown at pH 7.0. The dotted horizontal line shows $P = 0.01$, with points above the line having a P value < 0.01 and points below the line having a P value > 0.01 . Numbers (“up” and “down”) indicate the numbers of genes for which expression was upregulated or downregulated in response to acidic pH. The results presented are based on analysis of 4 independent RNA samples from each culture condition (growth for 1 h or 6 h in pH 5.3 or pH 7.0 medium).

RESULTS

***H. pylori* strain 26695 transcriptional alterations in response to acidic pH.** To identify *H. pylori* genes that are differentially expressed in response to changes in environmental pH, we cultured *H. pylori* strain 26695 at pH 5.3 or pH 7.0 and performed RNA-seq analysis as described in Materials and Methods (see Fig. S1A in the supplemental material). For each gene, we calculated a transcript abundance ratio (normalized transcript abundance in bacteria cultured at pH 5.3 divided by normalized transcript abundance in bacteria cultured at pH 7.0) (Fig. 1). We identified 66 genes that were differentially expressed when comparing bacteria grown at pH 5.3 with bacteria grown at pH 7.0 (Table 1 and Fig. S2), based on analysis of data from both the 1 h and 6 h time points (44 upregulated and 22 downregulated in response to low pH). Numerous additional genes were differentially expressed at only one of the two time

TABLE 1 pH-responsive genes in wild-type *H. pylori* strain 26695^{a,e}

	1h		6 h		
	FDR ^b	Fold ^c	FDR ^b	Fold ^c	Description
Upregulated genes ^d					
HP0078*	8.61E−12	3.00	3.72E−32	8.28	Hypothetical protein
HP0079*	1.93E−09	3.12	4.01E−43	9.85	Outer membrane protein (<i>horA</i>)
HP0101	1.92E−06	3.49	8.72E−07	3.38	Hypothetical protein
HP0106*	2.06E−23	5.96	7.55E−10	2.70	Cystathionine gamma-synthase (<i>metB</i>)
HP0117	2.01E−09	3.87	9.82E−09	2.51	Hypothetical protein
HP0118*	3.12E−25	8.52	4.56E−17	3.68	Hypothetical protein
HP0206	1.04E−08	4.87	6.71E−08	3.00	Hypothetical protein
HP0219*	4.15E−40	17.36	2.74E−42	14.99	Hypothetical protein
HP0228*	7.63E−15	5.65	4.39E−12	2.93	Hypothetical integral membrane protein
HP0241	2.50E−07	3.08	1.71E−13	3.64	Hypothetical protein
HP0287	8.94E−09	2.96	1.01E−10	2.85	Hypothetical protein
HP0307*	2.21E−07	2.53	4.48E−15	3.25	Hypothetical protein
HP0345	4.83E−30	17.33	9.78E−21	7.41	Hypothetical protein
HP0346	5.80E−20	5.79	2.23E−13	4.55	Hypothetical protein
HP0376*	1.90E−18	6.15	1.14E−13	4.06	Ferrochelatase (<i>hemH</i>)
HP0383	3.80E−11	5.85	4.30E−05	2.60	Hypothetical protein
HP0448	6.87E−03	3.04	2.23E−13	2.87	Hypothetical protein
HP0483	9.09E−18	5.94	3.94E−06	2.59	Cytosine specific DNA methyltransferase
HP0539	3.41E−06	3.24	2.30E−05	2.91	Cag pathogenicity island protein (<i>cagL</i>)
HP0641*	3.09E−09	5.47	2.03E−04	4.42	Hypothetical protein
HP0642*	2.15E−14	6.01	4.00E−16	5.11	NAD(P)H-flavin oxidoreductase
HP0704*	6.67E−13	6.40	2.42E−08	3.34	Hypothetical protein
HP0714	7.35E−06	3.68	4.14E−04	2.69	RNA polymerase sigma-54 factor (<i>rpoN</i>)
HP0755*	3.81E−12	3.65	1.20E−13	3.11	Molybdopterin biosynthesis protein (<i>moeB</i>)
HP0922*	1.94E−10	4.51	1.23E−08	3.29	Toxin-like outer membrane protein (<i>vlpC</i>)
HP1009	3.46E−05	3.02	8.42E−05	2.65	Site-specific recombinase
HP1066	7.45E−13	3.52	7.80E−15	3.09	Hypothetical protein
HP1169	3.58E−16	4.16	1.78E−08	2.81	Glutamine ABC transporter, permease protein (<i>glnP1</i>)
HP1187*	1.24E−32	9.54	1.79E−18	4.62	Hypothetical protein
HP1188*	1.32E−24	6.66	1.32E−12	3.00	Hypothetical protein
HP1220	5.13E−09	2.99	1.06E−11	2.95	ABC transporter, ATP-binding protein
HP1221*	1.53E−13	3.22	1.49E−09	2.79	Hypothetical protein
HP1222*	8.60E−26	5.32	6.03E−09	2.56	D-Lactate dehydrogenase (<i>dld</i>)
HP1235	1.17E−03	2.66	1.48E−04	2.79	Hypothetical integral membrane protein
HP1238*	4.69E−13	3.22	1.20E−13	3.57	Aliphatic amidase (<i>amiF</i>)
HP1282	9.05E−09	3.69	7.17E−08	2.90	Anthranilate synthase component I (<i>trpE</i>)
HP1330	3.39E−12	5.66	4.78E−12	4.23	Hypothetical integral membrane protein
HP1332*	3.99E−25	5.06	1.89E−13	3.29	Cochaperone and heat shock protein (<i>dnaJ</i>)
HP1336	1.44E−05	2.59	2.45E−12	2.67	Hypothetical protein
HP1432*	6.64E−34	15.85	4.07E−04	10.66	Histidine and glutamine-rich protein (<i>hpn</i>)
HP1499*	4.74E−25	11.52	2.34E−20	5.17	Hypothetical protein
HP1528	1.44E−04	44.12	1.79E−03	18.50	Hypothetical protein
HP1571	1.57E−10	3.45	3.98E−10	2.52	Rare lipoprotein A (<i>rlpA</i>)
HP1572*	2.55E−17	4.74	3.11E−15	3.13	Regulatory protein (<i>dniR</i>)
Downregulated genes ^d					
HP0009*	6.52E−04	0.33	2.95E−15	0.21	Outer membrane protein (<i>hopZ</i>)
HP0015	6.01E−05	0.35	1.72E−49	0.08	Hypothetical protein
HP0016	2.42E−03	0.34	2.97E−69	0.05	Hypothetical protein
HP0025*	4.16E−08	0.20	2.63E−90	0.01	Outer membrane protein (<i>hopD</i>)
HP0091*	4.27E−18	0.14	4.34E−32	0.12	Type II restriction enzyme R protein (<i>hsdR</i>)
HP0092*	2.74E−16	0.17	9.38E−42	0.12	Type II restriction enzyme M protein (<i>hsdM</i>)
HP0097*	2.45E−09	0.28	1.02E−34	0.11	Hypothetical protein
HP0229	1.03E−08	0.20	3.41E−14	0.26	Outer membrane protein (<i>hopA</i>)
HP0298	1.47E−09	0.33	1.63E−08	0.39	Dipeptide ABC transporter (<i>dppA</i>)
HP0408	1.01E−07	0.36	1.63E−11	0.33	Hypothetical protein
HP0996	2.06E−07	0.30	9.41E−36	0.16	Hypothetical protein
HP1027*	2.79E−09	0.19	2.31E−44	0.08	Ferric uptake regulation protein (<i>fur</i>)
HP1177*	6.56E−81	0.06	6.15E−45	0.06	Outer membrane protein (<i>hopQ</i>)
HP1180*	2.39E−05	0.25	2.50E−17	0.31	Pyrimidine nucleoside transport protein (<i>nupC</i>)

(Continued on next page)

TABLE 1 (Continued)

	1 h		6 h		Description
	FDR ^b	Fold ^c	FDR ^b	Fold ^c	
HP1211	4.67E-04	0.34	1.91E-12	0.16	Hypothetical protein
HP1212*	3.59E-04	0.22	6.63E-07	0.26	ATP synthase F0, subunit c (<i>atpE</i>)
HP1290	1.06E-03	0.39	1.90E-44	0.12	Nicotinamide mononucleotide transporter (<i>pnuC</i>)
HP1469*	1.01E-08	0.27	3.76E-10	0.34	Outer membrane protein (<i>horJ</i>)
HP1500	1.25E-13	0.07	3.00E-10	0.26	Hypothetical protein
HP1501*	1.11E-27	0.08	5.85E-12	0.30	Outer membrane protein (<i>hork</i>)
HP1512*	1.47E-24	0.10	4.11E-11	0.30	Iron-regulated outer membrane protein (<i>frpB3</i>)
HP1527*	1.55E-06	0.30	4.11E-48	0.10	Hypothetical protein

^a*H. pylori* 26695 was grown at either pH 5.3 or pH 7.0. Cultures were analyzed at 1 h and 6 h time points. RNA-seq was performed as described in Materials and Methods. Genes considered differentially expressed demonstrated fold change values (pH 5.3 versus pH 7.0) either >2.5 or <0.4, with a false-discovery rate <0.01. The table lists genes differentially expressed at both the 1 h and 6 h time points.

^bFDR, false-discovery rate.

^c"Fold" indicates fold change values. The fold change values are a ratio of RNA-seq reads from cultures grown at pH 5.3 compared to RNA-seq reads from cultures grown at pH 7.0.

^dTranscript levels of genes designated "upregulated" were higher in cultures grown at pH 5.3 than in cultures grown at pH 7.0. Transcript levels of genes designated "downregulated" were lower in cultures grown at pH 5.3 than in cultures grown at pH 7.0.

^eAsterisks denote pH-responsive genes identified in previous studies (15, 17, 18, 20).

points (Fig. S2 and Table S1 and S2). Many of the differentially expressed genes identified in these experiments have previously been reported to be acid-responsive (15–21, 50), whereas others are identified here for the first time. Interestingly, two transcriptional regulators were regulated in response to pH changes. Expression of *fur*, encoding a transcriptional regulator involved in iron homeostasis, was significantly repressed at low pH (both 1 h and 6 h time points). Expression of *nikR*, encoding a transcriptional regulator involved in nickel homeostasis, was upregulated in response to low pH conditions at the 1 h time point (Table S1).

We validated the RNA-seq data for several of the differentially expressed genes using quantitative reverse transcription-PCR (RT-qPCR). Consistent with the RNA-seq data, the RT-qPCR analyses showed upregulated expression of HP0079 (*horA*, encoding an outer membrane protein), HP1238 (*amiF*, encoding amidase), and HP1432 (*hpn*, encoding a histidine-rich protein) when *H. pylori* was grown at pH 5.3 compared to when it was grown at neutral pH (Fig. S3). Similarly, RT-qPCR confirmed the downregulated transcription of three genes encoding outer membrane proteins (HP0025 [*hopD*], HP1177 [*hopQ*], and HP1512 [*frpB3*]) in response to acidic pH (Fig. S3).

Comparative proteomic analysis of *H. pylori* 26695 cultured at neutral pH or acidic pH. We also determined the effect of acidic pH on the *H. pylori* proteome (Fig. S1B). For these experiments, we cultured *H. pylori* at pH 5.3 or pH 7.0 for a longer time period (24 h) than the time periods used for RNA-seq experiments (1 to 6 h) in order to optimize the likelihood of detecting changes in protein abundance. The bacteria were processed as described in Materials and Methods, yielding subcellular fractions predicted to be enriched in membrane proteins and fractions predicted to be enriched in soluble cytosolic and periplasmic proteins. The protein composition of these fractions was then analyzed by high-resolution LC-MS/MS to identify proteins that were differentially abundant in acidic pH cultures compared to those in neutral pH cultures. Figure 2 illustrates differences in the abundance of proteins in bacteria cultured at pH 5.3 compared to those cultured at pH 7.0. Among 706 proteins identified in the soluble (cytoplasm/periplasm) fractions, 69 were differentially abundant in bacteria cultured at pH 5.3 compared to bacteria cultured at pH 7.0, based on the criteria described in Materials and Methods (Table S3). Among 901 proteins identified in the insoluble (membrane) fractions, 90 were differentially abundant (Table S3).

We next evaluated correlations between the observed transcriptional changes (identified by RNA-seq) and changes in protein abundance (detected by proteomic analysis). As shown in Table S3 (highlighted with an asterisk) and Table 2, 33 of the 69 changes in protein abundance detected in analyses of cytoplasm/periplasm fractions matched corresponding transcriptional changes. In addition, 30 of the 90 changes

FIG 2 *H. pylori* proteomic changes in response to acidic pH. Seed cultures of *H. pylori* strain 26695 grown overnight in BB-Chol medium (pH 7.0) were inoculated into BB-Chol medium (pH 5.3 or pH 7.0) and were cultured for 24 h as described in the Materials and Methods. Bacterial lysates were fractionated into preparations enriched in cytoplasmic proteins (A) or membrane proteins (B), and proteomic analysis was done as described in Materials and Methods. The y axis shows the statistical significance of differences in protein abundance when comparing bacteria cultured at pH 5.3 with bacteria cultured at pH 7.0, and the x axis shows the magnitude of differences ($\log_2$ fold change values). The vertical dotted lines indicate 2-fold changes when comparing cultures grown at pH 5.3 versus pH 7.0. The dotted horizontal line shows $P = 0.01$, with points above the line having a P value < 0.01 and points below the line having a P value > 0.01 . The results presented are based on analysis of 4 independent samples from each condition (growth for 24 h in pH 5.3 or pH 7.0 medium).

detected in analyses of membrane fractions matched corresponding transcriptional changes (Table 2 and Table S3). Fourteen of the pH-responsive changes in protein abundance exhibiting matching transcriptional changes were detected by analysis of both the membrane and cytoplasmic fractions (Table 2), while 19 and 16 proteomic changes with matching transcriptional changes were identified uniquely in the cytoplasmic and membrane fractions, respectively. The results of proteomic analyses were thus concordant with RNA-seq transcriptional data for 49 proteins/genes (Table 2). The lack of concordance between proteomic data and RNA-seq data for other genes is potentially attributable to multiple factors, as considered in the Discussion. Many of the acid-responsive proteins identified in these experiments were reported to be acid-responsive in previous studies (15–21, 50), including upregulated production of AmiE (HP0294), AmiF (HP1238), outer membrane protein HorA (HP0079), and Hpn (HP1432) at low pH and downregulated production of several outer membrane proteins (HopD [HP0025], HopQ [HP1177], and an iron-regulated outer membrane protein [FrpB3, HP1512]) at low pH (Table 2 and Table S3). Other acid-responsive proteins identified in these experiments are reported here for the first time.

TABLE 2 pH-dependent transcriptional changes corroborated by proteomic analysis^a

Protein/ gene	Proteomic analysis ^b				RNA-seq ^d				Description
	Cytoplasmic		Membrane		1h		6 h		
	P value	Fold ^c	P value	Fold ^c	FDR	Fold	FDR	Fold	
HP0025	2.18E−08	0.02	5.32E−06	0.19	8.65E−09^e	0.21	6.66E−53	0.01	Outer membrane protein (<i>hopD</i>)
HP0056			1.49E−03	0.30	1.75E−06	0.49	1.01E−05	0.36	Delta-1-pyrroline-5-carboxylate dehydrogenase
HP0079			3.93E−05	14.08	2.22E−10	3.33	4.57E−28	9.85	Outer membrane protein (<i>horA</i>)
HP0080	9.58E−03	11.78	5.40E−04	5.16	7.05E−25	4.16	6.13E−01	1.14	Hypothetical protein
HP0089	6.47E−03	0.34	8.95E−03	0.41	9.84E−01	1.01	1.48E−12	0.25	pfs protein
HP0091	3.85E−03	0.42	3.75E−03	0.36	2.64E−19	0.14	2.80E−20	0.12	Type II restriction enzyme (<i>hsdR</i>)
HP0092	2.10E−04	0.12	5.33E−05	0.29	7.66E−21	0.17	9.81E−26	0.12	Type II restriction enzyme (<i>hsdM</i>)
HP0097	4.89E−07	0.08			2.30E−10	0.30	8.68E−25	0.11	Hypothetical protein
HP0106	7.76E−03	2.08			4.09E−28	6.16	2.78E−06	2.68	Cystathionine gamma-synthase (<i>metB</i>)
HP0153	4.37E−03	0.13	3.78E−04	0.24	1.02E−03	0.37	2.82E−04	0.46	Recombinase (<i>recA</i>)
HP0229			5.02E−03	0.35	8.34E−10	0.19	2.36E−10	0.25	Outer membrane protein (<i>hopA</i>)
HP0248			9.78E−03	0.29	1.04E−14	0.35	1.60E−04	0.44	Hypothetical protein
HP0294	2.57E−03	2.26			1.53E−14	8.50	5.74E−01	0.86	Aliphatic amidase (<i>amiE</i>)
HP0301	4.16E−03	0.32			1.30E−02	0.45	2.39E−17	0.19	Dipeptide ABC transporter (<i>dppD</i>)
HP0302	2.23E−03	0.22			4.38E−03	0.40	5.61E−18	0.19	Dipeptide ABC transporter (<i>dppF</i>)
HP0312	8.26E−03	2.39			2.06E−12	3.20	3.51E−02	0.64	Hypothetical ATP-binding protein
HP0409			8.05E−03	0.25	3.49E−08	0.42	1.58E−05	0.39	GMP synthase (<i>guaA</i>)
HP0605			8.63E−04	0.23	2.64E−19	0.34	2.55E−05	0.39	Efflux protein (<i>hefA</i>)
HP0642	4.40E−03	3.16			5.99E−16	6.10	4.79E−12	4.94	NAD(P)H-flavin oxidoreductase
HP0681			8.24E−05	0.19	2.31E−02	0.37	4.35E−17	0.20	Hypothetical protein
HP0709	2.08E−03	0.15	7.12E−03	0.44	2.29E−01	1.29	3.64E−48	0.05	Hypothetical protein
HP0710			1.64E−05	0.29	3.98E−01	1.21	7.67E−18	0.15	Outer membrane protein (<i>homA</i>)
HP0871	1.51E−03	0.28	1.61E−03	0.10	8.45E−01	1.06	1.56E−39	0.06	CDP-diglyceride hydrolase (<i>cdh</i>)
HP0876			2.06E−07	0.07	9.58E−03	0.52	1.02E−11	0.20	Fe-regulated outer membrane protein (<i>frpB1</i>)
HP0887	5.32E−05	0.05	5.03E−06	0.12	4.61E−07	0.47	8.72E−35	0.02	Vacuolating cytotoxin (<i>vacA</i>)
HP0914			1.64E−03	0.36	8.58E−01	0.97	5.93E−06	0.37	Hypothetical protein
HP1083			3.63E−04	0.28	2.34E−02	0.57	4.58E−28	0.09	Outer membrane protein (<i>hofB</i>)
HP1168			4.19E−05	0.28	5.12E−02	0.68	3.10E−24	0.13	Carbon starvation protein (<i>cstA</i>)
HP1174			4.74E−03	0.35	3.71E−03	1.70	6.65E−18	0.19	Glucose/galactose transporter (<i>gluP</i>)
HP1177	7.32E−07	0.03	4.64E−04	0.29	7.97E−97	0.07	5.70E−26	0.06	Outer membrane protein (<i>hopQ</i>)
HP1186	6.77E−03	0.21			1.38E−05	2.16	1.47E−34	0.09	Carbonic anhydrase (<i>cah</i>)
HP1200	1.98E−03	0.10			3.78E−07	0.38	5.33E−01	0.84	Ribosomal protein L10 (<i>rpl10</i>)
HP1222	3.79E−03	4.31	1.04E−04	3.51	1.41E−34	5.39	7.47E−05	2.50	D-Lactate dehydrogenase (<i>dld</i>)
HP1238	6.75E−08	11.75	1.96E−05	11.26	3.70E−13	3.40	1.92E−08	3.56	Aliphatic amidase (<i>amiF</i>)
HP1304	9.52E−03	0.12			1.07E−03	0.33	4.16E−02	0.62	Ribosomal protein L6 (<i>rpl6</i>)
HP1307	2.96E−03	0.06			8.80E−05	0.36	8.50E−03	0.55	Ribosomal protein L5 (<i>rpl5</i>)
HP1309	7.00E−03	0.06			1.17E−05	0.32	2.51E−03	0.51	Ribosomal protein L14 (<i>rpl14</i>)
HP1312	7.01E−03	0.07			1.85E−07	0.27	3.26E−03	0.52	Ribosomal protein L16 (<i>rpl16</i>)
HP1316	5.48E−03	0.03			3.06E−06	0.29	2.51E−03	0.50	Ribosomal protein L2 (<i>rpl2</i>)
HP1319	9.81E−03	0.06			3.06E−06	0.33	1.63E−02	0.58	Ribosomal protein L3 (<i>rpl3</i>)
HP1338	2.14E−03	2.13			1.10E−15	3.28	5.11E−05	2.19	Nickel responsive regulator (<i>nikR</i>)
HP1398	3.24E−03	0.38			8.58E−01	1.05	5.23E−20	0.17	Alanine dehydrogenase (<i>ald</i>)
HP1399	2.19E−06	0.17	1.47E−06	0.09	1.03E−21	5.08	6.44E−24	0.11	Arginase (<i>rocF</i>)
HP1432			1.48E−05	9.94	1.67E−39	16.54	1.97E−16	9.85	Histidine and glutamine-rich protein (<i>hpn</i>)
HP1469	5.82E−03	0.13			2.07E−09	0.28	1.16E−06	0.33	Outer membrane protein (<i>hopV</i>)
HP1512	3.17E−04	0.03			1.71E−26	0.11	2.47E−06	0.29	Fe-regulated outer membrane protein (<i>frpB3</i>)
HP1526			2.00E−04	0.16	9.40E−01	0.99	5.00E−09	0.31	Exodeoxyribonuclease (<i>lexA</i>)
HP1527	4.00E−10	0.02	1.20E−09	0.03	5.24E−15	0.37	2.80E−20	0.13	Competence protein (<i>comH</i>)
HP1588			6.04E−04	0.26	4.38E−08	0.39	1.84E−04	0.41	Hypothetical protein

^aFor proteomic analysis, *H. pylori* strain 26695 was grown for 24 h at either pH 5.3 or pH 7.0. Subcellular fractions enriched in either cytoplasmic/periplasmic or membrane proteins were prepared and evaluated by proteomic analysis. For transcriptional analysis, *H. pylori* was grown for either 1 h or 6 h at either pH 5.3 or pH 7.0. The table lists 49 differentially abundant proteins for which corresponding transcriptional changes were detected at either the 1 h or 6 h time points.

^bA total of 69 cytoplasmic and 90 membrane proteins were differentially abundant between bacteria cultured under pH 5.3 versus pH 7.0 conditions. Proteins were considered differentially abundant if both the following criteria were met: P value < 0.01 and fold change > 2 or < 0.5.

^cPeptide spectra for each protein were quantified as described in Materials and Methods. "Fold" indicates fold change values. The fold change values indicate a ratio of MS/MS spectra from cultures grown at pH 5.3 compared to normalized spectra from cultures grown at pH 7.0.

^dTranscript levels were determined by RNA-seq, as described in Materials and Methods. Genes considered differentially expressed in response to pH demonstrated fold change values (pH 5.3 versus pH 7.0) of either > 2.5 or < 0.4, with an FDR (false-discovery rate) of < 0.01.

^epH-responsive transcriptional changes at either the 1 h or 6 h time points corroborated by the proteomic analysis are shown in bold font.

pH-responsive gene expression in ArsRS TCS mutants. Several previous studies reported that the ArsRS TCS mediates alterations in *H. pylori* gene transcription in response to changes in pH (16, 33–38, 40, 42, 45). A current model proposes that low pH triggers autophosphorylation of the histidine kinase ArsS, with the phosphorylated histidine of the histidine kinase serving as a phosphodonator for autophosphorylation of the cognate response regulator ArsR at a conserved aspartate residue (D52) (44, 46). To facilitate further studies of the role of the ArsRS TCS in acid-responsive gene expression, we generated a $\Delta arsS$ mutant as well as strains containing point mutations in *arsR*, as described in Materials and Methods. Specifically, we analyzed two strains producing ArsR mutant proteins (ArsR-D52N, a nonphosphorylatable form of ArsR, or ArsR-D52E, a possible phosphomimetic form of ArsR). As a control, we generated a control strain containing a silent mutation (GAT to GAC) that still encodes amino acid D52. We also generated a strain containing not only a $\Delta arsS$ mutation but also mutations in the sensor kinases of two other *H. pylori* two-component systems that potentially contribute to pH-responsive gene expression (CrdRS and FlgRS).

Volcano plots illustrating pH-responsive changes in gene expression in these strains are shown in Fig. 3. Fewer genes were upregulated or downregulated in response to acidic pH in a $\Delta arsS$ mutant (26695 $\Delta rdxA \Delta arsS$, Fig. 3B) than in 26695 $\Delta rdxA$ (Fig. 3A), a strain used for the construction of the $\Delta arsS$ mutant. The number of pH-responsive genes in the triple mutant ($\Delta arsS \Delta crdS \Delta flgS$, Fig. 3C) was similar to the number of pH-responsive genes in the $\Delta arsS$ mutant. Acid-responsive gene expression remained intact in a control *arsR* strain (*H. pylori* 26695 *arsR-D52D*, Fig. 3D). *H. pylori* strains with mutations to the phosphorylation site of ArsR (*arsR-D52E* [Fig. 3E] or *arsR-D52N* [Fig. 3F]) each showed a pattern of gene expression similar to that of the $\Delta arsS$ mutant and markedly different from that of strains containing an intact ArsRS TCS. In analyses restricted to bacteria cultured at neutral pH (discussed subsequently), gene expression in the *arsR-D52E* mutant was similar to gene expression in strains containing an intact ArsRS TCS. Therefore, the *arsR-D52E* mutant did not exhibit detectable phosphomimetic activity.

Delineating the pH-responsive ArsRS regulon. In our initial RNA-seq analysis of wild-type *H. pylori* 26695, 237 genes were differentially expressed when comparing bacteria grown for 1 h at pH 5.3 versus pH 7.0 (Fig. 1A and Fig. 4). These include 171 genes (107 upregulated, 64 downregulated) that were differentially expressed exclusively at the 1 h time point and 66 genes (44 upregulated, 22 downregulated) that were differentially expressed at both the 1 h and 6 h time points. Similar numbers of genes were pH-responsive in additional strains containing an intact ArsRS system (*H. pylori* 26695 $\Delta rdxA$ and *H. pylori* 26695 *arsR-D52D*) following 1 h of growth at pH 5.3 or pH 7.0 (Fig. 4). A set of 114 genes was pH-responsive in all three strains (88 upregulated and 26 downregulated at low pH) (Fig. 4 and Table S4). We designate these as “group 1 genes” in the pH-responsive transcriptome of *H. pylori* 26695. Many of these genes are cotranscribed in operons (Fig. S4). In addition to the group 1 genes, 98 genes were pH-responsive in 2 of the 3 strains tested. These are designated “group 2 genes” in the pH-responsive transcriptome (Fig. 4 and Table S5). A lack of perfect concordance is likely attributed to multiple factors, including the choice of cutoff values for genes to be considered pH-responsive (upregulated 2.5-fold or downregulated 0.4-fold in response to low pH). If the criteria were changed to an upregulation of 2-fold or downregulation of 0.5-fold, 54 of the 98 genes listed in Table S5 would be considered pH-responsive in all 3 *H. pylori* strains. Based on the current criteria (upregulated 2.5-fold or downregulated 0.4-fold in response to low pH), a total of 212 genes (114 group 1 and 98 group 2) were pH-responsive in at least two of the three tested strains. Of these 212 genes, 25 were altered at both the transcriptional and proteomic levels in response to pH changes (Table 2). These results, based on analysis of multiple *H. pylori* strains (Fig. 4 and Table S4 and S5), corroborated and extended the results obtained with the analysis of a single *H. pylori* strain (Table 1 and Table S1 and S2).

We next compared the pH-responsive transcriptomes in the three *H. pylori* strains

FIG 3 ArsS and ArsR mutations alter pH-responsive transcription. *H. pylori* cultures grown overnight in BB-Chol medium (pH 7.0) were inoculated into BB-Chol medium (pH 5.3 or pH 7.0) and were cultured for 1 h. RNA was isolated and transcript levels were quantified by RNA-seq as described in Materials and Methods. Volcano plots illustrate differences in transcription when comparing bacteria cultured for 1 h in acidic or neutral pH. Parameters for the x and y axes are described in Fig. 1. The vertical dotted lines correspond to 2.5-fold changes and the horizontal dotted line corresponds to a *P* value of 0.01. The number of genes exhibiting pH-responsive changes in transcription is markedly lower in the $\Delta arsS$ mutant (26695 $\Delta rdxA$ $\Delta arsS$, B) than in 26695 $\Delta rdxA$ (containing an intact ArsRS TCS, A). A similar reduction in the number of pH-responsive genes was observed in a triple mutant (C) containing null mutations in three genes encoding sensor kinases (*arsS*, *crdS*, and *flgS*). Similarly, the number of genes undergoing pH-responsive changes in transcription was markedly lower in *arsR-D52E* (E) and *arsR-D52N* (F) mutants than in a control strain (*arsR-D52D*, D). The results presented for each panel are based on analysis of 4 independent RNA samples from each *H. pylori* strain and culture condition (pH 5.3 or pH 7.0).

harboring the wild-type ArsRS system (*H. pylori* strains 26695, 26695 $\Delta rdxA$, and 26695 *arsR-D52D*) with the pH-responsive transcriptomes in *H. pylori* strains containing mutations to either the sensor kinase ArsS (26695 $\Delta rdxA$ $\Delta arsS$) or the ArsR response regulator. Since the *arsR-D52N* mutant and *D52E* mutant exhibited similar transcriptional profiles (Fig. 3), we included both the *D52E* and *D52N* mutants in this analysis. We focused on 114 group 1 genes (pH-responsive in all three strains containing an intact ArsRS system, Table S4) and 212 genes that were pH-responsive in at least two of the three

FIG 4 Delineation of a pH-responsive transcriptome. pH-responsive gene expression was analyzed in 3 *H. pylori* strains containing an intact ArsRS TCS (26695, 26695 Δ *rdxA*, and 26695 *arsR*-D52D). These *H. pylori* strains were each grown for 1 h in BB-Chol medium buffered to either pH 5.3 or pH 7.0. Differentially expressed genes were identified as described in Materials and Methods. The Venn diagram illustrates the number of pH-responsive genes in each of the strains analyzed. The 114 genes that were pH-responsive in all three strains tested (strains 26695, 26695 Δ *rdxA*, and 26695 *arsR*-D52D) are designated group 1 genes in the pH-responsive transcriptome. The 98 genes that were pH-responsive in two of the 3 strains analyzed (25, 34, and 39 genes depicted in the Venn diagram) are designated group 2 genes. In total, 212 genes were pH-responsive in at least two of three strains tested.

strains (combined group 1 and group 2) (Table S4 and S5). As shown in Fig. 5, most of the pH-responsive genes (98 of the 114 group 1 genes [A] and 178 of 212 group 1 and 2 genes [B]) were not pH-responsive in Δ *arsS*, *arsR*-D52E, or *arsR*-D52N mutants, thus indicating a defect in pH-regulated transcription in the *arsS* and *arsR* mutants.

In a related analysis, we evaluated whether mutation of the ArsS sensor kinase and mutation of the ArsR response regulator have similar impacts on pH-responsive gene expression in *H. pylori*. Specifically, we evaluated whether genes that were pH-responsive in strains with an intact ArsRS system (group 1 and group 2 genes in the pH-responsive transcriptome) were pH-responsive in Δ *arsS*, *arsR*-D52N, or *arsR*-D52E mutants (see Table 1, Table 3, and Table S6). The Venn diagrams shown in Fig. 5 and Fig. S5 reveal that mutations to *arsS* and *arsR* have a similar effect on the expression of pH-responsive genes.

The ArsRS TCS appears to play a more prominent role in the upregulation of genes in response to low pH than in downregulation of genes in response to low pH. For example, of the 178 ArsRS target genes, 143 were upregulated in response to low pH and only 35 were downregulated in response to low pH. Among the 178 identified genes in the ArsRS regulon, 56 are distributed in 21 operons that are upregulated in response to acidic pH (Fig. 6, left panel), and 12 are distributed in 5 operons that are downregulated in response to acidic pH (Fig. 6, right panel).

Ten genes (7 group 1 genes [Fig. 5A] and 3 group 2 genes [Fig. 5B]) were pH-responsive not only in *H. pylori* strains possessing an intact ArsRS TCS (strains 26695, 26695 Δ *rdxA*, 26695 *arsR*-D52D) but also in all 3 *H. pylori* strains harboring mutations to the ArsRS TCS (strains 26695 Δ *arsS*, 26695 *arsR*-D52E, 26695 *arsR*-D52N). These include 3 genes predicted to encode heme-binding outer membrane proteins (HP0915 [*frpB1*], HP0916 [*frpB2*], and HP1512 [*frpB3*]) and 2 encoding other outer membrane proteins (HP0009 [*hopZ*] and HP1501 [*horkI*]) (Table S7). We presume that transcriptional regulators other than ArsRS mediate pH-responsive changes in transcription of these genes.

Functions of genes in the ArsRS TCS regulon. We next classified the 178 ArsRS-regulated genes according to known or proposed cellular functions (Fig. 7). Hypothetical proteins with unknown functions comprised the largest proportion of

FIG 5 Venn diagram illustrating the number of pH-responsive genes in strains with an intact ArsRS system or in strains with ArsRS mutations. pH-responsive genes were identified in six *H. pylori* strains (three containing intact ArsRS and three containing mutations in ArsRS), using criteria described in Materials and Methods. A total of 114 genes (designated group 1 genes) were pH-responsive in all three strains containing wild-type ArsRS. A total of 212 genes were pH-responsive in at least two of three strains containing wild-type ArsRS (combined group 1 and group 2), as shown in Fig. 4. A compares the number of pH-responsive genes in strains containing mutations to the ArsRS TCS (26695 Δ *arsS*, 26695 *arsR-D52E*, or 26695 *arsR-D52N*) with the 114 group 1 pH-responsive genes (tan oval). B compares the number of pH-responsive genes in strains containing mutations to the ArsRS TCS with the larger group of 212 pH-responsive genes (114 group 1 and 98 group 2 genes, tan oval). A shows 98 genes that were pH-responsive only in 3 *H. pylori* strains containing an intact ArsRS TCS (but not ArsRS mutant strains), and B shows 178 genes that were pH-responsive in at least 2 of the 3 *H. pylori* strains containing an intact ArsRS TCS (but not ArsRS mutant strains).

genes that were differentially expressed in response to low pH (41% of the upregulated genes and 34% of the genes downregulated at low pH). Genes associated with transport functions in *H. pylori* comprised another large group in the ArsRS regulon (13% of the upregulated genes and 14% of the genes downregulated at low pH). These genes encode proteins involved in iron transport (HP0687 [*feoB*], HP0889 [*fecD*]) and glutamine transport (HP1169 [*glnP1*], HP1170 [*glnP2*], HP1171 [*glnQ*], HP1172 [*glnH*]) (Table 3 and Table S6). Several genes in the ArsRS regulon are known to play a role in acid acclimatization (e.g., ammonia-producing enzymes such as amidases HP0294 [*amiE*] and HP1238 [*amiF*] and urease [*ureF*, *ureA*, and *ureB*]), consistent with an important role of the ArsRS TCS in promoting *H. pylori* survival or growth at low pH. Members of the ArsRS regulon also include numerous genes encoding proteins associated with the cell envelope, including outer membrane proteins (OMPs; 7% of the upregulated genes and 26% of the downregulated genes). Three genes encoding OMPs (HP0079 [*horA*], HP0923 [*hopK*], and HP1066 [*horD*]) were upregulated by the ArsRS TCS at acidic pH. Seven OMP-encoding genes were downregulated by the ArsRS TCS (HP0025 [*hopD*, also known as *labA* {51}], HP0253 [*hopG*], HP0725 [*sabA*], HP0912 [*alpA*], HP0913 [*alpB*], HP1083 [*hofB*], HP1395 [*horL*]). As shown in Fig. S5B, there were 11 genes (including *hopQ* and *horE*, which encode outer membrane proteins) whose

TABLE 3 pH-responsive genes regulated by the ArsRS system^{a,d}

	Fold change in the indicated strains ^c						Description
	26695	26695 <i>ΔrdxA</i>	26695 <i>arsR-D52D</i>	26695 <i>ΔarsS</i>	26695 <i>arsR-D52E</i>	26695 <i>arsR-D52N</i>	
Upregulated genes ^b							
HP0018	3.27	2.96	4.88	1.33	1.31	1.21	Hypothetical protein
HP0060	3.77	4.07	4.32	1.36	1.10	1.25	Hypothetical protein
HP0069*	2.76	3.04	3.44	1.36	1.31	1.38	Urease accessory protein (<i>ureF</i>)
HP0078*	3.00	5.61	4.68	0.63	1.00	0.83	Hypothetical protein
HP0079*	3.12	5.30	6.86	1.03	1.33	1.11	Outer membrane protein (<i>horA</i>)
HP0080	4.44	6.59	7.01	1.38	1.44	1.35	Hypothetical protein
HP0101	3.49	2.65	3.18	0.87	1.15	1.12	Hypothetical protein
HP0105	4.02	4.13	4.61	0.99	0.85	0.99	Hypothetical protein
HP0106	5.96	7.12	9.28	0.91	0.96	0.97	Cystathionine gamma-synthase (<i>metB</i>)
HP0107	2.66	4.27	3.47	1.10	1.05	1.02	Cysteine synthetase (<i>cysK</i>)
HP0117	3.87	3.24	4.00	1.01	1.15	1.12	Hypothetical protein
HP0118*	8.52	12.57	12.88	0.65	0.93	0.65	Hypothetical protein
HP0119	6.16	7.53	5.06	0.70	0.99	0.86	Hypothetical protein
HP0149	3.00	5.01	5.87	1.00	1.04	1.09	Hypothetical protein
HP0206	4.87	3.56	4.28	1.25	1.19	1.37	Hypothetical protein
HP0219	17.36	18.45	18.49	1.20	1.18	1.15	Hypothetical protein
HP0228*	5.65	5.97	8.02	1.62	1.55	1.40	Hypothetical integral membrane protein
HP0241	3.08	2.57	3.06	1.36	1.41	1.50	Hypothetical protein
HP0260	4.75	2.74	3.48	1.80	1.50	1.37	Adenine specific DNA methyltransferase (<i>mod</i>)
HP0261	5.14	2.81	3.99	1.90	1.63	1.71	Hypothetical protein
HP0285	3.58	4.39	3.40	1.29	1.21	1.21	Hypothetical protein
HP0286	3.35	3.98	3.45	1.53	1.32	1.35	Cell division protein (<i>ftsH</i>)
HP0287	2.96	2.70	2.97	1.44	1.17	1.21	Hypothetical protein
HP0294*	8.01	16.69	16.66	0.66	0.83	0.62	Aliphatic amidase (<i>amiE</i>)
HP0304	4.92	4.50	5.15	0.85	0.80	0.87	Hypothetical protein
HP0307*	2.53	2.88	3.18	1.08	1.12	1.22	Hypothetical protein
HP0312	3.04	3.58	2.74	1.45	1.11	1.35	Hypothetical ATP-binding protein
HP0313	3.27	3.88	4.05	1.15	0.95	0.96	Nitrite extrusion protein (<i>nark</i>)
HP0345	17.33	8.80	6.37	1.38	1.25	0.99	Hypothetical protein
HP0346	5.79	3.72	5.75	1.52	1.30	1.30	Hypothetical protein
HP0376	6.15	5.73	6.74	1.14	1.21	1.38	Ferrochelatase (<i>hemH</i>)
HP0380*	3.53	5.70	4.44	1.47	1.07	1.07	Glutamate dehydrogenase (<i>gdhA</i>)
HP0383	5.85	9.77	10.03	1.29	1.45	1.37	Hypothetical protein
HP0384	3.48	2.97	2.94	1.26	1.57	1.49	Hypothetical protein
HP0415	2.63	2.53	2.59	1.80	1.48	1.65	Hypothetical integral membrane protein
HP0455	7.48	2.92	3.31	1.18	1.24	1.09	Hypothetical protein
HP0483	5.94	3.36	3.50	0.89	0.93	0.86	Cytosine specific DNA methyltransferase
HP0484	10.20	4.91	6.35	0.99	0.87	0.89	Hypothetical protein
HP0489	10.95	3.49	3.32	2.68	1.95	1.11	Hypothetical protein
HP0519	6.13	4.80	6.59	1.28	1.17	0.94	Hypothetical protein
HP0586	3.15	2.51	2.59	1.39	1.37	1.41	Hypothetical protein
HP0641*	5.47	6.25	7.19	1.08	1.08	0.97	Hypothetical protein
HP0642*	6.01	6.50	9.66	1.11	1.19	1.12	NAD(P)H-flavin oxidoreductase
HP0687	2.51	2.69	3.22	1.31	1.24	1.15	Iron(II) transport protein (<i>feoB</i>)
HP0693*	2.56	3.41	2.54	1.36	0.81	0.87	Hypothetical integral membrane protein
HP0768	6.55	4.91	6.51	2.08	1.36	1.51	Molybdenum cofactor biosynthesis protein A (<i>moaA</i>)
HP0769	3.09	3.75	2.93	1.73	1.58	1.52	Molybdopterin dinucleotide biosynthesis protein A (<i>mobA</i>)
HP0811	3.63	3.09	5.70	0.80	0.67	0.85	Hypothetical protein
HP0844	2.54	2.50	3.38	1.17	1.17	1.24	Thiamine biosynthesis protein (<i>thi</i>)
HP0867	2.75	3.05	3.05	1.08	1.01	0.93	Lipid A disaccharide synthetase (<i>lpxB</i>)
HP0868	2.98	3.56	3.49	1.02	1.03	1.01	Hypothetical protein
HP0869*	3.22	5.21	3.72	1.03	0.90	0.83	Hydrogenase (<i>hypA</i>)
HP0873	3.25	3.36	2.74	0.99	0.82	0.97	Hypothetical protein
HP0874	2.63	2.97	3.38	0.92	0.78	1.05	Hypothetical protein
HP0875*	3.04	4.00	3.81	0.97	0.81	1.13	Catalase (<i>katA</i>)
HP0890*	2.77	3.85	4.94	1.31	1.35	1.39	Hypothetical protein
HP0922	4.51	4.63	5.24	2.17	2.21	2.33	Toxin-like outer membrane protein (<i>vlpC</i>)
HP0924*	2.53	4.85	3.33	0.94	0.99	0.97	4-Oxalocrotonate tautomerase (<i>dmpI</i>)

(Continued on next page)

TABLE 3 (Continued)

	Fold change in the indicated strains ^c						Description
	26695	26695 Δ rdxA	26695 <i>arsR-D52D</i>	26695 Δ arsS	26695 <i>arsR-D52E</i>	26695 <i>arsR-D52N</i>	
HP0948	3.56	4.30	3.30	1.40	1.15	1.38	Hypothetical protein
HP0962	3.82	3.45	2.77	1.70	1.02	1.16	Acyl carrier protein (<i>acpP</i>)
HP0963*	4.40	4.20	4.48	1.20	0.87	0.77	Hypothetical protein
HP1022*	4.57	4.80	4.05	1.50	1.30	1.16	Hypothetical protein
HP1080	9.01	2.94	6.53	0.82	0.96	0.99	Hypothetical integral membrane protein
HP1082	3.29	3.19	2.89	0.65	0.78	0.84	Multidrug resistance protein (<i>msbA</i>)
HP1166*	3.98	7.61	6.50	1.30	1.24	1.23	Glucose-6-phosphate isomerase (<i>pgi</i>)
HP1171	3.30	3.21	3.54	1.08	1.20	1.30	Glutamine ABC transporter (<i>glnQ</i>)
HP1185	2.88	3.37	4.21	1.28	1.14	1.15	Hypothetical integral membrane protein
HP1187*	9.54	16.12	14.42	0.76	0.96	0.75	Hypothetical protein
HP1188*	6.66	8.88	7.26	1.17	1.04	1.01	Hypothetical protein
HP1220	2.99	3.28	3.32	1.11	1.09	1.24	ABC transporter, ATP-binding protein (<i>yhcG</i>)
HP1221	3.22	3.56	3.44	1.00	1.14	1.24	Hypothetical protein
HP1222*	5.32	8.34	10.18	0.98	1.24	1.11	D-Lactate dehydrogenase (<i>ldd</i>)
HP1225*	2.84	2.71	3.12	0.94	1.01	0.94	Hypothetical integral membrane protein
HP1238*	3.22	6.08	7.96	0.84	0.95	0.79	Aliphatic amidase (<i>amiF</i>)
HP1282	3.69	3.67	3.20	1.34	1.17	1.29	Anthranilate synthase component I (<i>trpE</i>)
HP1330	5.66	6.04	4.18	1.44	1.39	1.60	Hypothetical integral membrane protein
HP1331*	4.09	3.31	4.93	1.54	1.17	1.38	Hypothetical integral membrane protein
HP1332*	5.06	6.22	7.40	1.44	1.22	1.24	Cochaperone and heat shock protein (<i>dnaJ</i>)
HP1337	2.97	3.21	3.36	1.02	1.06	1.00	Hypothetical protein
HP1338	3.64	4.80	4.20	1.32	1.07	1.01	Nickel responsive regulator (<i>nikR</i>)
HP1399*	5.16	3.48	2.55	1.16	0.54	1.63	Arginase (<i>rocF</i>)
HP1402*	2.68	3.44	3.17	2.26	2.14	2.07	Type I restriction enzyme R protein (<i>hsdR</i>)
HP1432*	15.85	34.86	22.56	1.67	1.14	0.64	Histidine and glutamine-rich protein (<i>hpn</i>)
HP1466*	3.28	3.39	4.14	1.24	1.23	1.08	Hypothetical integral membrane protein
HP1499*	11.52	11.97	10.58	1.01	1.12	0.78	Hypothetical protein
HP1571	3.45	3.77	3.83	0.86	1.09	1.14	Rare lipoprotein A (<i>rlpA</i>)
HP1572	4.74	4.81	5.13	1.18	1.29	1.20	Regulatory protein (<i>dniR</i>)
Downregulated genes ^b							
HP0015	0.35	0.23	0.25	0.44	0.42	0.59	Hypothetical protein
HP0016	0.34	0.16	0.13	0.49	0.46	0.69	Hypothetical protein
HP0025	0.20	0.13	0.06	0.70	0.59	0.69	Outer membrane protein (<i>hopD</i>)
HP0029	0.38	0.37	0.37	0.51	0.62	0.60	Dethiobiotin synthetase (<i>bioD</i>)
HP0097	0.28	0.24	0.22	0.74	0.71	0.81	Hypothetical protein
HP0298	0.33	0.32	0.36	0.60	0.67	0.62	Dipeptide ABC transporter (<i>dppA</i>)
HP0375	0.36	0.22	0.20	0.99	1.12	1.11	Hypothetical protein
HP0605	0.35	0.40	0.37	0.78	0.84	0.76	Hypothetical protein
HP1027	0.19	0.30	0.23	0.43	0.43	0.51	Ferric uptake regulation protein (<i>fur</i>)
HP1180*	0.25	0.25	0.26	1.27	0.98	0.81	Pyrimidine nucleoside transport protein (<i>nupC</i>)
HP1212	0.22	0.36	0.36	0.48	0.50	0.44	ATP synthase F0, subunit c (<i>atpE</i>)

^a*H. pylori* strains were grown at either pH 5.3 or pH 7.0 for 1 h. RNA-seq was performed as described in Materials and Methods. Genes ($n = 114$) classified in group 1 of the pH transcriptome of *H. pylori* (Table S4) were examined for pH-responsive expression in *H. pylori* strains harboring ArsRS mutations (26695 Δ arsS, 26695 *arsR-D52E*, and 26695 *arsR-D52N*). Genes considered differentially expressed demonstrated fold change values (pH 5.3 versus pH 7.0) either >2.5 or <0.4 , with an FDR (false-discovery rate) value <0.01 . The genes listed in the table were differentially expressed at the two pH conditions in strains harboring an intact ArsRS TCS but not in strains containing ArsRS mutations.

^bTranscript levels of genes designated "upregulated" were higher in *H. pylori* strains containing an intact ArsRS TCS grown at pH 5.3 than in cultures grown at pH 7.0.

"Downregulated" genes were lower in *H. pylori* strains containing an intact ArsRS TCS grown at pH 5.3 than in cultures grown at pH 7.0.

^c"Fold" indicates fold change values. The fold change values are a ratio of RNA-seq reads from cultures grown at pH 5.3 compared to RNA-seq reads from cultures grown at pH 7.0.

^dAsterisks denote genes that were previously shown to be regulated by the ArsRS TCS in microarray studies (33, 34).

expression was affected by 2 of the 3 described *arsRS* mutations. Genes associated with *H. pylori* stress responses were also upregulated by the ArsRS TCS at low pH. These include genes encoding 2 key components of the oxidative stress response (HP0875 [catalase *kataA*] and HP0389 [*sodB*]) (Table 3 and Table S6). Motility-related genes (e.g., *flaB* and HP1192) were also upregulated by the ArsRS system.

Gene regulation by the ArsRS TCS at neutral pH. In addition to examining the pH-responsive ArsRS TCS regulon, we examined the effect of ArsRS mutations on the

FIG 6 ArsRS-regulated genes within operons. The figure illustrates operons in which at least two genes are differentially expressed at pH 5.3 versus pH 7.0 in strains containing an intact ArsRS TCS but not in ArsRS mutants. Operon maps are based on a previous study of *H. pylori* strain 26695 (73). Numbers in the figure correspond to *H. pylori* strain 26695 gene numbers. Green arrows indicate genes that are upregulated at low pH, red arrows indicate genes that are downregulated at low pH, and black arrows indicate genes for which transcription did not meet the criteria for differential expression. Darker shades of green or red indicate genes that were differentially expressed in all 3 of the *H. pylori* strains containing intact ArsRS systems (i.e., group 1 genes). Lighter shades of green or red indicate genes that were differentially expressed in 2 of the 3 *H. pylori* strains containing intact ArsRS systems (group 2 genes). For comparison, Fig. S4 shows pH-responsive operons.

FIG 7 Pie charts showing putative functions of 178 differentially expressed genes regulated by the ArsRS TCS. Putative functions are based on the Clusters of Orthologous Groups of proteins (COGs) annotation (ncbi.nlm.nih.gov/research/COG). The two pie charts illustrate genes that are (A) upregulated or (B) downregulated at low pH. The relative abundance of genes in each category is indicated as a percentage of the total number of genes that were upregulated or downregulated at low pH.

H. pylori transcriptome at neutral pH. As shown in Table S8, a total of 41 genes were differentially expressed at neutral pH when comparing *H. pylori* strains containing an intact ArsRS TCS with corresponding ArsRS TCS mutants (Δ arsS, *arsR-D52E*, or *arsR-D52N*). For example, in comparison to expression in the parental strain *H. pylori* 26695 Δ rdxA containing a wild-type ArsRS system, expression of 13 genes was increased and expression of 11 genes was decreased in the Δ arsS mutant (Table S8). Five of these genes (i.e., HP0681, HP0682, HP1288, HP1289, HP1432) were previously reported to be regulated by the ArsRS TCS at neutral pH (39). Similar numbers of genes were differentially expressed when comparing the *arsR-D52N* mutant with a corresponding strain containing an intact ArsRS (i.e., 26695 *arsR-D52D*) (Table S8) or when comparing the *arsR-D52E* mutant with 26695 *arsR-D52D*. Only 3 genes (HP0722 [*sabB*], HP0871 [*cdh*, CDP-diglyceride hydrolase], and HP1192 [encoding a secreted protein]) were differentially expressed at neutral pH in all three ArsRS mutant backgrounds compared to strains containing an intact ArsRS TCS. Thus, the ArsRS system primarily functions to regulate gene transcription at low pH.

Finally, we compared the set of 41 genes affected by at least one of the ArsRS TCS mutations at neutral pH (Table S8) with the 178 genes in the ArsRS pH-responsive regulon (98 group 1 genes and 80 group 2 genes). Of the 178 genes that comprise the pH-responsive ArsRS regulon (Fig. S6), only 17 genes were differentially expressed at neutral pH when comparing the wild-type strain with *H. pylori* mutants harboring a Δ arsS, *arsR-D52E*, or *arsR-D52N* mutation (Table S9).

DISCUSSION

In this study, we delineate *H. pylori* transcriptional and proteomic alterations that occur in response to low pH. We confirm that the ArsRS two-component system has a key role in mediating pH-responsive changes in transcription. By analyzing Δ arsS and *arsR* mutant strains in comparison to strains containing an intact ArsRS system, we provide a comprehensive and robust definition of the ArsRS regulon.

Several previous studies analyzed pH-responsive gene expression in *H. pylori*, mainly using microarray methodologies (slide arrays with fluorescently labeled probes or membrane arrays with radiolabeled probes) (15, 17–19, 21, 28, 33, 38). One previous study used RNA-seq methodology (20). There has been relatively limited agreement in the results of these studies. The limited agreement is likely due in part to differences among the studies in experimental design and methods. For example, the previous studies varied in the choice of *H. pylori* strain, the type of culture media (including variable concentrations of urea, a substrate for *H. pylori* urease), the acidic pH conditions tested (ranging from pH 4.5 to 6.2), and the acid exposure times (ranging from 30 min to 48 h) (15, 17–19). Many of the previous studies did not add any buffering reagents to the culture medium to ensure maintenance of an acidic environment throughout the experiment. Previous studies of the effects of environmental pH on the *H. pylori* proteome also varied in methods, including variations in the length of time that the bacteria were exposed to acidic pH and variations in the acidic pH conditions tested (ranging from pH 2.0 to pH 6.0) (25, 26).

In the present study, we analyzed *H. pylori* grown at either pH 5.3 or pH 7.0 in MES [2-(*N*-morpholino)ethanesulfonic acid]-buffered medium, which minimized changes in the medium pH. By using RNA-seq methodology, we identified 114 genes that were differentially expressed at acidic pH compared to neutral pH in all three strains containing an intact ArsRS system (representing the acid-responsive genes identified with highest confidence) and an additional 98 genes that were differentially expressed in 2 of the 3 strains analyzed. As an additional means of examining how *H. pylori* responds to changes in pH, we analyzed pH-responsive changes to the *H. pylori* proteome. In analyses of strain 26695, 49 of the 136 (36%) pH-responsive proteomic changes were concordant with transcriptional changes identified by RNA-seq. One possible reason for the limited congruence between transcriptional and proteomic results might be the difference in the time periods that *H. pylori* was exposed to low pH. In the transcriptional analyses, *H. pylori* were exposed to low pH for either 1 h or 6 h, whereas protein levels were analyzed in cultures grown for 24 h. In addition, levels of steady-state RNA are not reliable indicators of protein levels (52, 53). Rapid degradation of transcripts compared to a slower turnover of proteins, or other posttranscriptional changes, may contribute to this incongruence. For example, sRNAs can alter mRNA stability, resulting in increased or decreased protein translation (54).

The ArsRS two-component system is known to have a role in regulating *H. pylori* transcription in response to changes in pH (16, 21, 27, 33–37). In previous studies, the ArsRS regulon was defined by comparing gene expression in a wild-type strain with gene expression in a $\Delta arsS$ sensor kinase mutant, using microarray methods (33, 34, 38, 39) and other methods, including proteomic analysis (16, 27, 35, 40–43). Deletion mutants of the response regulator ($\Delta arsR$ mutants) have not been analyzed because *arsR* is reported to be an essential gene in *H. pylori* (44). In the current study, we used RNA-seq methods to define the ArsRS regulon, and we analyzed not only a $\Delta arsS$ sensor kinase mutant but also strains containing mutations to the phospho-accepting residue of the ArsR response regulator (36, 47). We identified 178 genes that were pH-responsive in strains containing an intact ArsRS TCS but not in ArsRS mutant strains. Because *arsS* and *arsR* mutations each abrogated the pH-responsiveness of a high proportion of the genes that were pH-responsive in strains containing a wild-type ArsRS TCS (86% and 82%, respectively), we infer that the ArsRS TCS plays a key role in pH-responsive transcription in *H. pylori*.

To evaluate the possibility that CrdRS or FlgRS TCSs might contribute to pH-responsive gene regulation, we constructed a triple mutant containing deletion mutations in *arsS*, *crdS*, and *flgS* sensor kinases. The triple mutant exhibited a loss of pH-responsive transcription that was similar to that of the $\Delta arsS$ mutant. The similar transcriptional profiles of the triple mutant and the $\Delta arsS$ mutant suggest that the CrdRS or FlgRS TCSs do not independently contribute to pH-responsive gene regulation. In addition, these experiments suggest that there is no cross talk between CrdS and FlgS and the

ArsRS TCS (i.e., no evidence to suggest a role of noncognate sensor kinases in phosphorylation of ArsR).

The loss of pH-associated transcriptional control in the *arsR-D52N* mutant is attributed to the lack of phosphorylation of the ArsR-D52N protein. Despite the loss of pH regulatory function, ArsR-D52N is reportedly capable of binding to target genes in gel mobility shift assays (34). Previous studies have suggested that an aspartate to glutamate substitution at this site in some response regulators can result in phosphomimetic activity (49, 55, 56). The Glu (E) residue is thus thought to function as a phosphomimetic by mimicking the phosphorylated aspartate residue in the absence of the relevant sensor kinase. Prior to the current study, nothing was known about how the ArsR-D52E mutation affected *H. pylori* transcriptional regulation. Therefore, we tested the hypothesis that a D52E substitution would result in a constitutively active ArsR whose activity is unaffected by pH changes. If the ArsR-D52E mutation functioned as a phosphomimetic, we would expect many pH-responsive genes (Table S4 and S5) to be differentially expressed when comparing an *arsR-D52E* mutant with the wild-type strain at neutral pH. In contrast, only 8 genes were differentially expressed when comparing the *arsR-D52E* mutant with the wild-type strain at neutral pH (Table S8). Thus, rather than functioning as a phosphomimetic, the D52E mutation dysregulates pH-responsive gene transcription in the same manner as the D52N mutation (Fig. S5). It is likely that ArsR-D52E is not phosphorylatable by its sensor kinase, similar to the properties of ArsR-D52N.

Among 178 genes that comprise the pH-responsive ArsRS regulon, 143 were upregulated and 35 were downregulated in response to low pH. Fifty-five of the upregulated genes and 7 of the downregulated genes were reported to be regulated by the ArsRS TCS in previous studies in *H. pylori* strains G27 and 26695 (17, 33–35, 37–40, 42, 43). Therefore, about a third of the ArsRS-regulated genes identified in this study were also identified in previous studies. The current transcriptional study provides a more comprehensive view of the pH-responsive ArsRS regulon compared to what has been reported previously.

Several of the genes upregulated at low pH by the ArsRS TCS are involved in motility (e.g., *flaB* and HP1192 [encoding a secreted protein]), suggesting that low pH may alter the motility of *H. pylori*. Previous studies (15, 17) revealed that low pH is associated with an increased abundance of flagellar components, such as FlaB and HP1192. Consistent with this, video microscopy of *H. pylori* cells revealed not only a larger proportion of motile *H. pylori* cells at low pH but also showed that *H. pylori* grown at low pH travel significantly faster than the counterparts grown at pH 7.0 (15). The ability of the ArsRS TCS to sense the surrounding acidic environment and alter motility may enhance the ability of *H. pylori* to colonize the stomach.

In the current study, multiple genes encoding OMPs were found to be transcriptionally altered in response to low pH. The expression of 9 genes encoding OMPs (HP0079 [*horA*], HP0472 [*horE*], HP0009 [*hopZ*], HP0025 [*hopD*], HP0229 [*hopA*], HP0915 [*frpB2*], HP1177 [*hopQ*], HP1501 [*horK*], HP1512 [*frpB3*]) was altered by acidic pH in all 3 of *H. pylori* strains containing an intact ArsRS TCS. The expression of an additional 8 genes encoding OMPs (HP0923 [*hopK*], HP0725 [*sabA*], HP0912 [*alpA*], HP0913 [*alpB*], HP1395 [*horL*], HP0253 [*hopG*], HP1083 [*hofB*], HP1066 [*horD*]) was altered in at least 2 of the 3 *H. pylori* strains containing an intact ArsRS TCS. Expression of several of these genes (e.g., HP0009 [*hopZ*], HP0912 [*alpA*], HP0913 [*alpB*], HP0229 [*hopA*], HP0725 [*sabA*]) was previously reported to be altered by acidic pH (15, 17, 18, 20, 40, 42, 43). Thus, about 17 (27%) of the ~63 *H. pylori* genes predicted to encode OMPs (57) are differentially expressed in response to low pH. The regulation of 12 of the 17 pH-responsive OMP-encoding genes identified in this study (i.e., *horA*, *hopK*, *hopD*, *sabA*, *alpA*, *alpB*, *horL*, *hopG*, *hofB*, *horD*, *hopQ*, *horE*) is affected by mutations to the ArsRS TCS. Several of these OMP genes (e.g., *sabA*, *hopD*, *hopQ*) have previously been shown to be regulated by the ArsRS TCS (40, 42, 43, 58). While not identified in this study, additional OMPs (e.g., *homB*, *imaA*) were previously reported to be regulated by the ArsRS TCS (41, 59).

Several of the acid-responsive genes identified in this study encode OMPs that have adhesin properties (e.g., *alpAB*, *sabA*, *labA* [*hopD*] [51, 60, 61]). Therefore, we speculate that pH-responsive transcriptional changes in these genes, mediated by the ArsRS TCS, influence *H. pylori* interactions with host cells in various pH conditions. pH-responsive transcriptional changes in these genes also may influence *H. pylori* biofilm formation (41, 62).

While our current results indicate that the ArsRS TCS is a key regulator of pH-responsive gene transcription in *H. pylori*, several other regulatory systems, including metal-responsive regulators such as NikR (63–65) and Fur (50, 66), as well as a TCS associated with flagellar synthesis (FlgRS) (67, 68), can contribute to pH-responsive gene expression. NikR controls transcription of genes involved in nickel homeostasis, while Fur regulates transcription of genes involved in iron homeostasis (50, 63–66). Of 10 pH-responsive genes that were not regulated by the ArsRS TCS (Fig. 5B and Table S7), 8 were previously reported to be regulated by either Fur or NikR (63, 65, 66). In the present study, both Fur and NikR were identified as members of the ArsRS regulon. The complexity of transcriptional control in *H. pylori* is apparent when examining the overlap in genes regulated by multiple regulatory systems. For example, 44 genes in the NikR transcriptome (69) and 51 genes in the Fur transcriptome (66, 70, 71) are also members of the ArsRS regulon. Furthermore, 16 genes common to both the Fur and NikR transcriptomes (66, 69–71) are also regulated by the ArsRS TCS. Evidence of cross talk and interactions among NikR, ArsRS, and Fur at target promoters has been reported (65, 72).

Putative ArsR binding sites have been previously identified upstream of a number of genes (16, 33–35, 37, 42, 43, 45), including several that were identified as members of the ArsRS regulon in this study (e.g., *ureA*, HP0118, HP0119, HP1187, HP1188, HP0294 [*amiE*], HP1238 [*amiF*], HP1399 [*rocF*], and *sabA*), based on DNase protection assays. Curiously, the sequences protected from DNase treatment vary greatly when comparing these promoter regions and do not align well using tools such as k-align or MaliN. Similarly, we are not able to identify a potential consensus ArsR binding site upstream of transcriptional start sites (73) for members of the ArsRS regulon identified in this study (or other studies) for which ArsR binding sites have not been experimentally identified. We speculate that ArsR may interact with conserved secondary structural features that are not easily detected by simple sequence alignments. In addition, the involvement of additional regulators (e.g., NikR and Fur, discussed above) in the regulation of some ArsRS-regulated genes might contribute to the variability in ArsR DNA-binding sequences. For example, the NikR binding site is known to overlap the ArsR binding site at the *ureA* and *amiE* promoters (16, 63), while the site for Fur binding to the *arsR* promoter overlaps the *arsR* operator sequence required for auto-repression of *arsR* transcription by ArsR (72).

A previous study reported that an ArsS mutant was unable to colonize the stomach in a mouse model of infection (74). The colonization defect of this mutant might be primarily attributable to the loss of regulatory functions required for bacterial survival or replication within the acidic gastric environment (including regulation of genes encoding proteins involved in pH homeostasis, such as UreA, AmiE, AmiF, and RocF). The ArsRS system might also contribute to colonization or persistence by regulating genes encoding proteins involved in motility or proteins that enhance *H. pylori* adherence to epithelial gastric cells. Genes encoding proteins with high levels of sequence similarity to ArsR and ArsS are found in numerous gastric and enterohepatic species, consistent with an important role of this system in regulating gene expression in response to pH and regulating genes required for bacterial colonization of the gastrointestinal tract. Several of the ArsRS-regulated genes identified in this study are known to have roles in the pathogenesis of gastric disease states. These include CagL (a component of the Cag T4SS) (75–78) and the vacuolating cytotoxin VacA (79–82) (Table S6).

In summary, the current study provides a robust definition of the pH-responsive ArsRS regulon in *H. pylori* 26695. In future studies, it will be important to develop an

TABLE 4 Plasmids and strains used in this study^a

Plasmid or strain	Description	Reference
Plasmids		
p26ArsS3	pGEMT containing <i>arsS</i> and <i>arsR</i>	This study
pArsS::cat-rdxA	p26ArsS3, contains <i>arsS</i> ::cat-rdxA	This study
pArsSΔ1090	p26ArsS3, contains 1,090 bp deletion of <i>arsS</i>	This study
pCrdRS	pGEMT containing <i>crdS</i> and <i>crdR</i>	This study
pCrdR::cat-rdxA	pCrdRS, contains <i>crdR</i> ::cat-rdxA	This study
pΔCrdS	pCrdRS, contains 837 bp deletion of <i>crdS</i>	This study
pFlgS	pGEMT containing <i>flgS</i>	This study
pFlgS::cat-rdxA	pFlgS, contains <i>flgS</i> ::cat-rdxA	This study
pFlgSΔ988	pFlgS, contains 988 bp deletion of <i>flgS</i>	This study
p166	pGEMT containing <i>arsR</i> ORF	This study
p166cat	p166 containing <i>cat</i> insertion upstream of <i>arsR</i>	This study
p166ArsRD52E	p166cat, contains <i>arsR</i> -D52E mutation	This study
p166ArsRD52N	p166cat, contains <i>arsR</i> -D52N mutation	This study
p166ArsRD52D	p166cat, contains <i>arsR</i> -D52D mutation	This study
H. pylori strains		
26695	Wild-type <i>H. pylori</i>	(91)
26695 ΔrdxA	26695 containing deletion of <i>rdxA</i> gene, Mtz ^r	(84)
26695 <i>arsS</i> ::cat-rdxA	26695 ΔrdxA containing <i>arsS</i> ::cat-rdxA, Cm ^r	This study
26695 ΔarsS	26695 ΔrdxA containing deletion of <i>arsS</i>	This study
26695 ΔarsS	26695 ΔarsS containing <i>crdR</i> ::cat-rdxA, Cm ^r	This study
<i>crdR</i>::cat-rdxA		
26695 ΔarsS ΔcrdS	26695 ΔrdxA containing deletion of <i>arsS</i> , <i>crdS</i>	This study
26695 ΔarsS ΔcrdS	26695 ΔarsS ΔcrdS containing <i>flgS</i> ::cat-rdxA, Cm ^r	This study
<i>flgS</i>::cat-rdxA		
26695 Triple mutant	26695 ΔrdxA containing deletion of <i>arsS</i> , <i>crdS</i> , <i>flgS</i>	This study
26695 <i>arsR</i> -D52E	26695 containing <i>arsR</i> -D52E mutation, Cm ^r	This study
26695 <i>arsR</i> -D52N	26695 containing <i>arsR</i> -D52N mutation, Cm ^r	This study
26695 <i>arsR</i> -D52D	26695 containing <i>arsR</i> -D52D mutation, Cm ^r	This study

^aMtz^r, metronidazole resistance; Cm^r, chloramphenicol resistance.

improved understanding of the DNA motifs recognized by ArsR and the relationships and hierarchy of the ArsRS, Fur, and NikR regulatory systems in pH-responsive gene regulation. It will be important to determine whether the ArsRS regulon of *H. pylori* 26695 closely matches the ArsRS regulons of other *H. pylori* strains or if there are strain-specific differences. In addition, it will be important to develop a better understanding of the effects of ArsRS-mediated gene regulation on *H. pylori* acid resistance, colonization of the stomach, *H. pylori* interactions with host cells, and the pathogenesis of gastric diseases.

MATERIALS AND METHODS

H. pylori culture methods. The *H. pylori* strains used in this study are listed in Table 4. For routine culture, *H. pylori* strains were grown on Trypticase soy agar plates containing 5% sheep blood (blood agar plates) at 37°C in room air supplemented with 5% CO₂. When necessary, *H. pylori* was cultured on Brucella Broth agar plates containing 5% fetal bovine serum (BB-FBS) supplemented with metronidazole (7.5 μg/ml) or chloramphenicol (2.5 to 5 μg/ml). Broth cultures of *H. pylori* were grown in bisulfite-free Brucella broth (BB) (83) containing supplemental cholesterol (BB-Chol medium). After autoclaving the BB medium, 2-(*N*-morpholino)ethanesulfonic acid (MES) (50 mM) was added and the pH was adjusted to either 7.0 or 5.3 using NaOH or HCl, respectively. The broth was filter-sterilized, and cholesterol (250× cholesterol lipid concentrate, Gibco) was added to a final concentration of 1×. In both proteomic and RNA-seq experiments (see Fig. S1 in the supplemental material), broth cultures grown overnight at pH 7.0 were subcultured into BB-Chol medium buffered to either pH 5.3 or pH 7.0 and incubated with shaking for 1 h or 6 h (RNA-seq experiments) or 24 h (proteomics).

Generation of ΔarsS mutant. To mutate the gene encoding the ArsS sensor kinase (HP0165), a 3.2 kb PCR fragment containing *arsS* and *arsR* ORFs was PCR-amplified from *H. pylori* strain 26695 with primers 5'-CTTGCAATACCAATTGCGCACGC-3' and 5'-TGAGCGTGTGAGCATGGCGTA-3'. Following cloning of the PCR product into pGEM-T Easy (Promega), the resulting plasmid (p26ArsS3) was next used as a template for inverse PCR, using primers containing BamHI sites (underlined): 5'-GGGGATCCAGTGGAACAACTGGTAATGGCG-3' and 5'-GGGGATCCGAAGAGGATAATGAAGAGCTACCC-3'. The deleted region of *arsS* was replaced by a

BamHI fragment from pMM674 containing a *cat-rdxA* cassette (84) by ligation with T4 DNA ligase (NEB). The resulting plasmid contains a modified *arsS* gene with a 1,090 bp deletion (encoding amino acids 50 to 413). The *cat-rdxA* cassette confers resistance to chloramphenicol mediated by the chloramphenicol acetyltransferase (*cat*) gene from *Campylobacter coli* and contains an intact *rdxA* gene (HP0954) from *H. pylori* 26695. The pArsS::cat-rdxA plasmid, which is unable to replicate in *H. pylori*, was transformed into *H. pylori* 26695 Δ rdxA (84), and strain 26695 *arsS*::cat-rdxA was isolated based on resistance to chloramphenicol and sensitivity to metronidazole.

To generate an unmarked Δ arsS mutant, a separate inverse PCR was performed with the plasmid described above (p26ArsS3) to generate a plasmid containing the same 1,090 bp deletion described above. However, instead of the 5' BamHI sequences (see above), the iPCR primers 5'-AGTGG TAACAAACGTTGAATGGCG-3' and 5'-GAAGAGGATAATGAAGAGCTACCC-3' were 5' phosphorylated to facilitate ligation. The resulting plasmid pArsS Δ 1090, which is unable to replicate in *H. pylori*, was then used to transform *H. pylori* strain 26695 *arsS*::cat-rdxA (described above). The transformed bacteria were plated onto BB-FBS agar plates containing metronidazole, and metronidazole-resistant clones were isolated. The desired metronidazole-resistant colonies result from recombination events in which the *cat-rdxA* cassette is replaced with the transformed DNA sequences. To confirm that the desired Δ arsS mutation had been introduced into the chromosome, the relevant region was PCR-amplified using primers 5'-GCATTGATGTGATCATTGGCCG-3' and 5'-GCGTCCTTATGTTAGGAATGCC-3', and the amplicon was sequenced.

Construction of a Δ arsS Δ crdS Δ flgS mutant. To generate a mutant in which three genes encoding histidine kinases were deleted, we introduced a *crdS* (HP1364) deletion into the 26695 Δ arsS mutant, followed by a *flgS* (HP0244) deletion into the 26695 Δ arsS Δ crdS double mutant. To generate plasmids for *crdS* deletion mutagenesis, the *crdR*-*crdS* locus was amplified as a 2,498 bp amplicon using primers 5'-GCCTTTATGCTTGGCTC-3' and 5'-CAAAGCATACGAAGAAAACGC-3' and cloned into pGEM-T Easy. The resulting plasmid, pCrdRS, was used as a template to create both a plasmid containing *crdR*::cat-rdxA and a plasmid containing a Δ crdS mutation. Cloning of the *cat-rdxA* cassette took advantage of the naturally occurring unique BglII site within the coding sequence of *crdR*. The gel-purified BamHI fragment of pMM674 harboring *cat-rdxA* was cloned into the BglII site of *crdR* in plasmid pCrdRS based on compatible overhangs, and the resulting plasmid was transformed into *H. pylori* 26695 Δ rdxA Δ arsS (Mtx^r/Cm^r). *H. pylori* 26695 (Δ rdxA Δ arsS *crdR*::cat-rdxA) was selected based on chloramphenicol resistance.

Plasmid pCrdRS (discussed above) was then used as a template for PCR using 5' phosphorylated primers 5'-ATCCCCATGCGTTTCATGTGC-3' and 5'-GCGCGTGGGTTATGGTATAGGG-3'. The self-ligated inverse PCR product yielded p Δ crdS, harboring a deletion of coding sequence for amino acids 46 through 325 of the *crdS* gene. Natural transformation of *H. pylori* 26695 Δ rdxA Δ arsS *crdR*::cat-rdxA with p Δ crdS yielded Mtx^r clones (i.e., *H. pylori* 26695 Δ rdxA Δ arsS Δ crdS) that were screened by PCR and sequencing to confirm deletion of *crdS*.

To generate the desired *flgS* deletion plasmids, primers 5'-CAAGCCCTAAAAAAGTGGCGCG-3' and 5'-GACTAAGGGGTGATGCC-3' were used to amplify a 1,913 bp *flgS* amplicon that was cloned into pGEM-T Easy and designated pFlgS. To create pFlgS::cat-rdxA, the *cat-rdxA* cassette was excised from pMM674 as a SmaI/EcoRV fragment and blunt-end cloned into the Klenow-filled unique HindIII site in *flgS*. pFlgS::cat-rdxA was next used to transform *H. pylori* 26695 Δ rdxA Δ arsS Δ crdS with selection for chloramphenicol-resistant colonies. The resulting chloramphenicol-resistant mutant was named *H. pylori* 26695 Δ rdxA Δ arsS Δ crdS *flgS*::CAT-rdxA.

To delete a portion of *flgS* encoding amino acids 16 through 344, pFlgS was used as a template in an inverse PCR, using primers 5'-GGGGATCCGGAACGCTTCAAATAAGGGCGT-3' and 5'-GGGGATCCAA CGGCCTAGGGTTAGCCTTGTC-3'. The resulting deletion plasmid, pFlgS Δ 988, was used in natural transformation of the chloramphenicol-resistant strain *H. pylori* 26695 Δ rdxA Δ arsS Δ crdS *flgS*::CAT-rdxA. The selection for metronidazole resistance yielded the triple histidine kinase mutant, *H. pylori* 26695 Δ rdxA Δ arsS Δ crdS Δ flgS.

Generation of *arsR* mutants. Using primers 5'-GAAAAATGGCTGGTAGAATGG-3' and 5'-CCCTAAAGATATTCGCATCGCC-3' and *H. pylori* 26695 genomic DNA as a template, a 1.8 kb PCR product containing 500 bp of the *arsR* (HP0166) ORF was amplified. This fragment was cloned into pGEM-T Easy, resulting in plasmid p166. Plasmid p166 was then used in an inverse PCR with 5'-CCAGATCTCCATG AAAACAAAGCC-3' and 5'-GGAGATCTGCTTTGTTTCATGG-3' and the PCR product was ligated with a *cat* cassette (*Escherichia coli*) excised from plasmid pCM7 (ATCC 37173 [85]). This introduced a *cat* cassette upstream of HP0166. Targeted mutagenesis of the resulting plasmid (p166cat) was next carried out to introduce mutations into nucleotides encoding the aspartate residue at amino acid 52 (D52), the phosphorylation site within ArsR. This mutagenesis was carried out using the Quick-Change mutagenesis kit (Agilent Technologies). The nucleotide changes were GAT to AAT (encoding a D52N change), GAT to GAA (D52E), and GAT to GAC (D52D). The introduction of mutations into plasmids was confirmed by DNA sequencing. These plasmids were then used to transform *H. pylori* strain 26695, and transformants were selected on BB-FBS agar plates supplemented with chloramphenicol. To confirm successful introduction of the desired mutations, the *arsR* gene was amplified by PCR and nucleotide sequences of amplicons were determined.

Proteomics methodology. Seed cultures of *H. pylori* strain 26695 were initially prepared by inoculating bacteria from 1-day-old plates into flasks containing MES-buffered BB (BB-Chol, pH 7.0) (Fig. S1B). The seed cultures were grown at 37°C in room air supplemented with 5% CO₂. After 24 h of growth, aliquots of the seed cultures were used to inoculate broth cultures (either pH 7.0 or pH 5.3) with a starting optical density at 600 nm (OD₆₀₀) of ~0.05 to 0.1. The cultures were grown as described above for an additional 24 h, and bacteria were harvested by centrifugation at 4,500 × g for 15 min. The optical

densities (OD_{600}) at the time of harvest were slightly higher for cultures grown at neutral pH than for cultures grown at pH 5.3, but after 24 h of growth, there was at least a 10-fold increase in OD_{600} in all cultures (data not shown). The final pH of the cultures was similar to the pH at the time of initial inoculation, indicating that MES was an effective buffer.

The bacterial pellets were resuspended in 10 ml of TNKCM buffer (50 mM Tris, 100 mM NaCl, 27 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ [pH 7.4]) and centrifuged at $4,500 \times g$ for 15 min. The resulting pellets were resuspended in 5 ml resuspension/lysis buffer (50 mM Tris, 1 mM $MgCl_2$ [pH 7.4]) containing protease inhibitor (Roche cOmplete Mini Protease Inhibitor Cocktail Tablets). The bacteria were then lysed by sonication with 3 pulses, 20 s on/40 s off, 25% amplitude of maximum power, using a 1/8-inch tip (Scientific Sonicator, Fisher). Lysates were centrifuged at $4,500 \times g$ at 4°C for 10 min. The supernatant was ultracentrifuged at $100,000 \times g$ for 1 h at 4°C, resulting in a soluble fraction (predicted to be enriched in soluble cytoplasmic and periplasmic proteins) and an insoluble fraction (predicted to be enriched in membrane proteins) (86). The insoluble membrane fraction was resuspended in 0.5 ml radioimmunoprecipitation assay (RIPA) buffer (87) containing cOmplete protease inhibitor cocktail (Roche). Following a 60 min incubation (rotisserie, $3 \times$ for 20 min), the resuspended membrane fraction was centrifuged at $10,000 \times g$ (4°C) for 10 min, and the supernatant was retained. Protein concentrations of the subcellular fractions were determined using a Pierce BCA Protein assay kit (Thermo Scientific).

Aliquots of each subcellular fraction, standardized by protein concentration, were analyzed to determine protein content (88). Samples were partially resolved, ~ 2 cm, by SDS-PAGE using a 10% NuPAGE gel, and peptides were recovered via in-gel tryptic digestion. 750 ng of these peptides were analyzed by nano-flow LC-MS/MS using a self-packed 20 cm C18 column and eluting directly into a QExactive mass spectrometer (ThermoFisher). MS/MS spectra were acquired data-dependently over a 70 min aqueous to organic gradient (2% to 40% acetonitrile) with a 90 min total cycle time. Resulting spectra were searched against a database containing *H. pylori* 26695 sequences, and peptide intensities were integrated, normalized, and aggregated back to the protein level using MaxQuant-LFQ (89). A total of 706 and 901 proteins were identified in the soluble (cytoplasmic/periplasmic) fractions and membrane fractions, respectively. Relative quantitative comparisons and statistical analysis were performed using the ProStaR package in R (90). A *P* value cutoff of 0.01 and a >2 -fold or <0.5 -fold difference in protein abundance were used as criteria to identify proteins that were differentially abundant in *H. pylori* cultures grown at neutral or acidic pH.

Preparation of samples for RNA-seq analysis. Overnight cultures of *H. pylori* were grown in BB-Chol (pH 7.0) to an OD_{600} of ~ 0.5 and pelleted by centrifugation. The bacteria were resuspended and inoculated into BB-Chol (pH 7.0 or pH 5.3) at an initial OD_{600} of ~ 0.25 and then cultured for either 1 h or 6 h. Bacteria were pelleted and resuspended in RNAlater (Ambion) for 40 min. The cell suspensions were centrifuged at $3,500 \times g$, supernatants were decanted, and the pellets were stored at -80°C . Four independent samples from each growth condition were analyzed (e.g., four RNA preparations from bacterial cultures grown under pH 5.3 conditions and four from cultures grown under routine conditions). Total RNA was next isolated as previously described (58) using TRIzol reagent, according to the manufacturer's instructions. All samples were subjected to DNase digestion (Turbo DNA free kit, Ambion) to remove contaminating DNA, followed by a cleanup step using RNeasy columns (Qiagen). Each RNA sample was eluted in 100 μl of water.

Preparation of RNA-seq libraries and sequence analysis. RNA quality was assessed using a 2100 Bioanalyzer (Agilent). At least 200 ng of the DNase-treated total RNA (RNA integrity number greater than 8) was used to generate rRNA-depleted/mRNA-enriched libraries using TruSeq Ribo-Zero bacterial RNA kits (Illumina). Library quality was assessed using a 2100 Bioanalyzer (Agilent) and the KAPA library quantification kits (KAPA Biosystems). Libraries were sequenced on a NovaSeq 6000 with paired reads of 150-bp length, according to the manufacturer's protocol. Bcl2fastq2 conversion software (Illumina) was used to generate demultiplexed Fastq files. A total of four independent RNA-seq libraries from four sets of *H. pylori* cultures grown under pH 5.3 or pH 7.0 conditions were sequenced. The number of sequence reads for each sample ranged from 24 to 33 million.

RNA-seq data were trimmed to remove all bases below a quality of Q3, and adapter sequences were removed using FastQ quality control software (FaQCs). Kallisto pseudocounting was applied to all annotated genes in the *H. pylori* 26695 reference genome (GenBank accession number GCA_000008525.1). The expected counts fields from Kallisto outputs were used for all analysis steps. Transcripts associated with a total of 1,589 *H. pylori* genes were identified by RNA-seq. The EdgeR package for R was used to analyze count files. Data from individual samples were normalized within EdgeR and analyzed using the generalized linear model (GLM).

To ensure that the RNA-seq results represented sequences corresponding to mRNA instead of contaminating DNA, sequence coverage of RNA-seq data for 8 putative nontranscribed intergenic regions and 9 regulatory genes was analyzed for all samples, using samtools mpileup as previously described (58). The average sequence coverage for the intergenic regions was 13 sequence reads, whereas the coverage for the panel of regulatory genes (predicted to be transcribed at low levels) was 1,544. The low coverage of putative noncoding regions provided evidence that the preparations used for RNA-seq analysis contained minimal contaminating DNA.

Fold change values were calculated by comparing the means of normalized pseudocounts for samples from each group (calculated by edgeR) (Data Set S1 to S3). A false-discovery rate (FDR), corresponding to a Benjamini-Hochberg adjusted *P* value, was calculated using the p.adjust program within EdgeR. Differentially expressed genes were defined as those exhibiting an FDR value <0.01 , as well as a >2.5 or <0.4 -fold difference in transcript abundance when comparing cultures grown at pH 5.3 with cultures grown at pH 7.0.

Real-time PCR methodology. For RT-qPCR analyses, seed cultures were grown overnight in BB-Chol (pH 7.0) and then transferred to BB-Chol medium (either pH 5.3 or 7.0). The cultures were grown for 1 h or 6 h at 37°C with shaking. The bacteria then were harvested, centrifuged, and resuspended in RNeasy lysis buffer. RNA was isolated according to the RNeasy RNA Isolation protocol. RNA was purified using the RNeasy Cleanup protocol from the Qiagen RNeasy minikit. To convert RNA to cDNA, 100 ng of purified total RNA was reverse transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad). Real-time RT-qPCR was carried out on 1:20 dilutions of the cDNA preparations using an ABI real-time PCR machine, with SYBR green as the fluorochrome (iQAA universal SYBR mix; Bio-Rad). The abundance of each transcript was calculated using the $\Delta\Delta CT$ method. Transcript levels of the housekeeping gene *gyrB* (DNA gyrase subunit B) and 16S rRNA were analyzed as controls. The transcript levels of all genes examined were normalized to levels of 16S rRNA. The expression of each gene in bacteria grown at pH 5.3 was compared to expression in bacteria grown under pH 7.0 conditions. The primer pairs used for the RT-qPCR are listed in Table S10.

Data availability. The proteomics data have been deposited in ProteomeXchange under identifier PXD023679, and the RNA-seq data have been deposited in the GEO database under accession numbers GSE165055 and GSE165056.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

SUPPLEMENTAL FILE 1, XLSX file, 0.6 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.9 MB.

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