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NOTE

The *Helicobacter pylori* CrdRS Two-Component Regulation System (HP1364/HP1365) Is Required for Copper-Mediated Induction of the Copper Resistance Determinant CrdA

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Here we describe that the *Helicobacter pylori* sensor kinase produced by HP1364 and the response regulator produced by HP1365 and designated CrdS and CrdR, respectively, are both required for transcriptional induction of the *H. pylori* copper resistance determinant CrdA by copper ions. CrdRS-deficient mutants lacked copper induction of *crdA* expression and were copper sensitive. A direct role of CrdR in transcriptional regulation of *crdA* was confirmed by in vitro binding of CrdR to the *crdA* upstream region. A 21-nucleotide sequence located near the *crdA* promoter was shown to be required for CrdR binding.

Helicobacter pylori colonizes the human gastric mucosa and can cause severe gastric diseases (2, 10). In the hostile ecological niche, maintaining proper metal ion metabolism (38) is of critical importance for the pathogen. This has previously been shown for the homeostasis of iron, which turned out to be required for effective gastric colonization in animal models (38, 39). Copper ions play an important role in bacterial metabolism, because they function as cofactors for electron transport, oxidases, and hydroxylases (18, 19). On the other hand, copper catalyzes the generation of toxic hydroxyl radicals via Fenton-like reactions (20), and this necessitates mechanisms to keep the concentration of cytoplasmic copper ions below toxic levels. Whereas copper import occurs nonspecifically (13, 38), *H. pylori* controls the cytoplasmic copper concentration by efflux via the P-type ATPase CopA (14), which transports copper ions from the cytoplasm into the periplasmic space. Accumulating copper ions are detoxified via the *c*opper *r*esistance *d*eterminants CrdA (from HP1326), CrdB (from HP1327), and CzcB (from HP1328), which form together with the CzcA homolog from HP1329 a Czc-like metal export system, which was shown to contribute substantially to *H. pylori* copper resistance (40). The genetic organization of the *H. pylori* Crd system is orthologous to the *Escherichia coli* four-component copper export system Cus, which is proposed to transport Cu(I) ions from the periplasm across the outer membrane (12). Genome-wide RNA profiling revealed that *H. pylori* responds actively to changes in the environmental copper concentration and that the copper resistance determinant CrdA is strongly induced by copper at the transcriptional level (40). However, the underlying regulatory mechanisms were not investigated (40). Homologs of *E. coli* (24, 33), *Pseudomonas* (21, 22), or *Ralstonia* (35) copper regulators are absent in the *H. pylori* genome (1, 34), and the *H. pylori* metal regulator proteins Fur (6, 7, 38) and NikR (36, 37) are also not involved in *crdA* regulation (B. Waidner, F. N. Stähler, S. Bereswill, A. H. M. van Vliet, unpublished results). However, the transcriptional copper induction of *E. coli* Cus by CusRS, a two-component regulatory system (24) composed of a histidine kinase sensor protein and a cognate response regulator (23, 32), supported the idea that copper regulation of *H. pylori crdA* might be mediated by a similar type of regulator. The *H. pylori* genome contains only a small set of two-component signal transduction systems (1, 29, 34), and to date the regulated target genes have been defined for two of them (5, 9, 11). In the present study, we used mutational analysis and in vitro DNA/protein binding experiments to show that the *H. pylori* two-component regulatory system HP1364 (sensor homolog)/HP1365 (regulator homolog) is essential for transcriptional copper induction of *crdA* and for *H. pylori* copper resistance. Thus, we designated the HP1364 and HP1365 proteins as CrdS and CrdR, respectively. The putative sensor CrdS (from HP1364) was classified earlier as a member of an orthodox histidine kinase family (5, 9), and CrdR (from HP1365) was grouped into the OmpR family of response regulators (1, 5, 9, 29, 34). The CrdRS system was

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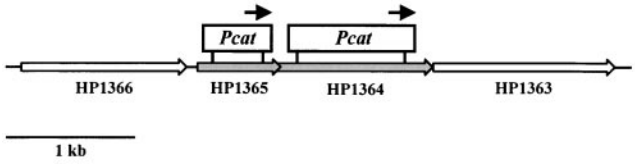

FIG. 1. Schematic overview and mutational analysis of HP1364 and HP1365. Genes are numbered according to the annotated genome sequence of *H. pylori* strain 26695 (32). HP1364 and HP1365 are indicated by grey arrows, and neighboring genes are indicated by white arrows. The insertion sites of the *Pcat* resistance cassettes are marked with white boxes. The directions of resistance genes are displayed by black arrows.

chosen because genes for bacterial copper regulators are often linked to their target genes and because the coding HP1364/HP1365 genes are located nearby the *crdA* locus in the *H. pylori* chromosome (1, 34, 40). Furthermore, the HP1364/HP1365 system was shown to be required for gastric colonization in a mouse infection model, but its target genes have not been determined so far (25).

To investigate possible functions of HP1364 and HP1365 in *crdA* regulation, we inactivated both genes separately in *H. pylori* strain 26695 (Fig. 1) and analyzed copper-mediated induction of *crdA* transcription in the resulting *crdS* and *crdR* mutants by Northern blot hybridization. Strains and plasmids used are listed in Table 1. Cloning was performed in *E. coli* according to standard protocols (3). The *cat* gene with its own promoter (*Pcat*) was fused to upstream and downstream DNA regions of mutagenized genes by using a modified megaprimer PCR protocol (26, 28). DNA regions flanking *Pcat* were amplified by PCR from DNA of *H. pylori* strain 26695 using primers carrying 5′ extensions complementary to the 5′ and 3′ ends, respectively, of the *Pcat* cassettes (Table 2). Plasmids carrying mutagenized versions of both genes (listed in Table 1) were used for the mutagenesis of the corresponding genes in the *H. pylori* chromosome. Marker exchange mutagenesis in *H. pylori* strain 26695 was performed by electroporation according to standard procedures (15). *H. pylori* mutants 26695-1364 and 26695-1365 carrying *Pcat* inserted into the chromosomal *crdS*

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Plasmids		
pZERO-2	Cloning vector, MCS in *lacZ′*, *neo*, Km^r	Invitrogen
p1364-PCAT	pZERO-2, Δ HP1364::*Pcat*, Cm^r, Km^r	This study
p1365-PCAT	pZERO-2, HP1365::*Pcat*, Cm^r, Km^r	This study
pASK-IBA3	Expression vector, *tetR*, P_{tet}, *bla*, Ap^r	IBA
pIBA3-1365	pASK-IBA3 carrying the HP1365 coding sequence under the control of the *tet* promoter cloned in the BsaI site	This study
H. pylori strains		
26695	wt, containing the entire *cag* PAI	32
26695-1364	26695, ΔHP1364::*Pcat*, Cm^r	This study
26695-1365	26695, ΔHP1365::*Pcat*, Cm^r	This study
G27	Clinical isolate	41
G27/HP1364::km	G27, ΔHP1364::*km*, Km^r	5
G27/HP1365::km	G27, ΔHP1365::*km*, Km^r	5

[a] MCS, multiple cloning site; PAI, pathogenicity island.

TABLE 2. Oligonucleotides used in this study

Gene[a]	Application	Primer	Sequence (5′→3′)	Primer	Sequence (5′→3′)[b]
HP1326	Antisense RNA probe	1326-L1	GGGTTATGGGTTTAAACGCA	1326-R1T7	T7-TTATAAATCCAGGCTTGTTT
	Promoter HP1326	P1326-L1	CTTGTTATCTTAATGTAAAG	1326-R2	TTCACTTCCAAGTCATTAGC
		P1326-L2	TATTATCCGTTCGCAACAAG	P1326-DIG	GGTTTGCTCCCATGCGTTTA
		P1326-L3	AAACACCATTTCCACAATTT		
HP1364	Mutagenesis, upstream	1364-L1	GGCAATTAGCGCTACAATAC	PCAT1364-R1	1-CTAATCACTAGCATCAACAC
HP1365	Mutagenesis, downstream	CAT1364-L1	2-ACAAGATCACAGAATTAAGC	1364-R1	CCTATCACGCAGTCTATTAA
	Mutagenesis, upstream	1365-L1	AGAGAGATTATCATAATTGG	PCAT1365-R1	1-CTCCTTAACGCTCTCGCTTA
	Mutagenesis, downstream	CAT1365-L1	2-TTCCACCTTGCGCACTTATA	1365-R1	GGCGGTGTTGTTGGTTGTCT
	Expression in *E. coli*	1365-ASK3-L1	TGGTAGGTCTCAAATGAATGCAAAAAAA AGATTTTTTTACTAGAAGACG	1365-ASK3-R1	ATGGTAGGTCTCAGCGCTTAGTGGGTTAAAGCG ATAGCCAAC
Pcat	*cat* gene with promoter	CATS1	TCCGGTTTTTGTTAATCCGCC	CATAS1	TTACGCCCCGCCCTGCCA

[a] Gene numbers refer to the *H. pylori* 26695 genome sequence (32).
[b] The 5′ extensions used for fusion of PCR products to the *cat* gene by megaprimer PCR are labeled as follows: 1, (5′-GGCGGATTAACAAAAACCGGA), complementary to the 5′ region of the *cat* gene with promoter; 2, (5′-TGGCAGGGCGGGGCGTAA), complementary to the 3′ end of the *cat* gene; T7, (5′-CTAATACGACTCACTATAGGGAGA) adds a T7 promoter sequence for creation of digoxigenin-labeled antisense RNA. The BsaI restriction site used for the HP1365 expression via the IBA system is underlined.

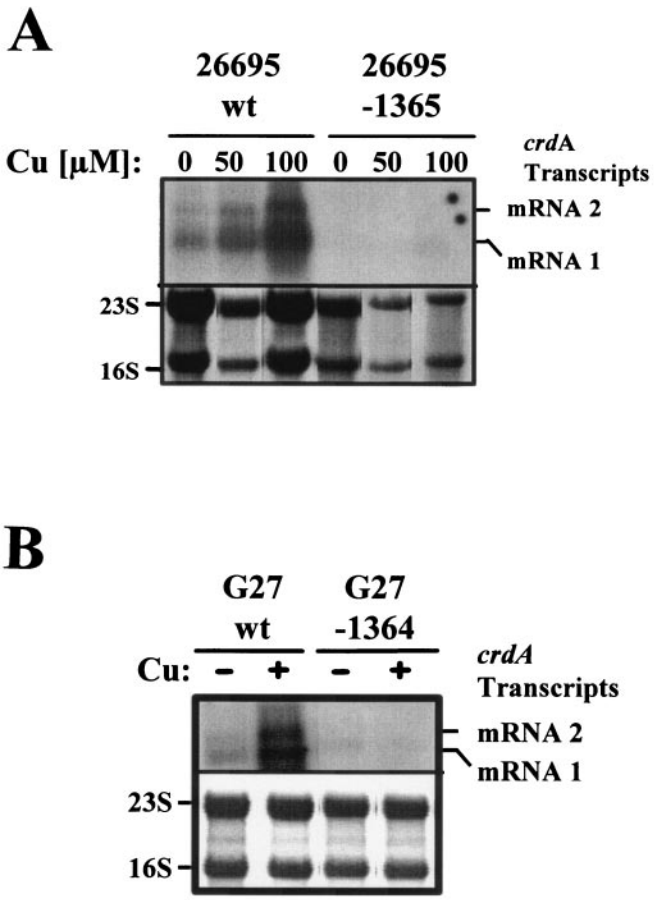

FIG. 2. The role of CrdR (HP1365) and CrdS (HP1364) in copper-mediated induction of *crdA* transcription. Copper-induced *crdA* transcripts, marked on the right (mRNAs 1 and 2), were visualized by hybridization of total RNA (15 μg), isolated from *H. pylori* strains that were grown in BBF supplemented with copper with a specific antisense RNA probe. (A) Induction of *crdA* transcription in the *H. pylori* wt strain 26695 and in its isogenic HP1365 mutant (26695-1365) grown for 48 h in BBF (0) and in BBF supplemented with 50 and 100 μM copper chloride. The lower panel shows methylene blue stains of the same RNA samples after blotting to the nylon membrane prior to hybridization. (B) Copper-induced *crdA* transcription in *H. pylori* strain G27-2 and its isogenic HP1364 mutant. The upper panel shows total RNA (15 μg) isolated from bacteria incubated without (−) or with (+) 1 mM copper chloride for 1 h. The lower panel shows methylene blue stains of the same RNA samples after blotting to the nylon membrane prior to hybridization. Sizes of *crdA* mRNAs were determined by using the digoxigenylated RNA length standards from Roche Diagnostics (Set I, No. 1526529).

and *crdR* genes, respectively, were selected on Dent blood agar with 20 mg/liter chloramphenicol. Analysis of copper-induced *crdA* transcription by Northern blot hybridization (performed according to standard procedures as described in references 3, 6, and 39) revealed that mutant strains 26695-1365 (Fig. 2A) and 26695-1364 (not shown) both completely lacked the copper-mediated increase of the small *crdA* mRNAs observed in the wild-type (wt) strain 26695 (Fig. 2A), indicating that CrdRS acts as a copper regulator of CrdA. Previous studies reported pronounced differences in the transcriptional regulation of two-component systems in different *H. pylori* strains (9, 11). Thus, we studied copper induction of *crdA* in the well-characterized *H. pylori* mutant strains G27/HP1364::km and G27/HP1365::km (5, 41; kindly provided by Dagmar Beier, Würzburg, Germany). Kinetic analyses revealed that copper-induced *crdA* transcription (40) occurred within minutes in the *H. pylori* wt strain G27 and was completely abolished in the *crdS* (Fig. 2B) and *crdR* (not shown) mutants.

Subsequently, we investigated by growth inhibition experiments whether the CrdRS-mediated copper induction of *crdA* transcription is required for *H. pylori* copper resistance. To this end, *H. pylori* wt strain 26695 and mutants that were normally cultured on Dent blood agar (36) were grown in brucella broth with 5% fetal calf serum (BBF) (total copper content, 0.5 μM [6]). At an optical density at 600 nm (OD_{600}) of 1.0, these precultures were diluted to a ratio of 1:150 in BBF supplemented with copper chloride ($CuCl_2$) (C6641; Sigma). Bacterial growth was determined by reading the OD_{600} after 48 h. Experiments were performed in triplicate and were repeated at least three times. Results from growth experiments demonstrated that *crdS* and *crdR* mutations generally do not limit bacterial fitness but that both genes are required for copper resistance (Fig. 3). Similar results were obtained for *crdS* (see above) and *crdR* (not shown) mutants of *H. pylori* strain G27.

To test whether CrdR can directly interact with the *crdA* promoter, we performed DNA-binding experiments with a purified recombinant version of the *H. pylori* CrdR protein produced in *E. coli* using the Strep-Tag (30) protein expression system from IBA (Göttingen, Germany) according to the manufacturer's instructions. The HP1365 coding sequence of *H. pylori* strain 26695 was amplified using the primer pair 1365ASK3-L1/1365ASK3-R1 (Table 2) and cloned via BsaI restriction sites added as 5′ extensions into the plasmid pASKIBA-3 (IBA-Göttingen). Solubilization and purification of the resulting inclusion bodies were performed according to the method of Sambrook et al. (27). Various parts of the DNA region upstream of *crdA* were amplified by PCR from genomic DNA of *H. pylori* strain 26695 with primers P1326-L1, -L2, and

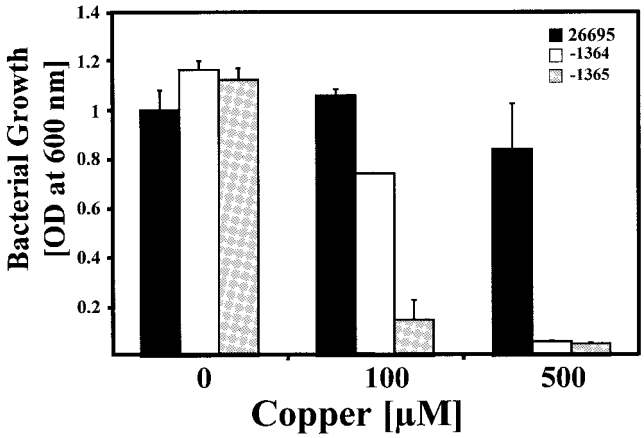

FIG. 3. The role of the HP1364 and HP1365 genes in copper resistance. The *H. pylori* wt strain 26695 (black) and the isogenic mutants 26695-1364 (white) and -1365 (grey) were cultivated for 48 h in BBF medium, and growth inhibition was determined by measuring the OD_{600}s. The medium was supplemented with copper at increasing concentrations as indicated on the *x* axis. The data represent mean values of three independent determinations. Standard deviations are indicated.

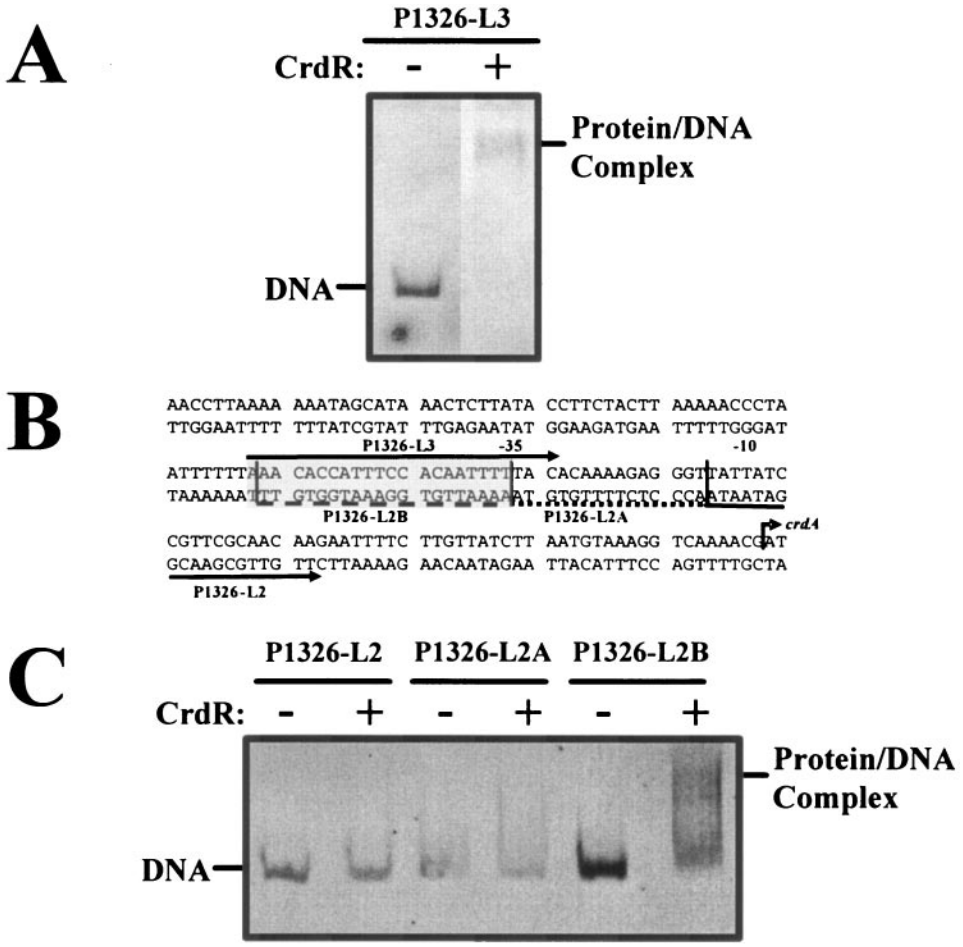

FIG. 4. Binding of the recombinant CrdR protein to the *crdA* promoter region. Different regions in front of the *crdA* gene were analyzed for binding of the recombinant CrdR (rCrdR) protein by EMSA. (A) EMSA with *crdA* upstream DNA generated by PCR with primers P1326-L3 and 1326-R2. The positions of the *crdA* DNA probe (DNA) and the protein complex with CrdR are indicated. PCR products were incubated without (−) and with (+) CrdR. The picture represents a black-and-white image of the ethidium bromide-stained agarose gel visualized under UV light. (B) Schematic overview of the sequence in front of the *crdA* gene. Primers P1326-L3 and -L2, depicted by arrows, and primers P1326-L2A and -L2B, depicted by dotted and dashed lines, respectively, were used for the generation of PCR products for determination of the CrdR binding site. Putative −35 and −10 binding sites for RNA polymerase are indicated. The ATG start codon is marked by a black arrowhead. The DNA region required for binding of CrdR is shown in grey. (C) Determination of the HP1365 binding site in the *crdA* promoter using PCR products generated with primers P1326-L2, -L2A, and -L2B (localizations and sequences are marked in panel B). The positions of the *crdA* DNA probe (DNA) and the protein complex with CrdR are indicated. PCR products were incubated without (−) and with (+) rHP1365 protein. The picture represents a black-and-white image of the ethidium bromide-stained agarose gel visualized under UV light.

-L3 (Fig. 4A; Table 2) in combination with primers 1326-R2 or P1326-DIG (Table 2). The formation of HP1365 protein-DNA complexes with the *crdA* DNA was analyzed by electrophoretic mobility shift assays (EMSA). Recombinant HP1365 protein (2,000 nM) was incubated for 20 min in 20 μl fivefold-concentrated binding buffer (10 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, 5% glycerol, 50 mM acetyl phosphate). Then, 0.14 pM of the target DNA, 30 μl glycerin (50%), and distilled water were added. After 30 min, the samples were electrophoresed on a 7% nondenaturing polyacrylamide gel, and protein-DNA complexes were visualized by staining with ethidium bromide or were blotted to a membrane and detected with the digoxigenin detection kit (Roche) if primer 1326-DIG was used for amplification. The results showed that the mobility of the PCR product generated with primer P1326-L3 was strongly retarded in the presence of CrdR (Fig. 4A), indicating that CrdR binds to *crdA* DNA. The addition of acetyl phosphate (50 mM), which can act as a phosphoryl donor for many response regulators (17), did not influence CrdR binding to the *crdA* DNA probe (not shown). The fact that CrdR binding was not observed with a shorter PCR product (Fig. 4B) generated with primer P1326-L2 and lacking the DNA region between primers P1326-L3 and -L2 (Fig. 4B and C) indicated that CrdR binding is sequence specific and that the target sequence is located in a short stretch of nucleotides directly upstream of the *crdA* coding sequence (Fig. 4B). Further analysis of the binding site by using PCR products generated with the prolongated primers P1326-L2A and -L2B (Fig. 4B) revealed that the binding of CrdR to the *crdA* promoter region depends on the presence of a 21-nucleotide region located directly upstream of the −35 RNA polymerase binding site (Fig. 4C).

We conclude that the transcriptional induction of the *H. py-*

lori CrdA copper resistance determinant by copper ions is directly mediated by the CrdRS two-component system encoded by the HP1365/1364 genes. The average daily copper intake, which is in the range of 1 mg (4), and the copper content of up to 200 mg/kg of body weight in copper-rich foods allow the estimate that *H. pylori* is exposed to copper ions in the micromolar range. Together with the finding that *H. pylori* CrdS and CrdR mutants are not able to colonize the stomach in mice (25), this indicates that *H. pylori* copper homeostasis plays a crucial role in the adaptation to the gastric environment. In this context, the first description of an *H. pylori* copper regulator and an environmental stimulus sensed by an *H. pylori* two-component regulatory system supports the importance of these regulation systems in gastric adaptation. The CrdR binding region is located directly upstream of the *crdA* promoter and contains a sequence signature in the form of a mirror repeat (AACACC-ATTT-CCACAA) (Fig. 4B). The CrdR binding region is not homologous to copper regulator binding sites in other bacteria and was not detected in other *H. pylori* promoters by a genome-wide screen, indicating that CrdR function might be limited to *crdA* regulation. The fact that in vitro binding of CrdR was not influenced by acetyl phosphate leads to the assumption that CrdR, like other response regulators (8, 16, 31), is able to interact with the *crdA* promoter even in the unphosphorylated form. However, the role of phosphorylation in CrdS and CrdR regulation remains to be analyzed in more detail. Earlier studies showed that the deletion of the input domain of HP1364 did not result in autophosphorylation and that HP1365 could be phosphorylated neither by HP1364 nor by the other *H. pylori* histidine kinases (5). In summary, the results presented here clearly support a direct functional relation between CrdR and CrdS (5) and demonstrate that CrdS most likely acts as a sensor for environmental copper ions. This is further supported by the fact that copper added at a concentration of 1 mM to CrdR DNA binding reactions did not influence the interaction of CrdR with its binding site (data not shown). The discovery of a copper regulator in *H. pylori* will support further studies on the role of metal ion homeostasis in gastric adaptation.

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