

# The Orphan Response Regulator HP1021 of *Helicobacter pylori* Regulates Transcription of a Gene Cluster Presumably Involved in Acetone Metabolism<sup>∇†</sup>

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***Helicobacter pylori* is a gastric pathogen for which no nonhuman reservoir is known. In accordance with the tight adaptation to its unique habitat, the human stomach, *H. pylori* is endowed with a very restricted repertoire of regulatory proteins. Nevertheless, the three complete two-component systems of *H. pylori* were shown to be involved in the regulation of important virulence traits like motility and acid resistance and in the control of metal homeostasis. HP1021 is an orphan response regulator with an atypical receiver domain whose inactivation has a considerable impact on the growth of *H. pylori*. Here we report the identification of HP1021-regulated genes by whole-genome transcriptional profiling. We show that the transcription of the essential housekeeping genes *nifS* and *nifU*, which are required for the assembly of Fe-S clusters, is activated by HP1021. Furthermore, we demonstrate that the expression of a gene cluster comprising open reading frames *hp0690* to *hp0693* and *hp0695* to *hp0697* which is probably involved in acetone metabolism is strongly upregulated by HP1021. Evidence is provided for a direct regulation of the *hp0695*-to-*hp0697* operon by the binding of HP1021 to its promoter region.**

*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 (6, 28, 36). Consistent with the human stomach being its unique habitat which is believed to provide a relatively stable environment lacking competition from other microorganisms, *H. pylori* has a remarkably small repertoire of transcriptional regulators. Containing only three histidine kinases and five response regulators involved in transcriptional regulation (35), *H. pylori* is among the organisms with the lowest number of two-component systems whose genomes have been sequenced so far. However, the two-component systems of *H. pylori* proved to play an important role in both in vitro growth of the organism and its ability to colonize in a mouse infection model (5, 26, 31). It was shown that the two-component systems HP0703-HP0244, named FlgRS, and HP0166-HP0165, named ArsRS, are involved in the regulation of flagellum-based motility and urease-dependent acid resistance, which are both essential virulence traits of *H. pylori* (5, 23, 29, 30). The two-component system HP1365-HP1364 (CrdRS) positively regulates the expression of the copper resistance determinant CrdAB-CzcAB in response to increasing concentrations of copper ions (37). Moreover, this two-component system was

recently reported to contribute to acid-responsive gene regulation (16).

Besides the aforementioned systems consisting of a histidine kinase and its cognate response regulator, *H. pylori* encodes two orphan response regulators with unique properties. HP1043, which belongs to the OmpR subfamily of response regulators, is essential for the growth of *H. pylori* under standard culture conditions. Deletion of the *hp1043* gene could only be achieved when a second gene copy had previously been integrated into the *H. pylori* chromosome (19, 31). Surprisingly, the receiver domain of HP1043 shows a high degree of degeneration since there is a lysine residue at the position corresponding to highly conserved D13 in the consensus sequence and the canonical receiver phosphorylation site (D57 in the consensus sequence) is lacking due to a four-amino-acid (aa) deletion. The target genes controlled by HP1043 are unknown; however, on the basis of in vitro DNA binding experiments it was hypothesized that HP1043 regulates its own expression, as well as the transcription of the *tlpB* gene encoding a methyl-accepting chemotaxis protein (9). Furthermore, the expression of HP1043 seems to be controlled on the posttranscriptional and/or posttranslational level (9, 22).

The second orphan response regulator, HP1021, is required for normal cell growth of *H. pylori*, since deletion of the response regulator gene caused a growth defect resulting in a small-colony phenotype (5, 19). HP1021 harbors a helix-turn-helix motif at the C terminus of its output domain, yet it cannot be grouped into any of the known response regulator subclasses. Interestingly, in the receiver domain of HP1021 and its orthologs in the other sequenced  $\epsilon$ -proteobacteria, i.e., *H. acinonychis*, *H. hepaticus*, *Campy-*

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† This paper is dedicated to Werner Goebel on the occasion of his retirement.

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TABLE 1. Oligonucleotides used in this study

Name	Sequence (5' to 3') <sup>a</sup>	Site <sup>b</sup>	Strand	Position <sup>c</sup>
hyuA-PE	TGCCACCGGCATCAATACC		–	745828–745846
fadA-PE	CCGCCACTACAACCCTTC		–	740565–740583
nifS-PE	GTCAATCCTAGTTGTAGCG		–	228368–228386
fadA-5	ATGAATGAAGTGGTTGTAGTGGCG		+	740559–740582
fadA-3	AAGCGGGTTTGAGCTTTGCAAGGG		–	741235–741258
katA-5	GTGGTGCATGCTAAAGGAAGC		–	926909–926929
katA-3	CAATGGATAATCTTGGAGATACC		+	926216–926238
16S-5	GCTAAGAGATCAGCCTATGTCC		–	1208855–1208876
16S-3	TGGCAATCAGCGTCAGGTAATG		+	1208356–1208377
BShyuA-5	ATGATATTTATATGATATTTTTGGG		+	745602–745626
BShyuA-3	TTGCGTCTTTCATTTAGACTCC		–	745792–745813
BSkatA-5	ATCCGTCAATGCATTGAGATAG		–	927348–927327
BSkatA-3	CTTCGTAATATGACACTAAGCC		+	927114–927135
Pcag-5	ataaaggatccCATTTTTAGCAAATTTTTGTTAATTGTGG	BamHI	+	579674–79702
Pcag-3	ttgaaatctagaGTTAGTGTCAAAGACTGCTAAAAATC	XbaI	–	579853–579878
PhyuA-5	ctaatactgcagCCTTAACATCTTCATTCAAGGC	PstI	+	745367–745388
PhyuA-3	atactaggatccTAGCTAAACTCTCATCTTCTGG	BamHI	–	745912–745933
PfadA-5	ctaatactgcagTATTGACAGCTATTGCCAAAGC	PstI	+	740082–740103
PfadA-3	atactaggatccCACATCGCTAGGCTTAAGGC	BamHI	–	740683–740702
PnifS-5	atattcgaaatcTCATTACCGCTCTTTATAACC	EcoRI	–	228011–228031
PnifS-3	ttgatctgcagGTCAATCCTAGTTGTAGCG	PstI	–	228368–228386
1021com-1	ttgcacgaatcTTAAAGGCCCATAGCGATGG	EcoRI	+	1083718–1083737
1021com-2	cttactggatccGGATTTTCTGTTATTTGCGCG	BamHI	–	1085018–1085038
1021com-3	caatgctgcagGGCGTTTGATCATAAATTGC	PstI	+	1085058–1085079
1021com-4	caatcagagctcTTGTAATGATCGCACAGCTGG	SacI	–	1085502–1085522
GST1021-5	caagaaggatccTGAAAATCTTAATCATTGAAGACG	BamHI	+	1084133–1084156
GST1021-3	aaggatgaatcTTATTTGCGCGTAAGTTATATTC	EcoRI	–	1085004–1085028

<sup>a</sup> Sequences in uppercase letters are derived from the genomic sequence of *H. pylori* 26695 (35). Sequences introduced for cloning purposes are given in lowercase letters, and restriction recognition sequences are underlined.

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

<sup>c</sup> Nucleotide positions refer to the genome sequence of *H. pylori* 26695 (35).

*lobacter jejuni*, *Wolinella succinogenes*, and *Thiomicrospira denitrificans* (4, 12, 27, 33; <http://cmr.tigr.org>), the highly conserved phosphate-accepting aspartic acid residue (D57) is replaced by serine. Though serine phosphorylation has been observed in response regulator mutants lacking an aspartic acid residue at the canonical phosphorylation site (3, 21), until now there are no reports on serine phosphorylation as a means of modulating the activity of wild-type response regulator proteins. Recently, we could show that phosphorylation of the serine residue is not required for the cell growth-associated function of HP1021 (31). However, it cannot be ruled out that the DNA binding activity of both HP1021 and HP1043 can be modulated by a so-far-unknown mechanism. Transcription of hp1021, which is directed from a promoter upstream of the *htrA* gene (hp1019) forming a transcriptional unit with open reading frames (ORFs) hp1020 and hp1021, is increased in response to an acidic pH (20, 29); however, this transcriptional induction is not mediated by the ArsRS two-component system (29, 30). *htrA* and hp1020 encode a serine protease and a protein of unknown function, respectively. Target genes regulated by HP1021 have not been reported.

In this study, we set out to characterize the regulon controlled by HP1021. By global transcriptional profiling, we identified 79 genes which are differentially expressed in an hp1021 null mutant of *H. pylori* 26695, including essential housekeeping genes and highly regulated genes which presumably are involved in the metabolism of acetone. Furthermore, we dem-

onstrate that these latter genes are regulated by direct binding of HP1021 to their promoter region.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *H. pylori* 26695 is a clinical isolate (35). *H. pylori* 26695/HP1021::km, lacking the response regulator gene hp1021, has been described previously (29). *H. pylori* strains were grown at 37°C under microaerophilic conditions (Oxoid) on Columbia agar plates containing 5% horse blood, 0.2% cyclodextrin, and Dent's antibiotic supplement. Liquid cultures were grown in brain heart infusion (BHI) broth containing Dent's antibiotic supplement and 10% fetal calf serum. When required, blood agar plates or liquid broth for *H. pylori* culture were supplemented with kanamycin or chloramphenicol in a final concentration of 20 µg/ml. *Escherichia coli* DH5α was grown in Luria-Bertani (LB) broth supplemented with antibiotics at the following final concentrations if necessary: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; chloramphenicol, 30 µg/ml.

**Construction of *H. pylori* strain 26695/Δ1021com.** Complemented strain 26695/Δ1021com is a derivative of 26695/HP1021::km into which ORF hp1021 was reintroduced via allelic-exchange mutagenesis by replacing the kanamycin resistance cassette with the response regulator gene and a downstream chloramphenicol acetyltransferase (*cat*) gene. The suicide plasmid used for the transformation of *H. pylori* 26695/HP1021::km was constructed by stepwise ligation of a 1,321-bp EcoRI-BamHI fragment encoding aa 277 to 406 of ORF hp1020 and ORF hp1021 and a 454-bp PstI-SacI fragment comprising the intergenic region between ORFs hp1021 and hp1022, as well as the region encoding aa 1 to 79 of ORF hp1022, into cloning vector pSL1180 (Amersham Biosciences). The cloned DNA fragments were PCR amplified from chromosomal DNA of *H. pylori* 26695 with primer pairs 1021com-1/1021com-2 and 1021com-3/1021com-4, respectively (Table 1). The resulting plasmid was linearized by restriction with BamHI and PstI, and a chloramphenicol resistance cassette from *Campylobacter coli* (38) was cloned between the *H. pylori*-specific DNA fragments, yielding suicide plasmid pSL-Δhp1021com, which was introduced into *H. pylori* 26695/HP1021::km by elec-

trotransformation (13). Chromosomal DNA of the resulting transformants was analyzed for the correct replacement of the kanamycin resistance cassette by the response regulator gene *hp1021* and the *cat* gene by PCR with primers specific for *hp1021* and *cat*, respectively, and with primers flanking the integration site.

**RNA isolation.** *H. pylori* RNA was isolated from bacteria grown in liquid broth to an optical density at 550 nm ( $OD_{550}$ ) of 0.7 to 0.75 by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA preparations intended to be used for global transcriptional profiling were further purified with 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 the 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 with a custom-made whole-genome microarray containing 1,649 PCR products generated with specific primer pairs derived from the genome sequences of *H. pylori* 26695 (35) and J99 (2), which were spotted in duplicate. Microarrays were produced as described previously (14). Synthesis of differentially labeled cDNAs (Cy3-dCTP and Cy5-dCTP; Amersham Biosciences) from RNAs extracted from *H. pylori* 26695 and 26695/HP1021::km, respectively; microarray hybridization; generation of raw data; and data analysis were performed essentially as described by Pflöck et al. (30).

**Primer extension and RNA slot blot analysis.** Primer extension analysis was performed essentially as described previously (29), with 0.5 pmol of  $\gamma$ - $^{32}$ P-end-labeled oligonucleotides *hyuA*-PE, *fadA*-PE, and *nifS*-PE (Table 1), respectively, and 30  $\mu$ g of RNA. Plasmids pSL-*hyuA*prom, pSL-*fadA*prom, and pSL-*nifS*prom, which were used as template DNAs in the sequencing reactions performed with primers *hyuA*-PE, *fadA*-PE, and *nifS*-PE, were constructed by ligating PstI-BamHI or EcoRI-PstI fragments of 566 bp, 620 bp, and 376 bp, respectively, which were amplified from chromosomal DNA of *H. pylori* 26695 with primer pairs *PhyuA*-5/*PhyuA*-3, *PfadA*-5/*PfadA*-3, and *PnifS*-5/*PnifS*-3 into cloning vector pSL1180 (Amersham Biosciences). Primer extension experiments were performed two times with independently prepared RNAs. Quantification of the signals from the primer extension products was performed with 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  $\mu$ g) was denatured in 1 $\times$  morpholinepropanesulfonic acid (MOPS) buffer containing 50% formamide and 6% formaldehyde. The samples were incubated at 65°C for 5 min and cooled on ice before addition of 1 volume of 20 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The denatured samples were filtered through a positively charged nylon membrane (Hybond N<sup>+</sup>; Amersham Biosciences) with a Bio-Dot chamber (Bio-Rad). After UV cross-linking, the nylon membrane was prehybridized for 1 h at 42°C in hybridization buffer (ECL gold hybridization buffer; Amersham Biosciences). The PCR products used as hybridization probes were amplified with primer pairs *fadA*-5/*fadA*-3, *cat*-5/*cat*-3, and 16S-5/16S-3, respectively, and labeled nonradioactively with the ECL Direct 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 $\times$  SSC, 0.4% sodium dodecyl sulfate [SDS]) for 20 min at 42°C and two times in wash solution II (2 $\times$  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.

**Preparation of whole-cell protein lysates and proteome analysis.** Bacteria from a liquid culture grown to an  $OD_{550}$  of 1.0 were washed twice with phosphate-buffered saline containing 20  $\mu$ g/ml chloramphenicol. The bacterial pellet was suspended in 1 ml of 10 mM Tris-HCl (pH 7.2)–5 mM MgCl<sub>2</sub> containing 40  $\mu$ g each of the proteinase inhibitors Pefabloc (Serva), pepstatin (Sigma), and leupeptin (Sigma), and the suspension was transferred to a Lysing Matrix B tube (MP Biomedicals). The tube was shaken six times for 30 s with a 1-min interval on ice after each shaking at a speed setting of 6.5 in a bead beater FP120 Fast Prep cell disrupter (Savant Instruments). Twenty-five units of benzonase (Merck) and 25 U of Rnase-it (Stratagene) were added, and the mixture was incubated for 10 min at room temperature. After centrifugation, urea and thiourea were added to the supernatant to final concentrations of 7 M and 2 M, respectively, and the mixture was incubated for 30 min at room temperature. For protein precipitation, trichloroacetic acid was added to a final concentration of 15% (vol/vol) and the mixture was incubated on ice for 2 h. The precipitated proteins were sedimented by centrifugation, the protein pellet was washed three times with ice-cold acetone, and the proteins were dissolved in urea buffer

containing CHAPS {7 M urea, 2 M thiourea, 4% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 70 mM dithiothreitol (DTT)} and stored at –20°C.

Protein extracts were loaded onto Immobiline DryStrips (Amersham Biosciences) covering a pH gradient ranging from 6 to 9 which had been reswollen in rehydration buffer (7 M urea, 2 M thiourea, 4% [wt/vol] CHAPS, some crystals of bromophenol blue) for at least 12 h. For isoelectric focusing, a total of approximately 120,000 Vh was applied with several stepwise increases in voltage to 8,000 V (IPGphor Isoelectric Focusing Unit; Amersham Biosciences). The second dimension was standard SDS-polyacrylamide gel electrophoresis with 12% polyacrylamide gels (ISODalt; Amersham Biosciences). Prior to SDS-polyacrylamide gel electrophoresis, the strips were subsequently treated for 15 min with equilibration buffer (50 mM Tris [pH 8.8], 6 M urea, 30% [vol/vol] glycerol, 2% [vol/vol] SDS, some crystals of bromophenol blue) containing freshly added 64 mM DTT and for 15 min with equilibration buffer supplemented with freshly added 135 mM iodoacetic acid. The gels were fixed and stained either with silver nitrate or with colloidal Coomassie blue G250 according to standard procedures.

Protein spots of interest were precisely excised from Coomassie blue-stained two-dimensional (2D) gels on which about 800  $\mu$ g of protein was separated. The gel pieces were cut into small cubes, rinsed several times with 100  $\mu$ l of water for 15 to 30 min each, and washed three times with 100  $\mu$ l of 50% (vol/vol) acetonitrile for 10 to 20 min. To shrink the gel and to extract residual water, pure acetonitrile was added dropwise for 10 min. After removal of the acetonitrile, 30 to 50  $\mu$ l of digestion buffer (50 mM *N*-methylmorpholine, pH 8.1) and 0.5  $\mu$ g of trypsin were added. Trypsin digestion of the protein was performed at 37°C for 6 to 12 h. The supernatant containing the generated peptides was recovered, and the gel pieces were extracted twice with 0.1% trifluoroacetic acid for 20 to 30 min. The volume of the combined extracts was reduced to 5  $\mu$ l in a SpeedVac concentrator. Liquid chromatography-mass spectrometry and collision-induced fragmentation spectra were recorded on a Finnigan LCQ ion trap mass spectrometer equipped with an electrospray ionization source. The grouping of fragment ion (collision-induced fragmentation) spectra that originated from the same precursor ion and cross-correlation analysis of the data were performed by using the Sequest program (10). The Sequest algorithm compares the measured fragment ion spectra of all selected peptides to the predicted spectra of tryptic peptides that are contained in protein databases (NCBI, OWL, and NRDB) and that exhibit the same molecular weight. Identification of multiple peptides derived from the same protein and evaluation of their cross-correlation scores result in unambiguous identification of the protein.

**Expression and purification of recombinant glutathione S-transferase (GST)-HP1021.** Plasmid pGEX-1021 was constructed by ligating an 895-bp BamHI-EcoRI fragment encoding HP1021 which was PCR amplified from chromosomal DNA of *H. pylori* 26695 with primer pair GST1021-5/GST1021-3 into BamHI-EcoRI-digested pGEX-3X vector DNA (Amersham Biosciences), creating an in-frame fusion to the gene encoding GST. The GST fusion protein derived from pGEX-1021 was produced in *E. coli* DH5 $\alpha$ . Bacteria were grown in 1 liter of LB broth at 37°C to an  $OD_{600}$  of 0.5. Protein expression then was induced by the addition of 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), followed by further incubation for 3 h at 30°C. Purification of GST-HP1021 by affinity chromatography on glutathione-Sepharose 4B (Amersham Biosciences) was performed essentially as described previously (5).

**Electrophoretic mobility shift assay (EMSA).** DNA fragments of 210 and 234 bp encompassing the upstream regions of *hyuA* (hp0695) and *katA* (hp0875), respectively, were PCR amplified with primer pairs BShyuA-5/BShyuA-3 and BSkatA-5/BSkatA-3 and chromosomal DNA of *H. pylori* 26695 as the template. The PCR fragments were 5' end labeled with [ $\gamma$ - $^{32}$ P]ATP with T4 polynucleotide kinase (MBI Fermentas) and purified with MicroSpin S-200 HR columns (Amersham Biosciences). The recombinant GST-HP1021 protein was diluted in dilution buffer (2 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM DTT, 0.1% Igepal CA 630). Increasing amounts of the protein were added to approximately 15,000 cpm of the labeled DNA probe in a final volume of 20  $\mu$ l of binding buffer (10 mM Tris-HCl [pH 8.0], 10 mM KCl, 5 mM EDTA, 1 mM DTT, 1% glycerol), and the samples were incubated for 30 min at room temperature and then loaded onto a nondenaturing 4% polyacrylamide gel. Gels were run for 2.5 h at 150 V. Gels were vacuum dried, and bands were visualized by phosphorimaging (Typhoon 9200 Variable Mode Imager). A DNA fragment comprising the promoter of the *cagA* gene which was amplified with primer pair P<sub>cag</sub>-5/P<sub>cag</sub>-3 from chromosomal DNA of *H. pylori* 26695 was used as a nonspecific competitor in the EMSA experiments.

**Nucleotide sequence accession number.** The microarray raw data obtained in this study were deposited in the NCBI Gene Expression Omnibus database (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE 5971.

TABLE 2. HP1021-activated and -repressed genes

Category and ORF HP no. <sup>b</sup>	ORF JHP no. <sup>b</sup>	$\Delta$ hp1021/ wild-type ratio	Function, gene <sup>c</sup>	Genome organization <sup>d</sup>	Regulation by acid or ArsR~P (reference[s]) <sup>e</sup>
<b>HP1021-activated genes<sup>a</sup></b>					
<b>Amino acid biosynthesis</b>					
HP0695	JHP0633	0.06	Hydantoin utilization protein A, <i>hvaA</i>	op hp0695-hp0697	+ (39), - (20)
HP0696	JHP0632	0.08	Predicted <i>N</i> -methylhydantoinase	op hp0695-hp0697	
<b>Biosynthesis of cofactors, prosthetic groups, and carriers</b>					
HP0220	JHP0206	0.39	Synthesis of [Fe-S] cluster, <i>ni/S</i>	op hp0220-hp0221	+ (39)
HP0221	JHP0207	0.37	NifU scaffold protein involved in [Fe-S] cluster assembly	op hp0220-hp0221	+ (30)
<b>Cell envelope</b>					
HP0025	JHP0021	0.19	Outer membrane protein, <i>omp2</i> ( <i>topD</i> )	m	+ (39)
HP1564	JHP1472	0.36	Outer membrane lipoprotein	m	+ (30)
<b>Cellular processes</b>					
HP0875	JHP0809	0.25	Catalase, <i>katA</i>	m	+ (30, 39)
HP0303	JHP0288	0.43	Predicted GTP-binding protein of the GTP1/Obg family involved in stress response	op hp0298-hp0304	
HP0630	JHP0573	0.48	Predicted modulator of drug activity	m	
<b>Energy metabolism</b>					
HP0691	JHP0637	0.18	Succinyl-CoA:acetoacetate-CoA-transferase subunit A, <i>scvA</i> ( <i>yyjD</i> )	op hp0690-hp0693	
HP0692	JHP0636	0.18	Succinyl-CoA:acetoacetate-CoA-transferase subunit B, <i>scvB</i> ( <i>yyjE</i> )	op hp0690-hp0693	+ (39)
HP1104	JHP1030	0.49	Predicted mannitol dehydrogenase	m	+ (30, 39)
HP1398	JHP1428	0.41	Alanine dehydrogenase, <i>ald</i>	m	- (30)
<b>Fatty acid and phospholipid metabolism</b>					
HP0090	JHP0083	0.36	Predicted malonyl coenzyme A-acyl carrier protein transacylase, <i>fabD</i>	op hp0090-hp0089	
HP0690	JHP0638	0.47	Predicted acetyl coenzyme A acetyltransferase, <i>fadA</i>	op hp0690-hp0693	+ (39)
<b>Central intermediary metabolism</b>					
HP0089	JHP0082	0.46	Predicted 5'-methylthioadenosine nucleosidase/ <i>S</i> -adenosylhomocysteine nucleosidase, <i>pfs</i>	op hp0090-hp0089	
HP1186	JHP1112	0.42	Carbonic anhydrase	m	+ (39), - (20)
<b>Protein fate</b>					
HP1299	JHP1219	0.39	Predicted methionine amino peptidase	op hp1320-hp1292?	
HP1300	JHP1220	0.41	Predicted preprotein translocase subunit	op hp1320-hp1292?	
<b>Transcription, transcription factors, and translation</b>					
HP1514	JHP1407	0.45	Transcription termination factor, <i>nusA</i>	m	
HP1301	JHP1221	0.44	Ribosomal protein, <i>rplI5</i>	op hp1320-hp1292?	
HP1302	JHP1222	0.47	Ribosomal protein, <i>rps5</i>	op hp1320-hp1292?	- (39)
HP1303	JHP1223	0.48	Ribosomal protein, <i>rplI8</i>	op hp1320-hp1292?	
HP1304	JHP1224	0.34	Ribosomal protein, <i>rpl6</i>	op hp1320-hp1292?	- (39)
HP1305	JHP1225	0.37	Ribosomal protein, <i>rps8</i>	op hp1320-hp1292?	
HP1306	JHP1226	0.45	Ribosomal protein, <i>rpsI4</i>	op hp1320-hp1292?	- (39)
HP1307	JHP1227	0.40	Ribosomal protein, <i>rpl5</i>	op hp1320-hp1292?	
HP1308	JHP1228	0.39	Ribosomal protein, <i>rpl24</i>	op hp1320-hp1292?	- (39)
HP1309	JHP1229	0.44	Ribosomal protein, <i>rplI4</i>	op hp1320-hp1292?	
HP1310	JHP1230	0.44	Ribosomal protein, <i>rpsI7</i>	op hp1320-hp1292?	- (39)
HP1313	JHP1233	0.34	Ribosomal protein, <i>rps3</i>	op hp1320-hp1292?	
HP1314	JHP1234	0.37	Ribosomal protein, <i>rpl22</i>	op hp1320-hp1292?	- (39)

HP1315	JHP1235	0.39	Ribosomal protein, <i>rps19</i>	op hp1320-hp1292?	- (39)
HP1316	JHP1236	0.35	Ribosomal protein, <i>rpl2</i>	op hp1320-hp1292?	- (39)
HP1318	JHP1238	0.47	Ribosomal protein, <i>rpl4</i>	op hp1320-hp1292?	- (39)
HP1319	JHP1239	0.48	Ribosomal protein, <i>rpl3</i>	op hp1320-hp1292?	
HP1554	JHP1445	0.28	Ribosomal protein, <i>rps2</i>	op hp1454-hp1455	
HP1555	JHP1444	0.36	Translation elongation factor EF-Ts	op hp1454-hp1455	
Transport and binding proteins					
HP0140	JHP0128	0.30	Predicted L-lactate permease, <i>lctP</i>	op 0140-hp0141?	- (7)
HP0299	JHP0284	0.35	Predicted dipeptide permease protein, <i>dppB</i>	op hp0298-hp0304	+ (39), - (7)
HP0300	JHP0285	0.37	Predicted dipeptide transport system permease protein, <i>dppC</i>	op hp0298-hp0304	+ (39)
HP0302	JHP0287	0.35	Predicted dipeptide transport system permease protein, <i>dppF</i>	op hp0298-hp0304	+ (39)
HP0693	JHP0635	0.24	Predicted short-chain fatty acid transporter	op hp0690-hp0693	+ (39)
HP1181	JHP1107	0.47	Predicted multidrug efflux transporter	op hp1181-hp1182	- (39)
Unknown					
HP0747	JHP0684	0.21	Predicted S-adenosylmethionine-dependent methyltransferase	op hp0745-hp0750?	
Hypothetical					
HP0697	JHP0631	0.07	Conserved hypothetical protein	op hp0695-hp0697	+ (39)
HP0902	JHP0839	0.42	Conserved hypothetical protein	op hp0902-hp0901	
HP0423	JHP0639	0.43	Hypothetical protein	op hp0427-hp0423?	+ (7, 30)
HP0699	JHP0808	0.48	Hypothetical protein	op hp0698-hp0703	
HP0874	JHP0808	0.29	Hypothetical protein	m?	+ (39)
HP1412	JHP1307	0.40	Hypothetical protein	op hp1408-hp1412?	+ (30, 39)
HP1021-repressed genes <sup>6f</sup>					
Ammonia production					
HP0070	JHP0065	2.94	Urease accessory protein, <i>ureE</i>	op hp0071-hp0067	+ (30, 39)
HP1238	JHP1559	2.80	Aliphatic amidase, <i>amtF</i>	m	+ (7, 20, 30)
Biosynthesis of cofactors, prosthetic groups, and carriers					
HP0306	JHP0291	2.24	Glutamate-1-semialdehyde 2,1-aminomutase, <i>hemL</i>	op hp0305-hp0308	+ (20, 30, 39)
Cell envelope					
HP0229	JHP0214	2.49	Outer membrane protein, <i>omp6 (hopA)</i>	m	- (7, 20, 30, 39)
HP0722	JHP0659	2.69	Outer membrane protein, <i>omp16 (hopO)</i>	m	- (7, 20, 30)
HP0725	JHP0662	3.77	Outer membrane protein, <i>omp17 (hopP)</i>	m	- (7, 20, 30)
HP1083	JHP0342	2.40	Predicted outer membrane protein, <i>hopB</i>	m	- (30)
Energy metabolism					
HP0954	JHP0888	2.65	NAD(P)H-dependent nitroreductase	op hp0966-hp0954?	+ (30)
HP1399	JHP1427	2.52	Arginase, <i>rocF</i>	m	+ (39), - (30)
Fatty acid and phospholipid metabolism					
HP0950	JHP0844	2.87	Predicted acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, <i>accD</i>	op hp0950-hp0948	+ (39)
Transport and binding proteins					
HP0686	JHP0626	2.32	Iron(III) dicitrate transport protein, <i>fccA1</i>	m	- (30)
HP1400	JHP1426	4.33	Iron(III) dicitrate transport protein, <i>fccA3</i>	m	- (7)
HP1174	JHP1101	3.42	Glucose/galactose transporter	m	- (30)
Pathogenesis					
HP0967		2.13	Predicted virulence-associate protein D	m	

Continued on following page



TABLE 2—Continued

Category and ORF HP no. <sup>b</sup>	ORF JHP no. <sup>b</sup>	Alp1021/ wild-type ratio	Function, gene <sup>c</sup>	Genome organization <sup>d</sup>	Regulation by acid or ArsR~P (reference[s]) <sup>e</sup>
Hypothetical					
HP1242	JHP1163	2.59	Conserved hypothetical protein	m	
HP0966	JHP0900/JHP0901	2.42	Conserved hypothetical protein	op	– (39)
HP1334		3.86	Conserved hypothetical protein	m	– (39)
HP0937	JHP0872	2.22	Conserved hypothetical protein	m?	– (39)
HP0938	JHP0873	3.14	Conserved hypothetical protein	m?	– (39)
HP0948	JHP0882	2.07	Conserved hypothetical protein	op	– (39)
HP0946	JHP0880	2.12	Conserved hypothetical integral membrane protein	m	– (39)
HP0097	JHP0089	2.10	Hypothetical protein	m	– (20, 39)
HP0242	JHP0227	2.97	Hypothetical protein	op	– (30, 39)
HP0945		3.73	Hypothetical protein	m	– (30)
HP0947	JHP0881	2.83	Hypothetical protein	m	– (30)
HP0953	JHP0887	2.08	Hypothetical protein	m?	– (39)
HP0990	JHP0938	2.12	Hypothetical protein	m	– (39)
HP0992		2.41	Hypothetical protein	m	– (39)

<sup>a</sup> Genes listed are those whose transcription, according to microarray analysis, differed more than twofold (ratio of >2.0 and <0.5) in the HP1021-deficient mutant *H. pylori* 26695/HP1021::km compared to the 26695 wild-type strain.

<sup>b</sup> ORF numbers and prediction of transcriptional units are according to the genome sequences of *H. pylori* 26695 and J99 (2, 35).

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

<sup>d</sup> m indicates monocistronically transcribed genes, and op indicates putative transcriptional units. A question mark indicates that the proposed operon structure cannot be unambiguously deduced from the genome sequence.

<sup>e</sup> Where a reference(s) is given, pH-responsive transcription or ArsR~P-dependent regulation was reported by Merrell et al. (20), Wen et al. (39), Bury-Moné et al. (7), or Pflock et al. (30). A plus sign denotes positive regulation by a low pH or ArsR~P, and a minus sign denotes negative regulation by a low pH or ArsR~P.

## RESULTS

**Whole-genome transcriptional profiling of an hp1021 null mutant of *H. pylori* 26695.** In order to characterize the regulon controlled by the response regulator HP1021, a transcriptome analysis was performed with a whole-genome microarray containing 1,649 PCR products generated with specific primer pairs derived from the genome sequences of *H. pylori* 26695 (35) and J99 (2) and comprising 98% of the coding sequences present in both genomes (14). RNA was prepared from two independent cultures of *H. pylori* 26695 and the isogenic hp1021 null mutant 26695/HP1021::km, respectively, grown to an OD<sub>550</sub> of 0.75. Cy5- and Cy3-labeled cDNA was synthesized from these RNAs, and the differentially labeled cDNA pairs derived from the *H. pylori* wild-type and hp1021 null mutant strains were hybridized to four independent microarray slides, creating eight sets of hybridization data. A total of 79 genes were found to be differentially expressed in the hp1021 null mutant when a signal ratio cutoff of <0.5 and >2.0 was applied (Table 2). Transcription of 51 genes was reduced in the mutant including 17 genes encoding ribosomal proteins and the *tsf* gene encoding translation elongation factor EF-Ts, which is cotranscribed with *rps2*. The remaining genes which are positively controlled by HP1021 comprise mainly metabolic genes including *nifS* and *nifU*, which are involved in the synthesis of iron-sulfur clusters; genes encoding transport proteins; and genes encoding proteins of unknown function. The transcription of 28 genes, half of which encode proteins of unknown function, was derepressed in the mutant, suggesting negative regulation by the HP1021 protein. The regulatory effects detected in the microarray analysis were modest in the majority of cases, with only 10 genes showing an at least fourfold change in the amount of transcript in the HP1021 null mutant. Six of these highly regulated genes are part of two transcriptional units, i.e., hp0690 to hp0693 and hp0695 to hp0697, and are annotated as ORFs encoding succinyl coenzyme A (CoA): acetoacetate-CoA-transferase subunits A and B (hp0691 and hp0692), a predicted short-chain fatty acid transporter (hp0693), hydantoin utilization protein A (hp0695, *hyuA*), *N*-methylhydantoinase (hp0696), and a hypothetical protein (hp0697). Hydantoinases are microbial enzymes which catalyze the hydrolysis of the cyclic amide bonds of substituted pyrimidines and hydantoins to form the corresponding *N*-carbamyl amino acid (18), and therefore HP0695 and HP0696 were expected to be involved in amino acid biosynthesis (35). Transcription of the first gene in the hp0690-to-hp0693 operon encoding acetyl-CoA-acetyltransferase (hp0690, *fadA*) was downregulated about twofold in the hp1021 null mutant. *FadA* was suggested to be involved in fatty acid biosynthesis in *H. pylori* (17). The other highly regulated genes encode an outer membrane protein (hp0025), a predicted *S*-adenosylmethionine-dependent methyltransferase (hp0747), catalase (hp0875, *katA*), and the iron(III) dicitrate receptor protein *FecA* (hp1400), which is the only highly regulated gene under negative control of HP1021.

To confirm the data from the microarray analysis, transcription of *fadA* (hp0690), *hyuA* (hp0695), and *nifS* (hp0220) was investigated by primer extension experiments performed with RNA extracted from *H. pylori* wild-type strain 26695 and its

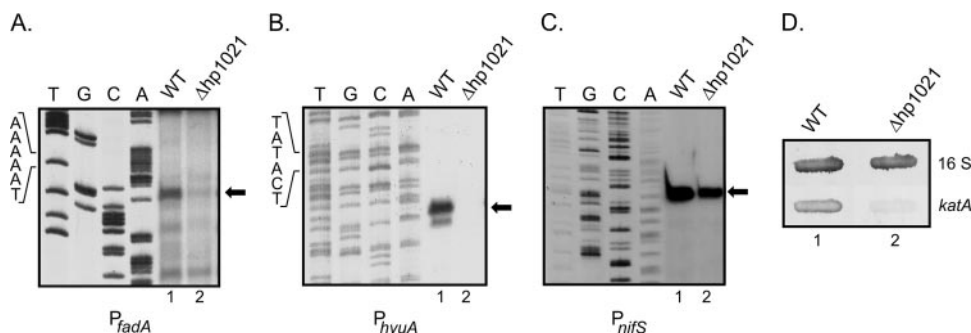


FIG. 1. Analysis of transcription of *fadA* (A), *hyuA* (B), *nifS* (C), and *katA* (D) by primer extension and RNA slot blot hybridization. (A, B, and C) Primer extension experiments with radiolabeled oligonucleotides *fadA*-PE (A), *hyuA*-PE (B), and *nifS*-PE (C), respectively, were performed on equal amounts of RNAs extracted from *H. pylori* 26695 (lane 1) and 26695/HP1021::km (lane 2), which were grown to the late logarithmic phase in BHI broth. The respective cDNAs are indicated by an arrow on the right. The sequences of the  $-10$  elements of the  $P_{fadA}$  and  $P_{hyuA}$  promoters are given on the left. The sequencing ladders (lanes T, G, C, and A) were obtained by annealing primers *fadA*-PE, *hyuA*-PE, and *nifS*-PE to plasmids pSL-*fadA*prom, pSL-*hyuA*prom, and pSL-*nifS*prom, respectively. (D) RNA slot blot hybridization carried out with equal amounts of RNAs extracted from *H. pylori* 26695 (lane 1) and 26695/HP1021::km (lane 2). Hybridization was performed with *katA*- and 16S rRNA-specific probes as indicated on the right. WT, wild type.

isogenic *hp1021* null mutant. As shown in Fig. 1, the transcriptional start site of *fadA* was mapped to position  $-106$  with respect to the translational start codon. Transcription of *hyuA* started at position  $-39$ . The upstream sequences of *fadA* and *hyuA* revealed  $-10$  promoter hexamers with the sequences AAAAAT and TATACT, respectively, exhibiting two mismatches and one mismatch compared to the *E. coli*  $-10$  consensus promoter element. The *nifS*-specific transcript was found to comprise a long untranslated leader sequence, since the transcription initiation site was mapped between positions  $-245$  and  $-252$  with respect to the translational start site. In the *hp1021* null mutant, transcription of *fadA* and *nifS* was clearly reduced and no *hyuA*-specific transcript could be detected, corroborating the role of HP1021 as a positive regulator of the *hp0690*-to-*hp0693*, *hp0695*-to-*hp0697*, and *nifS*-*nifU* operons (Fig. 1). Furthermore, transcription of the *katA* gene

was monitored by RNA slot blot analysis and, in agreement with the microarray data, was found to be strongly decreased in the *hp1021* null mutant, confirming positive regulation by HP1021.

**Analysis of the protein expression pattern of the *hp1021* null mutant of *H. pylori* 26695.** To further confirm the microarray data, comparative 2D gel electrophoresis was performed with whole-cell protein lysates prepared from *H. pylori* 26695 and its HP1021-deficient derivative 26695/HP1021::km grown in liquid culture to the late logarithmic growth phase with an immobilized pH gradient ranging from 6 to 9 for isoelectric focusing. In silver-stained gels, 12 protein spots were reproducibly found to be missing or appeared less prominent when samples from the *hp1021* null mutant were analyzed (Fig. 2). Five of the differentially expressed proteins could be identified by mass spectrometry following the excision of the respective spot from colloidal Coomassie-

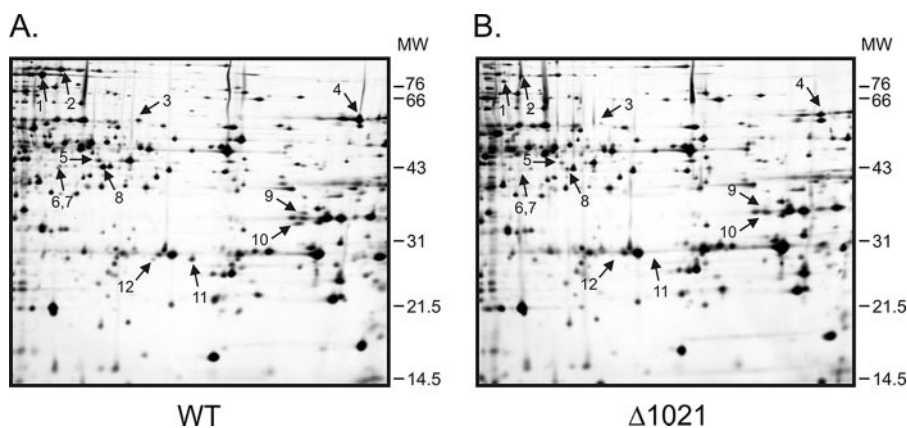


FIG. 2. Two-dimensional map of whole-cell protein lysates of *H. pylori* 26695 (A) and 26695/HP1021::km (B). Fifty micrograms of protein was separated by 2D gel electrophoresis with an immobilized pH gradient ranging from 6 to 9 for isoelectric focusing. After separation in the second dimension with 12% SDS polyacrylamide gels, the protein spots were visualized by silver staining. The positions of molecular weight (MW) marker proteins and their molecular masses (in kilodaltons) are indicated on the right. Proteins which are differentially expressed in *H. pylori* 26695 and 26695/HP1021::km are indicated by arrows. Proteins corresponding to the following spot numbers were identified by liquid chromatography-mass spectrometry: spot 1, hydantoin utilization protein A (HP0695, HyuA), 78.5 kDa; spot 4, catalase (HP0875, KatA), 58.6 kDa; spot 8, acetyl-CoA-acetyltransferase (HP0690, FadA), 41.1 kDa; spot 10, response regulator HP1021, 35.2 kDa; spot 11, 7- $\alpha$ -hydroxysteroid dehydrogenase (HP1014), 28.5 kDa. WT, wild type.

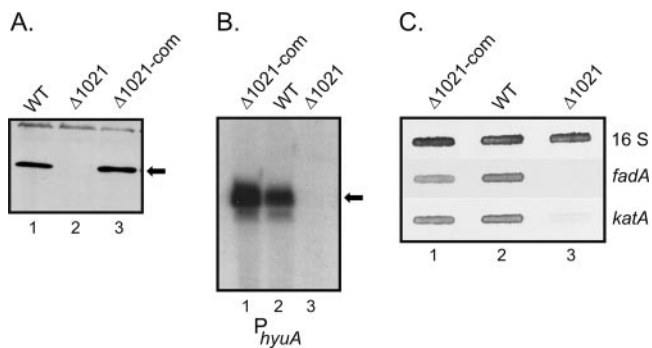


FIG. 3. Analysis of the expression of response regulator HP1021 (A) and of selected target genes (B and C) in complemented strain *H. pylori* 26695/Δ1021com. (A) Immunoblot analysis of equal amounts of whole-cell protein prepared from *H. pylori* 26695 (lane 1), 26695/HP1021::km (lane 2), and 26695/Δ1021com (lane 3) grown to the late logarithmic phase in BHI broth with a polyclonal rabbit antiserum directed against response regulator HP1021. The position of the HP1021 protein is indicated on the right. (B) Primer extension analysis with radiolabeled oligonucleotide *hyuA*-PE was performed with equal amounts of RNAs extracted from *H. pylori* 26695 (lane 2), 26695/HP1021::km (lane 3), and 26695/Δ1021com (lane 1), which were grown to the late logarithmic phase in BHI broth. The *hyuA*-specific cDNA is indicated by an arrow on the right. (C) RNA slot blot hybridization was performed with equal amounts of RNAs extracted from *H. pylori* 26695 (lane 2), 26695/HP1021::km (lane 3), and 26695/Δ1021com (lane 1) and *fadA*- and *katA*-specific probes. Hybridization with a 16S RNA-specific probe was carried out as a control. WT, wild type.

blue stained gels (legend to Fig. 2). As expected, the response regulator HP1021 was not produced in the deletion mutant 26695/HP1021::km (spot 10, Fig. 2). The two most intense spots were identified as hydantoin utilization protein A (HyuA; spot 1, Fig. 2) and acetyl-CoA-acetyltransferase (FadA; spot 8, Fig. 2). Furthermore, catalase (KatA; spot 4, Fig. 2) was identified as an HP1021-regulated protein. The fifth protein spot represented 7- $\alpha$ -hydroxysteroid dehydrogenase (HP1014; spot 11, Fig. 2). While differential expression of FadA, HyuA, and KatA on the protein level is in agreement with the results from transcriptional profiling of *H. pylori* 26695 and its isogenic hp1021 null mutant

(Table 2; Fig. 1), no significant differences in the amount of transcript of ORF hp1014 were noted in the microarray analysis, suggesting that posttranscriptional regulation is involved in the control of its expression.

**Reintegration of ORF hp1021 into the HP1021-deficient mutant restores the wild-type transcription profile.** To ensure that the differences in the transcription profile of the hp1021 null mutant of *H. pylori* 26695 are due to the deletion of the response regulator gene and not to secondary-site mutations, ORF hp1021 was reintegrated into the null mutant strain. For this purpose, the kanamycin resistance cassette replacing ORF hp1021 in parental strain 26695/HP1021::km was replaced with the response regulator gene flanked by a downstream chloramphenicol resistance gene by allelic-exchange mutagenesis, restoring the *htrA*-hp1020-hp1021 operon. Expression of HP1021 in complemented strain 26695/Δ1021com was checked by Western blot analysis with a polyclonal rabbit antiserum raised against recombinant response regulator protein HP1021 (22) and was found to be similar to that in wild-type strain 26695 (Fig. 3A). When transcription of *hyuA*, *fadA*, *nifS*, and *katA* was analyzed by primer extension or RNA slot blot analysis in the 26695 wild-type strain, the hp1021 null mutant strain, and its complemented derivative 26695/Δ1021com, similar amounts of transcript were detected in the wild-type and complemented strains (Fig. 3 and data not shown), demonstrating that the reduced expression of these genes in the hp1021 null mutant in fact is caused by the inactivation of the response regulator.

**HP1021 binds directly to the promoters of the *hyuA* and *katA* genes.** To investigate whether response regulator HP1021 directly controls the transcription of the highly regulated *hyuA* and *katA* genes, gel retardation experiments were performed. When increasing amounts of purified recombinant HP1021 C terminally fused to GST were added to a 210-bp DNA probe comprising the promoter region of the *hyuA* gene, four retarded complexes were observed in the presence of protein amounts ranging up to 200 ng and corresponding to a protein concentration of 160 nM. At higher protein concentrations, the two complexes exhibiting the highest electrophoretic mobility disappeared (Fig. 4A). The specificity of the binding of GST-

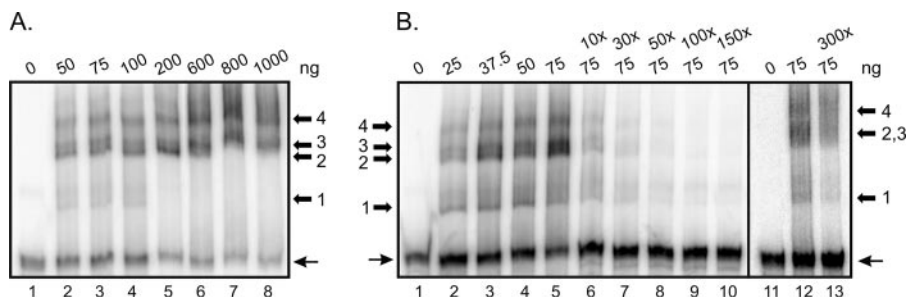


FIG. 4. Binding of GST-HP1021 to the promoter region of the *hyuA* gene. (A) EMSA of GST-HP1021 on a radioactively labeled 210-bp DNA probe comprising the upstream region of *hyuA*. Lane 1 contains the labeled DNA probe, while in lanes 2 to 8 increasing amounts of GST-HP1021 were added to the probe. The amounts (in nanograms) of protein used are indicated above the lanes and correspond to protein concentrations of 40, 60, 80, 160, 480, 640, and 800 nM. (B) Specific and nonspecific competition for the binding of GST-HP1021 to the *hyuA* promoter probe. Lanes 1 and 11 contain the labeled DNA probe. In lanes 2 to 5, 25, 37.5, 50, and 75 ng of GST-HP1021 were added, resulting in protein concentrations of 20, 30, 40, and 60 nM. Specific competition was performed by adding 10-fold, 30-fold, 50-fold, 100-fold, and 150-fold excesses of the unlabeled 210-bp *hyuA* promoter fragment to binding reaction mixtures containing 15,000 cpm (22 fmol) of the labeled probe and 75 ng of GST-HP1021 (lanes 6 to 10). In lane 13, a 300-fold excess of a 217-bp DNA fragment derived from the promoter of the *cagA* gene was added as a nonspecific competitor to a binding reaction mixture containing 15,000 cpm (22 fmol) of the labeled probe and 75 ng of GST-HP1021 (lane 12). Thin arrows to the right or left of the panels indicate the labeled DNA promoter probes. Thick arrows indicate protein-DNA complexes.



HP1021 to the *hyuA* promoter probe was assayed by adding increasing amounts of unlabeled DNA to the binding reaction mixture containing 75 ng of response regulator protein, corresponding to a protein concentration of 60 nM. Complexes 1 to 4 progressively disappeared upon addition of a specific DNA probe, while addition of a 300-fold excess of unspecific DNA caused only a slight decrease in the intensity of the retarded complexes (Fig. 4B), suggesting specific protein-DNA interactions. As a nonspecific competitor, a 217-bp DNA fragment derived from the upstream region of the *cagA* gene was used. Two retarded complexes were detected upon addition of GST-HP1021 to the 234-bp DNA probe encompassing the upstream region of the *katA* gene. These complexes were competed by the addition of a specific DNA probe but were largely retained when nonspecific competitor DNA was included in the binding reaction mixture (data not shown). From these results, we conclude that transcription of both *hyuA* and *katA* is positively controlled by the direct binding of response regulator HP1021 to the respective promoter regions.

## DISCUSSION

HP1021 is an orphan response regulator with a DNA-binding output domain which carries an atypical serine residue at the canonical receiver phosphorylation site. It is unclear whether the activity of the orphan response regulator HP1021 is modulated in response to external or internal stimuli. We have shown previously that the atypical serine residue at the canonical receiver phosphorylation site is not a prerequisite for the cell growth-associated functions of HP1021 (31). In order to identify genes which are controlled by HP1021, we performed comparative whole-genome transcriptional profiling of the *H. pylori* 26695 wild-type strain and the isogenic hp1021 null mutant grown under standard culture conditions. The amount of transcript of 79 genes differed more than twofold between the wild-type strain and the hp1021 null mutant with 51 and 28 genes, respectively, being more efficiently transcribed in either the wild-type strain or the hp1021 null mutant (Table 2), indicating that response regulator HP1021 is active as a transcriptional regulator in *H. pylori* strains grown under standard culture conditions. Among the 51 genes whose transcription is reduced in the hp1021 null mutant, 17 genes encoding ribosomal proteins and ORF hp1555 (*tfs*) encoding translation elongation factor EF-ts were found. Differential expression of these genes possibly is not due to their direct regulation by response regulator HP1021 but might be the consequence of a general growth retardation which is observed upon response regulator inactivation (5, 19). Downregulation of the ribosomal gene cluster hp1320-hp1292 and of *tfs* was also observed when *H. pylori* switches from the logarithmic to the stationary growth phase (34). However, only 5 (hp0229, hp0630, hp0140, hp0686, and hp1400) of the other 60 genes which were reported to undergo significant changes in their expression patterns upon entry into stationary phase (34) were also detected in the present study. Of these, *fecA1* (hp0686) and *fecA3* (hp1400) showed the opposite regulation upon entry into stationary phase and in the hp1021 null mutant. Interestingly, transcription of the *nifS* and *nifU* genes, whose products are involved in the formation of Fe-S clusters required for the proper function of several *H. pylori* enzymes, is positively reg-

ulated by HP1021. Inactivation of HP1021 reduced the amount of *nifS*-specific transcript about twofold (Table 2; Fig. 1). *NifS* donates sulfur via an L-cysteine desulfurase activity, while *NifU* provides a scaffold onto which the nascent Fe-S cluster is assembled (40). The cluster maturation proteins are considered "housekeeping" factors, and consequently, *nifS* and *nifU*, which encode the only such proteins of *H. pylori*, were shown to be absolutely required for its viability (25). Therefore, downregulation of these genes in the hp1021 null mutant might contribute to the observed growth defect. Recently, Alamuri et al. (1) reported that the *nifS-nifU* operon is upregulated in the presence of excess oxygen or iron and that this regulation is dependent on the Fur repressor. However, it should be noted that in the study of Alamuri et al. with *H. pylori* strain HP43504, a different transcription initiation site close to the translational start codon of the *nifS* gene was mapped (1).

Forty of the genes differentially expressed in the hp1021 null mutant (ribosomal genes excluded) were also reported to be regulated in response to an acidic pH (7, 20, 30, 39), including five ORFs encoding outer membrane proteins (hp0025, hp0229, hp0722, hp0725, and hp1564), as well as hp0875 (*katA*) encoding catalase. According to microarray analysis, the ArsRS two-component system is involved in the regulation of 19 of these genes (30). Expression of HP1021 itself is induced in response to an acidic pH (20; M. Bathon, M. Pflock, and D. Beier, unpublished data), and therefore elevated amounts of response regulator protein might cause an increased regulatory effect on its target genes under these conditions. However, there is no clear-cut correlation regarding the acid-responsive induction or repression of genes which are positively or negatively controlled by HP1021 (Table 2).

Only 10 genes showed more-than-fourfold regulation in the hp1021 null mutant. According to the genome annotation of *H. pylori* 26695, six of these strongly regulated genes belong to operons hp0690 to hp0693 and hp0695 to hp0697 and encode succinyl-CoA-transferase subunits A and B (hp0691 and hp0692), a predicted short-chain fatty acid transporter (hp0693), hydantoin utilization protein A (hp0695, *hyuA*), *N*-methylhydantoinase (hp0696), and a hypothetical protein (hp0697). Semiquantitative primer extension analysis confirmed the reduced transcription of *hyuA* and also of hp0690 (*fadA*) encoding acetyl-CoA acetyltransferase in the hp1021 null mutant (Fig. 1), which was decreased 16-fold and 2-fold, respectively, in the microarray experiments. By RNA slot blot hybridization, we detected clearly reduced amounts of *katA*-specific mRNA in the 26695 mutant lacking HP1021, which is in accordance with the fourfold decrease in *katA* transcription observed by whole-genome transcriptional profiling. These results were corroborated by 2D gel electrophoresis of whole-cell protein lysates of *H. pylori* 26695 wild-type and mutant strains grown under standard culture conditions where decreased expression of *HyuA*, *FadA*, and catalase was detected in the hp1021 null mutant (Fig. 2).

Complementation experiments with a derivative of the hp1021 null mutant of *H. pylori* 26695 into which the response regulator allele was reintegrated demonstrated that the reduced transcription of the hp0690-to-hp0693 and hp0695-to-hp0697 operons and of *katA* in the null mutant is due to the deletion of the response regulator gene hp1021 and not to secondary-site mutations which might have occurred during allelic-exchange mutagenesis, since transcription of *fadA*,

*hyuA*, and *katA* was fully restored in the complemented strain (Fig. 3). Moreover, microarray analysis of the complemented strain showed that, with the exception of four genes, all of the genes being differentially expressed in the hp1021 null mutant were transcribed in 26695/Δ1021com to a similar rate as in the 26695 wild-type strain (data not shown).

In vitro DNA binding experiments indicated that transcription of *hyuA* and *katA* is regulated directly by the binding of the response regulator HP1021 to the upstream regions of these genes (Fig. 4 and data not shown). Up to four protein-DNA complexes were detected in EMSA experiments with the *hyuA* promoter probe. Incubation of HP1021 with a *katA* promoter probe yielded two DNA-protein complexes which, however, were less distinct. These results suggest that HP1021 forms multimeric complexes when binding to the promoter DNA. Comparison of the promoter regions of *hyuA*, *fadA*, *nifS*, and *katA* mapped by primer extension analysis (Fig. 1) (24) revealed no pronounced similarity. Additional studies are required to unravel the DNA binding properties of response regulator HP1021.

Blast analysis showed that ORFs hp0695 (*hyuA*) and hp0696, which are annotated as hydantoin utilization protein A and a predicted *N*-methylhydantoinase, also share significant identity with the β and α subunits of acetone carboxylase, respectively. Furthermore, ORF hp0967, annotated as a hypothetical protein, shows similarity to acetone carboxylase subunit γ. Since in both *Xanthobacter autotrophicus* and *Rhodobacter capsulatus*, from which acetone carboxylases have been purified and characterized, the genes encoding the subunits of the enzyme are organized in an operon in the order β, α, and γ (32), and the same order of putative genes is present in the hp0695-to-hp0697 operon, it is very likely that this operon encodes the subunits of an acetone carboxylase of *H. pylori*, as previously suggested by Sluis et al. (32). Acetone carboxylase converts acetone to acetoacetate (11), which might be further metabolized to acetyl-CoA by enzymatic reactions catalyzed by the gene product of the hp0690-to-hp0693 operon, i.e., the A and B subunits of succinyl-CoA:acetoacetate-CoA-transferase (hp0691 and hp0692, respectively), which converts acetoacetate to acetoacetyl-CoA and acetyl-CoA acetyltransferase or thiolase (*fadA*, hp0690), cleaving acetoacetyl-CoA to acetyl-CoA. Succinyl-CoA:acetoacetate-CoA-transferase activity has already been detected in lysates of *H. pylori* (8). Interestingly, orthologs of the genes hp0690 to hp0693 and hp0695 to hp0697 are only present in the genomes of gastric *Helicobacter* species (2, 12, 35) but are missing in *H. hepaticus* and the closely related organisms *C. jejuni* and *W. succinogenes* (4, 27, 33). The presence of acetone, which is a toxic compound, is not a completely unphysiologic condition for *H. pylori*, since in fasting individuals and patients suffering from diabetes acetone can accumulate to millimolar concentrations in the blood (15) and probably also in the gastric juice. Therefore, it is conceivable that the gene products of the hp0690-to-hp0693 and hp0695-to-hp0697 operons might enable *H. pylori* to detoxify acetone and/or to use this compound as an additional carbon source under nutrient-limiting conditions. Studies addressing the influence of acetone on the growth of *H. pylori* 26695 and its isogenic hp1021 null mutant are currently ongoing in our laboratory.

In contrast to the homologous enzyme of *H. pylori*, the

acetone carboxylases of *X. autotrophicus* and *R. capsulatus* are highly expressed only upon exposure of the bacteria to acetone. Transcription of the acetone carboxylase genes of *X. autotrophicus* is dependent on σ<sup>54</sup> and a σ<sup>54</sup>-dependent transcriptional activator protein, while the homologous regulator protein in *R. capsulatus* is likely to interact with the σ<sup>70</sup>-dependent RNA polymerase. In both *X. autotrophicus* and *R. capsulatus*, the gene encoding the transcriptional regulators is located upstream of the respective acetone carboxylase operon and it is expected that acetone binds as a cofactor to the N-terminal regions of these regulators (32). Since close orthologs of the orphan response regulator HP1021 are present in all sequenced ε-proteobacteria while the genes hp0690 to hp0693 and hp0695 to hp0697 are only found in stomach-colonizing *Helicobacter* species, we hypothesize that in the course of their evolution these genes were integrated into a preexisting HP1021 regulon of a common ancestor.

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