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Phosphorylation-Independent Activity of Atypical Response Regulators of *Helicobacter pylori*

Jennifer Schär,[1] Albert Sickmann,[2] and Dagmar Beier[1]*

Theodor-Boveri-Institut für Biowissenschaften, Lehrstuhl für Mikrobiologie, Universität Würzburg, Am Hubland,[1] and Rudolf-Virchow-Zentrum für experimentelle Biomedizin,[2] Würzburg, Germany

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The genome of the gastric pathogen *Helicobacter pylori* harbors a remarkably low number of regulatory genes, including three and five open reading frames encoding two-component histidine kinases and response regulators, respectively, which are putatively involved in transcriptional regulation. Two of the response regulator genes, hp1043 and hp166, proved to be essential for cell growth, and inactivation of the response regulator gene hp1021 resulted in a severe growth defect, as indicated by a small-colony phenotype. The sequences of the receiver domains of response regulators HP1043 and HP1021 differ from the consensus sequence of the acidic pocket of the receiver domain which is involved in the phosphotransfer reaction from the histidine kinase to the response regulator. Using a genetic complementation system, we demonstrated that the function of response regulator HP166, which is essential for cell growth, can be provided by a mutated derivative carrying a D52N substitution at the site of phosphorylation. We found that the atypical receiver sequences of HP1043 and HP1021 are not crucial for the function of these response regulators. Phosphorylation of the receiver domains of HP1043 and HP1021 is not needed for response regulator function and may not occur at all. Thus, the phosphorylation-independent action of these regulators differs from the well-established two-component paradigm.

Helicobacter pylori is a gram-negative microaerophilic organism that colonizes the human gastric mucosa and is the causative agent of chronic active-type B gastritis and peptic ulcer disease (7, 31). Moreover, infection with *H. pylori* is regarded as a risk factor for the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (16, 29). Little information regarding the molecular mechanisms regulating the expression of housekeeping and virulence-related genes is available. Analysis of the genome sequences of two unrelated *H. pylori* isolates, 26695 and J99 (1, 38), revealed the presence of only a few transcriptional regulators, including several proteins belonging to the family of two-component systems.

Two-component systems are signal transduction systems for the regulation of cellular functions in response to environmental conditions (28). Usually they are composed of a sensor protein perceiving environmental stimuli via its N-terminal input domain and a cognate response regulator. In the presence of the appropriate stimulus the sensor protein autophosphorylates at a highly conserved histidine residue in the transmitter domain. Subsequently the phosphoryl group is transferred to an aspartic acid residue in the N-terminal receiver domain of the response regulator, resulting in a conformational change and activation of its C-terminal output domain, which frequently has DNA-binding properties.

The *H. pylori* genome contains five open reading frames (ORFs) encoding response regulators harboring DNA-binding motifs in their output domains. Two of the response regulator genes, hp166 and hp1365, are located adjacent to genes encoding a cognate histidine kinase, while the constituents of a third two-component system, response regulator HP703 and histidine kinase HP244, are encoded at unlinked loci (6). hp1043 and hp1021 are orphan response regulator ORFs. So far, regulated target genes have been identified only in the case of the HP703-HP244 and HP166-HP165 two-component systems. The NtrC-like response regulator HP703 controls the transcription of genes encoding components of the flagellar basal body and hook and the minor flagellin FlaB (35). In response to an acidic pH HP166 regulates the expression of the urease genes and of several genes encoding *H. pylori*-specific proteins with unknown functions (10, 12, 32). On the basis of in vitro DNA-binding experiments, it was hypothesized that HP1043 regulates its own expression, as well as transcription of the *tlpB* gene encoding a methyl-accepting chemotaxis protein (9).

Surprisingly, HP1043 and HP166, both of which belong to the OmpR subclass of response regulators, proved to be essential for cell growth. Inactivation of the encoding genes by allelic exchange mutagenesis could be achieved only in the presence of a wild-type copy of the respective ORF (6, 24). In contrast, ORF hp165 encoding the cognate histidine kinase of response regulator HP166 could be easily replaced by a kanamycin resistance cassette (6). Inactivation of ORF hp1021 resulted in a severe growth defect, as demonstrated by the small-colony phenotype of the mutant (24), or in the complete arrest of growth of transformants (6). The output domain of response regulator HP1021 contains a helix-turn-helix motif, but this response regulator could not be grouped in any of the well-known response regulator subclasses. Only a very few of the large number of two-component systems identified so far in prokaryotes have been shown to be essential. However, it is

* Corresponding author. Mailing address: Theodor-Boveri-Institut für Biowissenschaften, Lehrstuhl für Mikrobiologie, Universität Würzburg, Am Hubland, 97074 Würzburg, Germany. Phone: 49-931-8884421. Fax: 49-931-8884402. E-mail: d.beier@biozentrum.uni-wuerzburg.de.

now evident that some two-component systems are involved in vital processes like cell division and differentiation. In *Caulobacter crescentus* an essential two-component system consisting of the response regulator CtrA and its cognate histidine kinase CckA regulates the cell cycle by controlling DNA replication, DNA methylation, and flagellar biogenesis (17, 33). An essential homolog of CtrA is also present in *Sinorhizobium meliloti* (4). The essential *yycF-yycG* two-component system of *Bacillus subtilis* has recently been shown to be involved in the regulation of the cell division operon *ftsAZ* (11, 14) and of several genes involved in cell wall metabolism (15). Orthologs of *yycF* and *yycG* are present in other gram-positive bacteria (18, 20, 22, 37). In *Mycobacterium tuberculosis* the essential response regulator gene *mtrA* is located adjacent to the nonessential histidine kinase ORF *mtrB* (43). The target genes of the latter systems remain to be identified.

Interestingly, the receiver domains of the orphan response regulators HP1043 and HP1021 differ from the consensus sequence at the acidic pocket which is the active site for the phosphotransfer reaction. Considering the observation that these regulator proteins are not phosphorylated in vitro by the *H. pylori* histidine kinases and the observation that deletion of all histidine kinase genes has no effect on the in vitro growth of *H. pylori* (6), the question of whether phosphorylation is relevant at all for the function of HP1043 and HP1021 arises. The observation that the histidine kinase gene hp165 is not essential suggests that there are two sets of target genes of HP166; one group is essential for viability and is controlled by unphosphorylated HP166, while the second group is nonessential and is regulated by the phosphorylated response regulator (HP166~P). Target genes whose regulation requires HP166~P have been identified previously (10, 12, 32). In this study we demonstrated by genetic complementation that the essential function of HP166 can be provided by a mutated response regulator derivative which is not capable of phosphorylation. Furthermore, we found that the atypical receiver sequences of HP1043 and HP1021 are not crucial for response regulator function and that these proteins do not require phosphorylation to allow normal cell growth.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *H. pylori* strain G27 is a clinical isolate which has been described previously (42). *H. pylori* strains were grown under microaerophilic conditions (Oxoid) on Columbia agar plates containing 5% horse blood and Dent's or Skirrow's antibiotic supplement at 37°C. Liquid cultures were grown in brain heart infusion broth containing 10% fetal calf serum and Dent's or Skirrow's antibiotic supplement. When required, blood agar plates and liquid broth were supplemented with kanamycin or chloramphenicol at a final concentration of 20 μg/ml. Natural transformation of *H. pylori* G27 was performed as described previously (5). *Escherichia coli* strains were grown in Luria-Bertani broth. When necessary, antibiotics were added to the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 30 μg/ml.

General techniques. DNA manipulations, cloning procedures, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were carried out by using standard procedures. PCR amplifications were performed with a Techgene-02 thermocycler by using Deep Vent DNA polymerase (New England Biolabs) or *Taq* DNA polymerase (QBiogene). The primers used for the PCRs are listed in Table 2. All cloned PCR products were subjected to automated sequencing to ensure proper amplification. Site-directed mutagenesis was performed by using a QuickChange site-directed mutagenesis kit (Stratagene).

Purification of His_6-HP166, His_6-HP166-D52N, His_6-HP1365, His_6-HP1043, and His_6-HP1021-O. The recombinant His_6-tagged response regulator proteins His_6-HP166, His_6-HP1365, His_6-HP1043, and His_6-HP1021-R are encoded on plasmids pQE-166, pQE-1365, pQE-1043, and pQE-1021R, respectively (6). pQE-166D52N is a derivative of pQE-166 encoding a mutated HP166 response regulator with a D52N substitution. The proteins were overexpressed in *E. coli* and were purified by affinity chromatography on Ni^{2+}-nitrilotriacetic acid agarose essentially as described previously (6).

In vitro phosphorylation of response regulator proteins. The purified response regulator proteins (200 pM) were incubated in phosphorylation buffer (100 mM Tris-HCl [pH 7.5], 100 mM KCl, 20 mM $MgCl_2$) in the presence of 5 μCi of ^{32}P-labeled acetylphosphate (6,000 Ci/mmol; NEN Biosciences) in a 20-μl (final volume) mixture for 5 to 30 min at 30°C. Phosphorylation of His_6-HP166 and His_6-HP166-D52N in the presence of the cognate histidine kinase was performed as described previously (6). The phosphorylation reactions were stopped by addition of Laemmli buffer, and the samples were loaded on SDS—15 or 20% polyacrylamide gels. After electrophoresis the gels were autoradiographed.

Construction of *H. pylori* strains carrying additional copies of the genes encoding response regulators HP1043, HP1021, and HP166 integrated into the *cag* locus. Plasmids for integration of the response regulator genes hp1043, hp1021, and hp166 into the *cag* locus of *H. pylori* G27 were constructed as follows. An 830-bp EcoRI-BamHI DNA fragment encoding CagD, as well as amino acids 48 to 115 of CagC, was amplified from chromosomal DNA of *H. pylori* G27 with primers cagD-5 and cagC-3. PCR performed with primers cagA-5 and cagA-3 yielded an 862-bp PstI-SacI fragment encoding amino acids 192 to 478 of CagA. These PCR fragments were cloned into pSL1180 to obtain plasmid pSL*cag*AD. A BamHI-PstI fragment encoding the corresponding response regulator gene or a mutated allele was subsequently ligated into pSL*cag*AD. The resulting plasmids were linearized with PstI and ligated with an 805-bp chloramphenicol resistance cassette from *Campylobacter coli*.

ORFs hp1043 and hp166 were amplified with primer pairs 1043-5–1043-3 and 166-5–166-3, respectively, yielding 1,067- and 1,010-bp BamHI-PstI DNA fragments that contained the respective promoter regions. The fragments were cloned into pBluescript SK, yielding plasmids pSK-1043 and pSK-166, respectively, which were used as templates for site-directed mutagenesis of ORFs hp1043 and hp166. ORF hp1021 was amplified with primer pair 1021-5–1021-3, yielding a 973-bp XbaI-PstI DNA fragment which was cloned into pSL1180 together with a 300-bp BamHI-XbaI fragment containing the promoter region of hp1018 (amplified with primers 1018-5 and 1018-3) to obtain plasmid pSL-1021. pSL-1021 was used as the template DNA for site-directed mutagenesis of ORF hp1021.

The various plasmid constructs were then used for transformation of *H. pylori* G27. Correct integration of the response regulator genes into the *cag* locus was checked by PCR analysis with primer pairs flanking the integration site (see below).

Construction of plasmids used for allelic replacement of ORFs hp1043, hp1021, and hp166. The suicide plasmids used were derivatives of pSL1180 which carried DNA fragments flanking the ORF to be replaced on the *H. pylori* chromosome, as well as a kanamycin resistance cassette from *C. coli* inserted between these fragments. Plasmid pSL-1021::km has been described previously (6). pSL-1043::km2 contains a 629-bp EcoRI-BamHI fragment comprising the region encoding amino acids 330 to 348 of HP1042, as well as the intergenic region between ORFs hp1042 and hp1043 (PCR amplified with primers 1042-5 and 1042-3) and a 716-bp BamHI-PstI fragment encoding amino acids 110 to 318 of HP1044 (PCR amplified with primers 1044-5 and 1044-3). pSL-166::km2 harbors a 539-bp EcoRI-BamHI fragment encoding HP168 and amino acids 327 to 422 of HP169 (PCR amplified with primers 169-5 and 168-3) and a 501-bp BamHI-PstI fragment encoding amino acids 1 to 164 of HP165 (PCR amplified with primers 165-5 and 165-3).

Characterization of *H. pylori* strains resulting from transformation of merodiploid strains with the suicide plasmids pSL-166::km2, pSL-1043::km2, and pSL-1021::km. Chromosomal DNAs of the *H. pylori* strains resulting from transformation of the various merodiploid strains with the appropriate suicide plasmids were used as templates in PCRs with appropriate primer combinations, yielding DNA fragments which proved the presence of the respective response regulator genes in the *cag* locus and deletion of the response regulator genes in the wild-type locus. The following primer pairs were used: cagD-5 and cagA-3, cagC-seq and cagA-seq, 166-5 and cagA-3, cagD-5 and 166-3, 169-5 and 165-3, 169-52 and kan2, and 166-32 and kan3 for analysis of HP166; cagD-5 and cagA-3, cagC-seq and cagA-seq, 1043-5 and cagA-3, cagD-5 and 1043-3, 1044-32 and kan2, and 1042-52 and kan3 for analysis of HP1043; and cagD-5 and cagA-3, cagC-seq and cagA-seq, 1018-5 and cagA-3, cagD-5 and 1021-3, 1022-32 and kan2, and 1020-52 and kan3 for analysis of HP1021. To exclude the possibility that in the merodiploid strains the response regulator ORFs in the *cag* locus had

TABLE 1. Strains and plasmids

Bacterial strain or plasmid	Relevant features	Reference or source
H. pylori strains		
G27	Clinical isolate	42
G27/1043	G27 with an additional copy of ORF hp1043 integrated into the *cag* locus	This study
G27/1043Δ	G27/1043, but with the copy of ORF hp1043 located in the wild-type locus replaced by a kanamycin resistance cassette	This study
G27/1043-K8D	G27/1043, but with a K8D mutation in the additional copy of ORF hp1043	This study
G27/1043-K8DΔ	G27/1043-K8D, but with the wild-type copy of ORF hp1043 replaced by a kanamycin resistance cassette	This study
G27/1043-K8N9DD	G27/1043, but with a K8N9DD mutation in the additional copy of ORF hp1043	This study
G27/1043-K8N9DDΔ	G27/1043-K8N9DD, but with the wild-type copy of ORF hp 1043 replaced by a kanamycin resistance cassette	This study
G27/1043-S51N	G27/1043, but with an S51N mutation in the additional copy of ORF hp1043	This study
G27/1043-S51NΔ	G27/1043-S51N, but with the wild-type copy of ORF hp1043 replaced by a kanamycin resistance cassette	This study
G27/1043-D52N	G27/1043, but with a D52N mutation in the additional copy of ORF hp1043	This study
G27/1043-D52NΔ	G27/1043-D52N, but with the wild-type copy of ORF hp1043 replaced by a kanamycin resistance cassette	This study
G27/1043-S51D52AN	G27/1043, but with an S51D52AN mutation in the additional copy of ORF hp1043	This study
G27/1043-S51D52ANΔ	G27/1043-S51D52AN, but with the wild-type copy of ORF hp1043 replaced by a kanamycin resistance cassette	This study
G27/1021	G27 with an additional copy of ORF hp1021 integrated into the *cag* locus	This study
G27/1021Δ	G27/1021, but with the copy of ORF hp1021 located in the wild-type locus replaced by a kanamycin resistance cassette	This study
G27/1021-S47D	G27/1021, but with an S47D mutation in the additional copy of ORF hp1021	This study
G27/1021-S47DΔ	G27/1021-S47D, but with the wild-type copy of ORF hp1021 replaced by a kanamycin resistance cassette	This study
G27/1021-S47N	G27/1021, but with an S47N mutation in the additional copy of ORF hp1021	This study
G27/1021-S47NΔ	G27/1021-S47N, but with the wild-type copy of ORF hp1021 replaced by a kanamycin resistance cassette	This study
G27/1021-S48N	G27/1021, but with an S48N mutation in the additional copy of ORF hp1021	This study
G27/1021-S48NΔ	G27/1021-S48N, but with the wild-type copy of ORF hp1021 replaced by a kanamycin resistance cassette	This study
G27/1021-S47S48NL	G27/1021, but with an S47S48NL mutation in the additional copy of ORF hp1021	This study
G27/1021-S47S48NA	G27/1021, but with an S47S48NA mutation in the additional copy of ORF hp1021	This study
G27/1021-S47S48NAΔ	G27/1021-S47S48NA, but with the wild-type copy of ORF hp1021 replaced by a kanamycin resistance cassette	This study
G27/166	G27 with an additional copy of ORF hp166 integrated into the *cag* locus	This study
G27/166Δ	G27/166, but with the copy of ORF hp166 located in the wild-type locus replaced by a kanamycin resistance cassette	This study
G27/166-D52N	G27/166, but with a D52N mutation in the additional copy of ORF hp166	This study
G27/166-D52NΔ	G27/166-D52N, but with the wild-type copy of ORF hp166 replaced by a kanamycin resistance cassette	This study
E. coli strains		
DH5α	Strain used for high-efficiency transformation	Gibco
XL1-Blue	Strain used for high-efficiency transformation	Stratagene
M15	Strain used for overproducing His_6-tagged response regulators	Qiagen
Plasmids		
pBluescript SK	Cloning vector	Stratagene
pSL1180	Cloning vector	Amersham Biosciences
pILL600	Plasmid containing the kanamycin resistance cassette from *C. coli*	19
pDT2548	Plasmid containing the chloramphenicol resistance gene from *C. coli*	40
pQE-166	pQE30 expressing the His_6-tagged response regulator HP166	6
pQE-166D52N	pQE30 expressing a His_6-tagged derivative of response regulator HP166 with a D52N mutation	This study
pQE-1365	pQE30 expressing the His_6-tagged response regulator HP1365	6
pQE-1043	pQE30 expressing the His_6-tagged response regulator HP1043	6
pQE-1021R	pQE30 expressing the His_6-tagged receiver domain of response regulator HP1021	6
pSL-1021::km	pSL1180 containing the kanamycin resistance cassette flanked by 758- and 632-bp EcoRI-BamHI and BamHI-PstI fragments derived from ORFs hp1020 and hp1022, respectively	6
pSL-1043::km2	pSL1180 containing the kanamycin resistance cassette flanked by 629- and 716-bp EcoRI-BamHI and BamHI-PstI fragments derived from the intergenic region between ORFs hp1042 and hp1043 and ORF hp1044, respectively	This study
pSL-166::km2	pSL1180 containing the kanamycin resistance cassette flanked by 539- and 501-bp EcoRI-BamHI and BamHI-PstI fragments derived from ORFs hp168-hp169 and hp165, respectively	This study
pSK-1043	pBluescript SK containing a 1067-bp BamHI-PstI fragment comprising ORF hp1043 and its promoter region	This study

Continued on following page

TABLE 1—*Continued*

Bacterial strain or plasmid	Relevant features	Reference or source
pSL-P_{1018}1021	pSL1180 containing ORF hp1021 fused to a 300-bp BamHI-XbaI fragment comprising the P_{1018} promoter	This study
pSK-166	pBluescript SK containing a 1,010-bp BamHI-PstI fragment comprising ORF hp166 and its promoter region	This study
pSL*cag*AD	pSL1180 containing 830- and 862-bp EcoRI-BamHI and PstI-SacI fragments derived from the *cagCD* and *cagA* genes, respectively	This study
pSL*cag*1043cm	pSL*cag*AD containing the 1,076-bp BamHI-PstI fragment from pSK-1043 and the chloramphenicol resistance cassette	This study
pSL*cag*1043K8Dcm	pSL*cag*1043cm, but with a K8D mutation in ORF hp1043	This study
pSL*cag*1043K8N9DDcm	pSL*cag*1043cm, but with a K8N9DD mutation in ORF hp1043	This study
pSL*cag*1043S51Ncm	pSL*cag*1043cm, but with an S51N mutation in ORF hp1043	This study
pSL*cag*1043D52Ncm	pSL*cag*1043cm, but with a D52N mutation in ORF hp1043	This study
pSL*cag*1043S51D52ANcm	pSL*cag*1043cm, but with an S51D52AN mutation in ORF hp1043	This study
pSL*cag*1021cm	pSL*cag*AD containing the 1,247-bp BamHI-PstI fragment from pSL-P_{1018}1021 and the chloramphenicol resistance cassette	This study
pSL*cag*1021S47Dcm	pSL*cag*1021cm, but with an S47D mutation in ORF hp1021	This study
pSL*cag*1021S47Ncm	pSL*cag*1021cm, but with an S47N mutation in ORF hp1021	This study
pSL*cag*1021S48Ncm	pSL*cag*1021cm, but with an S48N mutation in ORF hp1021	This study
pSL*cag*1021S47S48NLcm	pSL*cag*1021cm, but with an S47S48NL mutation in ORF hp1021	This study
pSL*cag*1021S47S48NAcm	pSL*cag*1021cm, but with an S47S48NA mutation in ORF hp1021	This study
pSL*cag*166cm	pSL*cag*AD containing the 1,010-bp BamHI-PstI fragment from pSK-166 and the chloramphenicol resistance cassette	This study
pSL*cag*166D52Ncm	pSL*cag*166cm, but with a D52N mutation in ORF hp166	This study

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5′ to 3′)[a]	Site[b]	Strand	Position[c]
cagD-5	aaaattgaattcTAGATATACCGCTTCACATGTAATCG	EcoRI	+	578118–578144
cagC-3	tggttaggatccTCATTTCTGTTCTAGCGATCGTAGG	BamHI	−	578924–578948
cagA-5	atgggcctgcagGATGAATCCTTGAAAGAAAGGC	PstI	+	580494–580515
cagA-3	tctgctgagctcCCATAATCTTTGAGAGTGTAGCTC	SacI	−	581333–581356
1043-5	agccccggatccGATCGCATACCTTAGGCTTTTACC	BamHI	−	1105789–1105812
1043-3	aaaccactgcagTTACTCTTCACACGCCGGTTTGGG	PstI	+	1104745–1104769
PF1043-3	tatgacggtaccATTTAAGAGTAATTCAGGCAC	KpnI	+	1105433–1105453
1018-5	ccttatggatccCCCTTTCTACCCTTTTTGC	BamHI	+	1081210–1081228
1018-3	ctttggtctagaTTAGCTCTGGATTTGGATATTGCC	XbaI	−	1081491–1081511
1021-5	ttagtctctagaCATGTGAGCATGCGTTATAAAC	XbaI	+	1084077–1084098
1021-3	caaaaactgcagAACAGGGCTTAAGGATTTTCT	PstI	−	1085030–1085050
166-5	agaaactgcagCCATGAAGCGTTTGGATTTGG	PstII	−	174768–174788
166-3	agaataggatccTCAGTATTCTAATTTATAACCAATCC	BamHI	+	173778–173803
1042-5	tgatgggaattcGGAGAGTTTCAATTATAGGG	EcoRI	+	1104142–1104161
1042-52	GATTGCAGCGTTTATTACCAGGATA		+	1103899–1103924
1042-3	tatcgtggatccTACCCCAAACCGGCGTGTGAAG	BamHI	−	1104750–1104771
1044-5	aaggccggatccCTCACATAAATTTGAGTGGTGG	BamHI	+	1105864–1105885
1044-3	ttttaactgcagGGTTTTATCTGGCGTTATTAGGG	PstI	−	1106558–1106580
1044-32	GCTTGGCAAACGCTCTGTTAGG		−	1107148–1107169
1020-5	ccacacgaattcGCTCCAATCAGCCCTAAATC	EcoRI	+	1083462–1083481
1020-52	GGAATCTAGGCGTTTTTCTCAAACCATCAA		+	1083021–1083051
1022-3	catcggctgcagATTCAAAACAATGTTCGCCC	PstI	−	1085625–1085644
1022-32	ACGCATGTTTAAAAGAGCCTTTTCTTTA		−	1085977–1086003
169-5	accactgaattcAACACCGCTTATGAAATCATCG	EcoRI	−	175659–175680
169-52	GAAGGGCGCACCAAGTCCAGTTAC		−	175939–175962
168-3	gaaataggatccTTCAATTCCATTCTACCAGCC	BamHI	+	175142–175162
165-5	ttttttggatccGTCGCTTTGTTTATGATAACGC	BamHI	−	173704–173725
165-3	tcttttctgcagCTTTTAGAACTCACGCTTTTATCC	PstI	+	173224–173247
165-32	AAAGCATTTTTTCAATCTTGTCTA		+	172901–172924
cagA-seq	CTATATCTGGTTGGACATGG		−	580636–580656
cagC-seq	GCTAACATAGCTATGGGTTGC		+	578813–578832
kan2	CCAGCTTATATACCTTAGCAG			
kan3	CTGGATGAATTGTTTTAGTACC			

[a] Sequences in uppercase type were derived from the genome sequence of *H. pylori* 26695 (38). Sequences introduced for cloning purposes are indicated by lowercase type and restriction recognition sequences are underlined. The sequences of primers kan2 and kan3 were derived from the kanamycin resistance cassette of *C. coli* (19).

[b] Restriction recognition sites.

[c] Nucleotide position in the genome sequence of *H. pylori* 26695 (38).

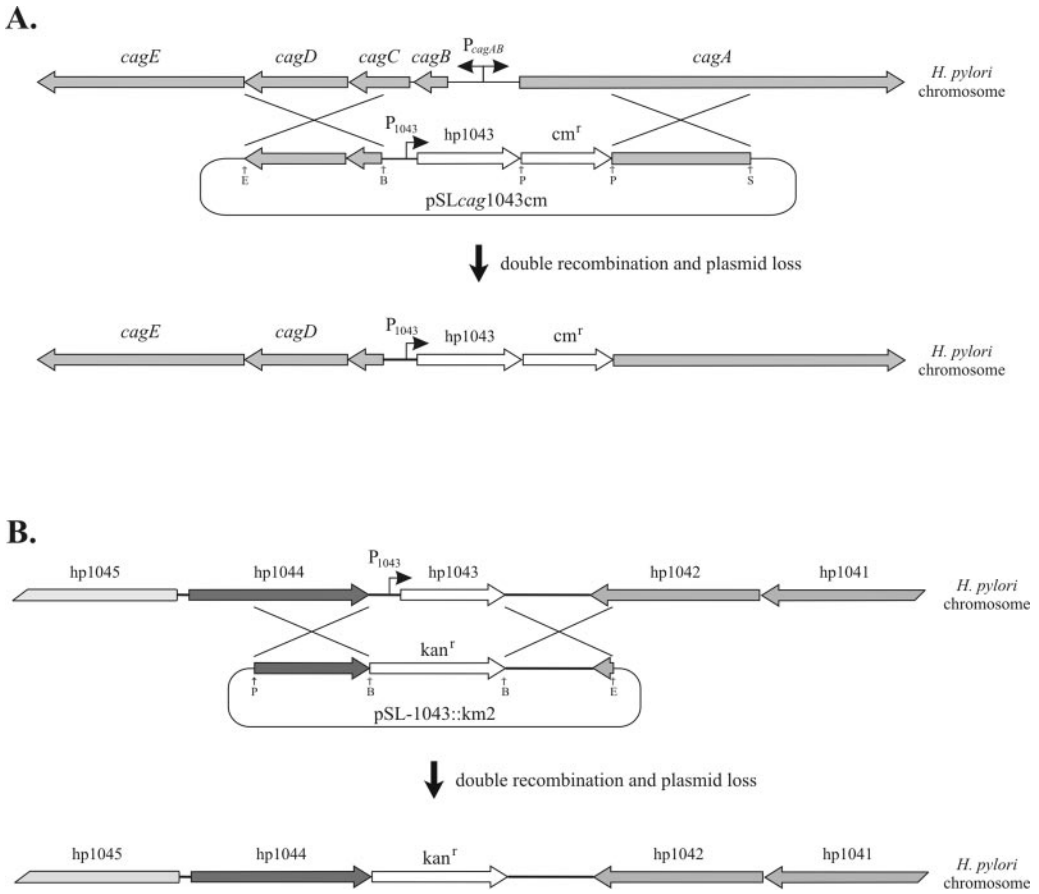

FIG. 1. Strategy for functional analysis of mutated derivatives of the response regulators HP166, HP1043, and HP1021 by in vivo complementation. The allelic exchange reactions performed for analysis of derivatives of ORF hp1043 are shown schematically. (A) Integration of an additional copy of the response regulator gene hp1043 into the *cag* pathogenicity island by homologous recombination, yielding an *H. pylori* strain which is merodiploid for hp1043. (B) Deletion of the hp1043 gene in the wild-type locus by transformation of the merodiploid strain with an appropriate suicide plasmid. ORFs are indicated by arrows or by trapezoids when they are incomplete, and noncoding DNA is indicated by solid bars. The positions of the P_{cagAB} and P_{1043} promoters are indicated by arrowheads. cm^r, chloramphenicol resistance gene; kan^r, kanamycin resistance gene; E, EcoRI; B, BamHI; P, PstI; S, SacI. See the text for details.

reverted to the wild-type sequence, these genes were amplified from chromosomal DNAs of several of the chloramphenicol- and kanamycin-resistant transformants by using primers cagC-seq and cagA-seq and sequenced.

RESULTS

Genetic complementation strategy for functional analysis of essential *H. pylori* response regulators. An effective approach for functional analysis of mutated derivatives of essential genes involves the stable chromosomal integration of a mutated gene in addition to the wild-type copy and the subsequent selective inactivation of the wild-type gene by allelic exchange mutagenesis. In this study the *cag* pathogenicity island, which encodes the components of a type IV secretion system (26), was chosen as the integration site. The response regulator genes were inserted by substituting a 1,545-bp DNA fragment harboring the divergently transcribed P_{cagAB} promoter (34) and part of the coding region of the *cagA* and *cagBC* genes to ensure that transcription of the response regulator genes was exclusively controlled by their own promoters.

The transcriptional start sites of the response regulator genes hp166 and hp1043 are located 66 and 34 bp upstream of the respective translational start codons (9, 10). Appropriate DNA fragments comprising both the promoter regions and ORFs hp166 and hp1043 were integrated into the *cag* locus of *H. pylori* G27 by allelic exchange reactions by making use of a chloramphenicol resistance cassette cloned adjacent to the response regulator ORFs (Fig. 1), yielding strains G27/166 and G27/1043. The response regulator gene hp1021 is part of an operon that also comprises *htrA* (ORF hp1018/hp1019) encoding a serine protease and ORF hp1020 encoding a protein with an unknown function. The transcriptional initiation site was mapped to −43 bp upstream of the translational start site of ORF hp1018 (32). A 300-bp DNA fragment harboring the P_{1018} promoter was fused to the response regulator gene hp1021. Integration of the resulting DNA fragment into the *cag* locus yielded strain G27/P_{1018}-1021. Correct integration of the response regulator genes into the *cag* pathogenicity island was checked by PCR analysis performed with chromosomal DNA of the transformants. On blood agar plates the *H. pylori* strains carrying additional copies of the wild-type response regulator ORFs hp166, hp1043, and hp1021 were indistinguishable from the G27 wild-type strain in their growth characteristics.

These strains were subsequently transformed with plasmids pSL-166::km2, pSL-1043::km2, and pSL-1021::km carrying a kanamycin resistance cassette cloned between DNA fragments derived from sequences which on the *H. pylori* chromosome flank the respective response regulator genes but which do not overlap the promoter regions contained in the DNA fragments integrated into the *cag* locus. In all cases selection for chloramphenicol and kanamycin resistance yielded viable transformants and, as demonstrated by PCR analysis, resulted exclusively in allelic exchange of the response regulator gene in the wild-type locus. After control transformation of *H. pylori* G27 with plasmids pSL-166::km2 and pSL-1043::km2, no transformants were obtained, and, as observed previously, the small colonies resulting from transformation of G27 with pSL-1021::km did not continue to grow when they were passaged on fresh blood agar plates. These results confirmed the finding of McDaniel et al. (24) that hp1043 and hp166 are essential genes and demonstrated the applicability of the complementation system for analysis of mutated response regulator genes.

Receiver phosphorylation of response regulator HP166 is not a prerequisite for its essential function. Since the response regulator ORF hp166 is essential (6, 24) but the cognate histidine kinase HP165 is dispensable for growth under in vitro culture conditions (6), we hypothesized that phosphorylation of the receiver domain is not involved in the control of essential target genes by HP166. To address this question, a derivative of ORF hp166 which resulted in a change in the probable phosphate-accepting aspartic acid residue at position 52 to asparagine was constructed. The mutated protein was expressed in *E. coli* as an N-terminal His_6 fusion and was purified by affinity chromatography. As expected, in contrast to the wild-type response regulator, HP166-D52N was not phosphorylated in vitro by the cognate histidine kinase HP165 (data not shown). The hp166-D52N allele was then integrated into the *cag* locus of *H. pylori* G27 via transformation with plasmid pSL*cag*166D52Ncm. Subsequently, strain G27/166-D52N was transformed with the suicide plasmid pSL-166::km2. Selection for kanamycin and chloramphenicol resistance yielded viable colonies with a normal growth phenotype (Table 3) on blood agar plates, and analysis of these colonies proved that the wild-type ORF hp166 was correctly replaced by the kanamycin resistance cassette and that the D52N mutation was present in the response regulator gene in the *cag* locus, thus demonstrating that the essential function of HP166 can be provided by the response regulator in its unphosphorylated state.

Atypical receiver sequences of HP1043 and HP1021 are not crucial for response regulator function. The sequences of the receiver domains of response regulators HP1043 and HP1021 show striking deviations from the consensus sequence (Fig. 2). In HP1043 the highly conserved aspartic acid residue D8 corresponding to position 13 in the sequence of CheY from *E. coli* is replaced by lysine, and the adjacent amino acid, which is an acidic amino acid or histidine in the consensus sequence, is asparagine. Furthermore, comparison with the homologous response regulator proteins of *Helicobacter hepaticus*, *Campylobacter jejuni*, and *Wolinella succinogenes* (3, 27, 36) showed that HP1043 has a four-amino-acid deletion which affects the canonical receiver phosphorylation site (D57 in *E. coli* CheY). In HP1021 the sequence at the phosphorylation site is VSSK, with serine at the position of the phosphate-accepting aspartic acid residue (Fig. 2B). In the HP1021 orthologs of *H. hepaticus*, *C. jejuni*, and *W. succinogenes* this aspartic acid residue is also replaced by serine. Since HP1043 and HP1021 are required for normal cell growth of *H. pylori*, while deletion of all histidine kinase genes individually has no effect on the growth phenotype (6, 13), it seems unlikely that these response regulators act in the two-component paradigm (i.e., receiver phosphorylation by a cognate histidine kinase) to exert their growth-associated function. It has been shown that a D13K substitution in *E. coli* CheY confers a constitutively active conformation to the protein (8). Therefore, to investigate whether the K8N9 receiver sequence of HP1043 is important for response regulator function, derivatives of ORF hp1043 encoding response regulators with the consensus receiver sequences K8D and K8N9DD were integrated into the *cag* pathogenicity island of *H. pylori* G27, yielding strains G27/1043-K8D and G27/1043-K8N9DD, respectively. Transformation of these strains with plasmid pSL-1043::km2 resulted in allelic replacement of the wild-type copy of the response regulator genes by the kanamycin resistance cassette, which did not affect the cell viability and growth characteristics of the strains. In all transformants analyzed the receiver mutations in the hp1043 alleles in the *cag* locus were still present, demonstrating that the atypical receiver sequence K8N9 in HP1043 is not a prerequisite for the function of this response regulator protein.

Similarly, the merodiploid strain G27/1021-S47D expressing a derivative of HP1021 with a consensus aspartic acid residue at the common receiver phosphorylation site was constructed. Subsequent deletion of the wild-type response regulator gene yielded transformants with a wild-type growth phenotype, demonstrating that the serine residue at position 47 is not crucial for the function of response regulator HP1021.

HP1043 and HP1021 are not phosphorylated in vitro by the low-molecular-weight phosphate donor acetylphosphate. Re-

TABLE 3. Replacement of ORFs hp166, hp1043, and hp1021 with mutated response regulator genes in *H. pylori* G27[a]

Strain	Growth on blood agar plates	Growth in liquid culture
G27/166-D52NΔ	+	+
G27/1043-K8DΔ	+	ND
G27/1043-K8N9DDΔ	+	ND
G27/1043-S51NΔ	+	ND
G27/1043-D52NΔ	+	ND
G27/1043-S51D52ANΔ	+	+
G27/1021-S47DΔ	+	ND
G27/1021-S47NΔ	+	ND
G27/1021-S48NΔ	+	ND
G27/1021-S47S48NAΔ	+	+
G27/1021-S47S48-NLΔ	−	

[a] Growth of the mutants was monitored on Columbia blood agar plates and, in case of selected mutants, in brain heart infusion supplemented with 10% fetal calf serum, 20 μg of kanamycin per ml, and 20 μg of chloramphenicol per ml. +, growth of the mutant was similar to growth of wild-type strain G27 (solid medium) or a derivative of G27 carrying a wild-type copy of the response regulator gene in the *cag* locus but having the response regulator ORF in the wild-type locus replaced by a kanamycin resistance cassette (liquid broth); −, either no transformants were obtained or the transformants showed the small-colony phenotype typical of G27/hp1021::kan. ND, not determined.

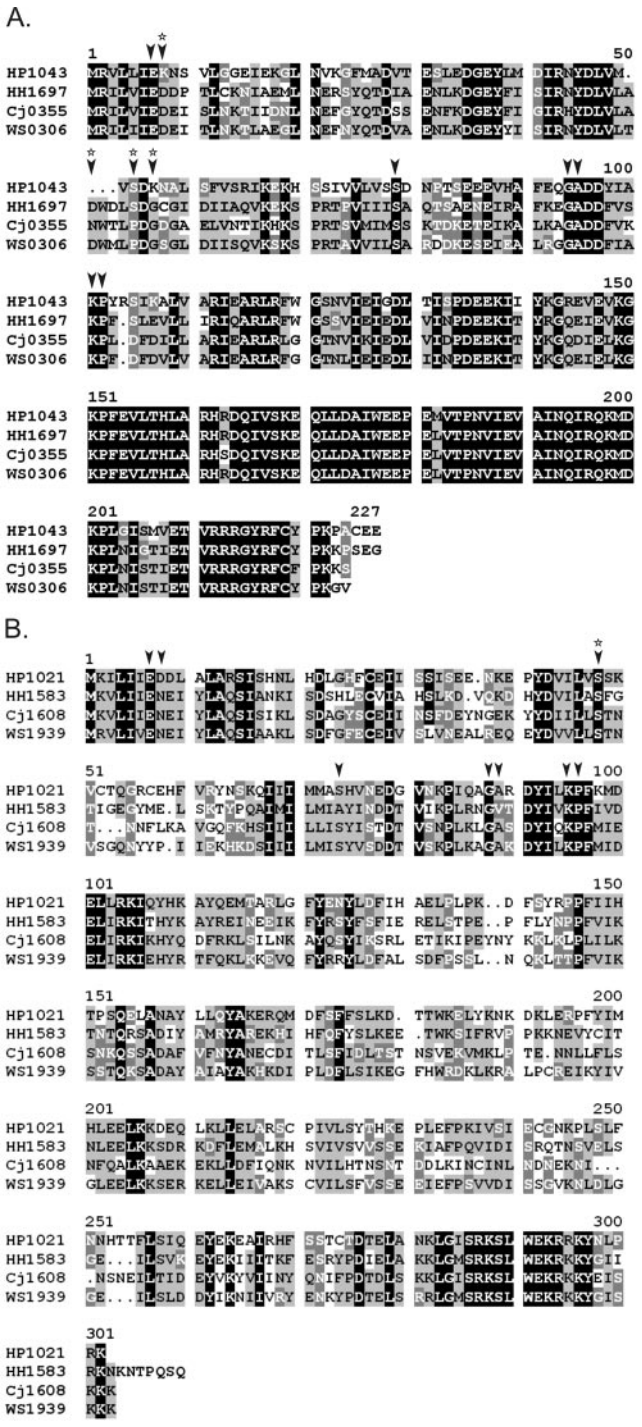

FIG. 2. Alignment of the amino acid sequences of the response regulators HP1043 (A) and HP1021 (B) of *H. pylori* and the homologous proteins of *H. hepaticus* (HH), *C. jejuni* (Cj), and *W. succinogenes* (WS). Gaps introduced to maximize the alignments are indicated by dots. The similarity among the three homologous proteins is indicated by shading, as follows: black background, all amino acids are identical; light grey background, more than one-half of the amino acids are identical or belong to a strong similarity group; dark grey background, more than one-half of the amino acids belong to a weak similarity group or the amino acid with the dark grey background could be grouped into a weak similarity group with every amino acid belonging to a strong similarity group indicated by a light grey background. The highly conserved positions corresponding to D/E12, D13, D57, P61, G65, T/S87, G102, G103, K109, and P110 in the receiver consensus sequence (39) are indicated by arrowheads. Deviations from the receiver consensus sequence in HP1043 and HP1021 are indicated by stars above the arrowheads. In HP1043 the positions corresponding to E12, S87, G102, A103, K109, and P110 are conserved, while in HP1021 the positions corresponding to E12, D13, S87, G102, A103, K109, and P110 are retained. The receiver sequence of response regulator HP166 corresponds perfectly to the consensus sequence.

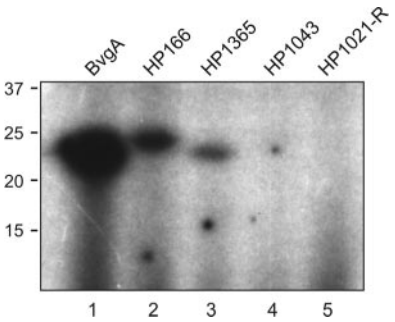

FIG. 3. In vitro phosphorylation of *H. pylori* response regulators with acetylphosphate. The recombinant proteins BvgA (23 kDa) (lane 1), His_6-HP166 (25 kDa) (lane 2), His_6-HP1365 (24 kDa) (lane 3), His_6-HP1043 (25 kDa) (lane 4), and His_6-HP1021-R (13 kDa) (lane5) were incubated with ^{32}P-labeled acetylphosphate for 30 min at room temperature. The samples were analyzed on an SDS–20% polyacrylamide gel. The sizes of the molecular weight standards (in kilodaltons) are indicated on the left.

ceiver domains exhibit a common secondary structure composed of five alternating β-strands and α-helices which are connected by loops of various sizes. The phosphate-accepting aspartic acid residue (D57) is located at the very end of β-strand 3. In HP1043 the serine residue at position 51 and the aspartic acid residue at position 52 which corresponds to D62 in the receiver consensus sequence come close to this position and, therefore, might be used as an alternative phosphorylation site. To test whether phosphorylation of HP1043 and HP1021 is possible in vitro, we incubated the purified proteins His_6-HP1043 and His_6-HP1021R comprising the receiver domain of HP1021 with the low-molecular-weight phosphate donor acetylphosphate. The BvgA protein of *Bordetella holmesii* was used as a positive control. BvgA has previously been shown to be efficiently phosphorylated by acetylphosphate in vitro (30). As shown in Fig. 3, within 30 min no transfer of the radioactive phosphoryl group from acetylphosphate to His_6-HP1043 and His_6-HP1021R was detected, while the recombinant *H. pylori* response regulators His_6-HP166 and His_6-HP1365 were phosphorylated by acetylphosphate, albeit with much lower efficiency than the BvgA protein. Likewise, HP1021R~P and HP1043~P could not be detected with longer time courses.

Modification at the common phosphorylation site is not required for the function of response regulators HP1043 and HP1021 in vivo. To investigate the requirement of phosphorylation of HP1043 and HP1021 in vivo, we constructed alleles that encode response regulators with receiver mutations at the putative phosphorylation sites. Since in response regulators HP1043 and HP1021 two amino acids which could serve as putative phosphorylation sites are located adjacent to each

other (i.e., S51 and D52 in HP1043 and S47 and S48 in HP1021) (Fig. 2), different sets of mutants were constructed with substitutions at either one or both of the putative phosphate-accepting amino acids. In the HP1043-S51N and HP1043-D52N mutants the serine residue at position 51 and the aspartic acid residue at position 52, respectively, were replaced by asparagine, while in HP1043-S51D52AN these amino acids were replaced by alanine and asparagine. In the case of response regulator HP1021 mutants HP1021-S47N and HP1021-S48N and mutants HP1021-S47S48NL and HP1021-S47S48NA proteins with changes at positions 47 and 48 were constructed. The mutated response regulator genes were integrated into the *cag* locus of *H. pylori* G27.

Viable transformants were obtained after transformation of the merodiploid strains G27/1043-S51N, G27/1043-D52N, and G27/1043-S51D52AN with plasmid pSL-1043::km2. Analysis of the transformants proved that there was correct replacement of wild-type ORF hp1043 by the kanamycin resistance cassette and that the desired mutations were present in the response regulator genes in the *cag* locus, indicating that phosphorylation of D52 or S51 in the receiver domain of HP1043 is not a prerequisite for its function in cell viability.

H. pylori strains G27/1021-S47N, G27/1021-S48N, G27/1021-S47S48NL, and G27/S47S48NA were transformed with plasmid pSL-1021::km, and again allelic exchange resulted in transformants with normal growth characteristics. Analysis of the resulting clones demonstrated that the wild-type ORF hp1021 was replaced by the kanamycin cassette and that receiver mutations were present in the remaining allele of ORF hp1021 in the case of *H. pylori* strains G27/1021-S47NΔ, G27/1021-S48NΔ, and G27/1021-S47S48NAΔ. Because the response regulator derivatives were able to functionally replace the wild-type protein, we concluded that phosphorylation of S47 or S48 is not required for the function of response regulator HP1021 in cell growth. However, when 15 colonies resulting from two independent transformations of the merodiploid strain G27/1021-S47S48NL with the suicide plasmid pSL-1021::km were characterized, these strains proved to carry exclusively the wild-type response regulator gene hp1021 in the *cag* locus. This observation was attributed to recombination between the mutated gene copy in the *cag* locus and the wild-type gene prior to loss of the hp1021 gene copy in the wild-type locus by allelic exchange with the kanamycin cassette. The fact that all the colonies that were analyzed resulted from this recombination event indicates that there was a nonrandom effect. Since immunoblot analysis demonstrated that HP1021-S47S48NL was expressed when the corresponding allele was integrated into the *cag* locus of *H. pylori* 26695/HP1021::km carrying a deletion of the hp1021 wild-type gene (32), we hypothesized that the S47S48NL mutation interfered with the function of HP1021 (data not shown).

DISCUSSION

In *H. pylori* the response regulator HP166 is essential for growth, while the gene encoding the corresponding histidine kinase, HP165, is not (6, 24). We hypothesized that HP166 controls transcription from two sets of target promoters that have different binding affinities for HP166. Promoters of essential genes should contain binding sites with high affinity for HP166 which can be recognized by the unphosphorylated protein. The promoters of nonessential genes, which are regulated by HP166 under environmental conditions that activate the cognate histidine kinase HP165, might harbor binding sites with lower affinity whose recognition requires phosphorylation of the HP166 protein. In line with this hypothesis, promoters which interact preferentially with phosphorylated HP166, resulting in the activation of transcription of nonessential genes (10, 12, 32), have been identified, and it has been shown recently that the autophosphorylation of histidine kinase HP165 is triggered by an acidic pH (32). Our observation that an *H. pylori* mutant carrying only the HP166-D52N allele is viable clearly supports the hypothesis that the transcriptional control of essential genes by response regulator HP166 is independent of its phosphorylation. A similar phenomenon was described for the response regulators AlgR and AlgB of *Pseudomonas aeruginosa*, which are required for the transcription of alginate synthesis genes. Both response regulators participate in the regulation of alginate synthesis in the unphosphorylated state since *P. aeruginosa* strains expressing *algR* and *algB* alleles with mutations at the predicted phosphorylation sites produced alginate (21). Both AlgR and the cognate histidine kinase, FimS, are needed for twitching motility, suggesting that AlgR~P plays a role in this process (41).

The orphan response regulators HP1043 and HP1021 are uncommon, as their sequences differ from the receiver consensus sequence at highly conserved sites. The presence of the atypical receiver sequences in HP1043 and HP1021 raises the question of whether these response regulators are activated by phosphorylation according to the two-component paradigm. *H. pylori* mutants in which the histidine kinase genes hp165, hp1365, and hp244 and also the *cheA* ortholog hp392 have been deleted show normal growth characteristics in culture media (6, 13), and phosphotransfer from the HP244, HP165, and HP392 kinases to the response regulators HP1043 and HP1021 could not be observed in vitro (6, 9; M.-A. Jiménez-Pearson and D. Beier, unpublished data). Therefore, at least phosphorylation by a cognate kinase might not be required for these response regulators to function in cell growth. In HP1043 the aspartic acid residue corresponding to position 13 in the consensus sequence is replaced by lysine, and there is a four-amino-acid deletion at the canonical phosphorylation site (Fig. 2A). However, this deletion brings the aspartic acid residue at position 52 corresponding to D62 in the receiver consensus sequence close to the end of β-strand 3, and, therefore, D52 might be used as an alternative phosphorylation site. In addition, a serine residue (S51) is located next to D52, which might be a phosphorylation target as well (see below). It has been reported that a D13K mutation in the *E. coli* chemotaxis response regulator CheY renders the protein active in vivo irrespective of the presence of the cognate histidine kinase CheA (8). Therefore, it was speculated that this mutation causes a conformational change of the CheY protein that results in its constitutive activity. Our observation that the mutated response regulators HP1043-K8D and HP1043-K8N9DD can replace the wild-type protein demonstrates that the atypical amino acids K8 and N9 replacing the aspartic or glutamic acid residues of the consensus acidic pocket are not crucial for establishing the active conformation of HP1043.

In HP1021 and the homologous response regulator proteins

of *H. hepaticus*, *C. jejuni*, and *W. succinogenes*, there is a serine at the position corresponding to the phosphate-accepting aspartic acid residue (D57), which might be used as an alternative phosphorylation site (Fig. 2B). It has been reported that in a D57N mutant of *E. coli* CheY the neighboring serine residue (S56) is phosphorylated by the histidine kinase CheA, although the efficiency is clearly reduced, demonstrating that the high-energy phosphoramidate of the kinase can serve as a phosphoryl source to generate serine phosphate (2). Phosphorylation of a D54N mutant of NtrC from *E. coli* which likewise harbors a serine residue N terminally adjacent to D54 was also observed, while D54T and D54Y mutants of NtrC were not phosphorylated by the cognate histidine kinase NtrB in vitro (25). Therefore, it was speculated that alternative-site phosphorylation of serine requires the presence of an amino acid containing a carbonyl group in the neighboring position that acts as a ligand for Mg^{2+}, which is important in catalyzing the phosphotransfer reaction (2). In neither HP1021 nor its orthologs from *H. hepaticus*, *C. jejuni*, and *W. succinogenes* is an amino acid with a carbonyl side chain located adjacent to S47 (Fig. 2B). The atypical serine residue S47 in HP1021 could be replaced by aspartic acid, thereby creating a consensus receiver sequence, without affecting the function of the response regulator protein in our in vivo complementation system.

Because there seems to be no cognate histidine kinase for HP1043 and HP1021, it could be hypothesized that these proteins might be phosphorylated by low-molecular-weight phosphate donors that accumulate in the cell under certain growth conditions. Acetylphosphate, which in vitro is a phosphate source for many response regulator proteins, is produced in the cell as an intermediate in the synthesis of acetate from acetyl coenzyme A and the reverse reaction (23). Both metabolic enzymes involved in these reactions, phosphotransacetylase (Pta) and acetate kinase (AckA), are encoded in the genome of *H. pylori* J99, whereas both the *pta* and *ackA* genes contain frameshift mutations in *H. pylori* 26695 (1, 38). The *pyrAa* (hp1237) and *pyrAb* (hp919) genes are present in both *H. pylori* 26695 and J99 (1, 38). Their products catalyze the biosynthesis of carbamoyl phosphate, which is another low-molecular-weight phosphate donor for response regulator proteins in vitro. Here we showed that the purified recombinant proteins His_6-HP1043 and His_6-HP1021R are not phosphorylated by acetylphosphate in vitro, while incubation of response regulators His_6-HP166 and His_6-HP1365 with acetylphosphate resulted in phosphorylation of these proteins (Fig. 3). Since the *pta* and *ackA* genes contain frameshift mutations in *H. pylori* 26695 and the two genes can be deleted in *H. pylori* G27 without any effect on the growth phenotype (unpublished data), it is unlikely that phosphorylation by acetylphosphate is important for the growth-associated functions of HP1043 and HP1021.

Because derivatives of HP1043 carrying either the S51N and D52N single-amino-acid substitutions or the S51D52AN double mutation can functionally substitute for the wild-type response regulator protein in vivo, we concluded that phosphorylation of S51 or D52 is not required for the essential function of the protein. Moreover, the observation that deletion of ORF hp1043 can be complemented by the *C. jejuni* ortholog cj355 encoding a response regulator protein with an asparagine residue replacing the consensus phosphate-accepting aspartic acid residue (data not shown) supports this hypothesis. In the case of HP1021, single-amino-acid replacement of S47 and S48 by asparagine and an S47S48NA double mutation resulted in functional response regulator proteins. These results support the hypothesis that phosphorylation of S47 or S48 in the receiver domain is not required for the function of response regulator HP1021 necessary for normal cell growth. Because the receiver sequence of HP1021 lacks an amino acid with a carbonyl side chain in the vicinity of the serine residues, this result is in line with our current understanding of the phosphotransfer reaction to the receiver domain (2, 25). Moreover, when whole-cell protein lysates of *H. pylori* were separated by two-dimensional gel electrophoresis and the spot corresponding to HP1021 was analyzed by mass spectrometry, no evidence of serine phosphorylation was obtained (data not shown). The observation that the HP1021 protein with the S47S48NL double mutation could not replace the wild-type response regulator might have been due to steric hindrance caused by the presence of amino acids that were not the proper size. It should be noted that in case of both HP1043 and HP1021 we cannot rule out the possibility that additional target genes which are not related to normal cell growth are regulated by the phosphorylated response regulator proteins. However, so far there is no experimental evidence for this hypothesis.

The finding that in an organism with a very restricted repertoire of regulatory genes, such as *H. pylori*, two response regulators are absolutely essential, while a third response regulator contributes to normal growth, was surprising. Our data suggest that the response regulators HP166, HP1043, and HP1021 have basal activity independent of their phosphorylation. The activity of these regulators might be controlled by strict regulation of expression, evidence for which has been obtained in the case of HP166 and HP1043 (9, 10), or by covalent modifications other than phosphorylation. It is interesting that the ortholog of HP1043 in *W. succinogenes*, WS306, is encoded by a gene adjacent to a histidine kinase gene (3) and that the HP1043 homolog in *H. hepaticus*, HH1697, which is an orphan response regulator, has a consensus receiver sequence (36), while in Cj355 of *C. jejuni* there is asparagine in place of the phosphate-accepting aspartic acid residue, and the HP1043 receiver has a deletion at the common phosphorylation site, as well as other nonconservative amino acid substitutions at usually conserved positions. Therefore, the receiver sequences of the HP1043 orthologs of these organisms might reflect the degeneration of a typical two-component system that was present in a common ancestor which had to cope with a wide spectrum of environmental conditions to a regulatory relic in a highly specialized bacterium like *H. pylori*, which is adapted to a single niche.

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