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# The CrdRS two-component system in *Helicobacter pylori* responds to nitrosative stress

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## Summary

***Helicobacter pylori*** **inhabits the gastric mucosa where it senses and responds to various stresses via a two-component systems (TCSs) that enable its persistent colonization. The aim of this study was to investigate whether any of the three paired TCSs (ArsRS, FleRS and CrdRS) in *H. pylori* respond to nitrosative stress. The results showed that the expression of *crdS* was significantly increased upon exposure to nitric oxide (NO). *crdS*-knockout (Δ*crdS*) and *crdR*/*crdS*-knockout (Δ*crdRS*) *H. pylori*, but not *arsS*-knockout (Δ*arsS*) or *fleS*-knockout (Δ*fleS*) *H. pylori*, showed a significant loss of viability upon exposure to NO compared with wild-type strain. Knockin *crdS* (Δ*crdS-in*) significantly restored viability in the presence of NO. Global transcriptional profiling analysis of wild-type and Δ*crdS H. pylori* in the presence or absence of NO showed that 101 genes were differentially expressed, including copper resistance determinant A (*crdA*), transport, binding and envelope proteins. The CrdR binding motifs were investigated by competitive electrophoretic mobility shift assay, which revealed that the two AC-rich regions in the *crdA* promoter region are required for binding. These results demonstrate that CrdR–*crdA* interaction enables *H. pylori* to survive under nitrosative stress.**

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## Introduction

Persistent *Helicobacter pylori* infection in the human stomach leads to chronic inflammation, which can cause peptic ulcers, gastric carcinoma and lymphoma (Marshall, 2002; Peek and Blaser, 2002). The small genome of *H. pylori* encodes relatively few sensor-regulator transducers or the so-called two-component systems (TCSs), which allow the bacterium to sense and respond to extracellular stimuli and enable adaptation to its exclusive ecological niche (Tomb *et al.*, 1997; Allan *et al.*, 2000). The CheA–CheY sensor-regulator system of *H. pylori* modulates chemotaxis and colonization of the gastric mucosa (Beier and Frank, 2000; Foynes *et al.*, 2000). Phosphotransfer reactions identified three pairs of histidine kinase-response regulator TCSs (Beier and Frank, 2000), designated ArsR–ArsS (ArsRS; HP0165–HP0166), FleR–FleS (FleRS; HP0703–HP0244), and CrdR–CrdS (CrdRS; HP1365–HP1364) (Niehus *et al.*, 2004; Pflock *et al.*, 2004; Waidner *et al.*, 2005).

The importance of the mentioned proteins mentioned earlier was shown by experiments using knockout mutants of the genes encoding ArsS, FleS and CrdS, which lost the capacity to colonize the stomachs of mice (Panthel *et al.*, 2003). Additionally, deletion of *arsR* results in a lethal phenotype (Beier and Frank, 2000). Whole-genome transcriptional profiling analysis of *H. pylori* 26695 and *arsS* knockout strains identified over 100 acid-responsive genes, including amidases and members of the urease gene cluster (Pflock *et al.*, 2005; 2006; Loh and Cover, 2006), and gel-shift and DNA-footprinting analyses showed that ArsR binds directly to the regions upstream of these genes (Pflock *et al.*, 2005; 2006), indicating that the

ArsRS TCS plays an important role in acid adaptation, ensuring successful colonization of the gastric mucosa by *H. pylori*.

The FleRS TCS activates the $\sigma^{54}$ factor, and plays a role in regulating the expression of flagellar structural genes (Niehus *et al*., 2004), whereas the CrdRS TCS is involved in copper-mediated induction of the copper resistance determinant, CrdA (Waidner *et al*., 2005). The CrdS in *H. pylori* J99, but not strains 26695 and G27, is also thought to respond to acidic stress (Loh and Cover, 2006; Muller *et al*., 2007), suggesting that there are strain-specific variations in *H. pylori*. FleS was recently identified as a pH-responsive regulon that induces the expression of urease genes (*ureA*, *ureB* and *ureI*) and amidase (*amiE*) (Wen *et al*., 2009; 2011). The results of these studies suggest that there may be cross-talk between different TCSs in *H. pylori*, which facilitates quick adaptation to the changing, hostile environment within the stomach.

Murine models of *H. pylori* 26695 infection have shown that the bacterium induces innate immune responses, which induce the recruitment of neutrophils, monocytes and macrophages to the site of infection, the expression of inducible nitric oxide (NO) synthase and production of NO (Wilson *et al*., 1996; Fu *et al*., 1999; Chaturvedi *et al*., 2007). NO generation in the stomach is a powerful defense against pathogens (Nathan and Shiloh, 2000), and the mechanisms in *H. pylori* that have evolved to promote its resistance to NO and enable life-long colonization within its gastric niche are intriguing. *H. pylori* arginase and macrophage arginase II, which is induced by *H. pylori*, reduce NO-dependent bactericidal activity and enhance immune system evasion (Gobert *et al*., 2001; Lewis *et al*., 2010). The potential role of *H. pylori* TCSs in the regulation of NO resistance has not been well investigated.

Therefore, the aim of the present study was to investigate whether any of the *H. pylori* 26695 TCSs are involved in the response to NO-induced stress. The results showed that *crdS* was upregulated in the presence of NO, and that the viability of Δ*crdS* and Δ*crdRS H. pylori* strains was significantly reduced under NO-induced stress conditions. A global transcriptional analysis was employed to investigate the putative NO-responsive genes regulated by *crdS*. Competitive electrophoresis mobility shift assay (EMSA) demonstrated the mode of CrdR–*crdA* interaction. The results of the present study suggest that the *H. pylori* CrdRS TCS is involved in its response to nitrosative stress by regulating the expression of metal-homeostasis and/or transport binding proteins.

## Results

### *The CrdRS TCS responds to nitrosative stress*

The sensitivity of the *H. pylori* response to nitrosative stress was first investigated. We utilized a NO-releasing agent, spermine NONOate (Calbiochem) which generates 2 mol of NO per mole of spermine NONOate. *H. pylori* were incubated with NONOate (0.2 μM–1 mM) at 37°C for 4 h. After incubation, the bacterial broth was plated onto Brucella blood agar plates and cultured for 3–4 days, after which the bacterial viability was evaluated by counting the colony forming units (CFUs). As shown in Fig. 1A, *H. pylori* survived and grew normally in the presence of 0.4–40 μM NO, but showed significantly reduced survival rates in the presence of 100–1000 μM NO. We also evaluated the response of *H. pylori* to NO using other NO-releasing agents, S-nitrosoglutathione and sodium nitroprusside (SNOP). A similar trend to reduce *H. pylori* growth was obtained, indicating that *H. pylori* was relatively resistant to nitrosative stress (below 40 μM) (Fig. S1). The expression levels of three histidine kinase genes, *arsS*, *fleS* and *crdS*, were then evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) to examine whether their expression was altered by exposure to 40 μM NO. As shown in Fig. 1B, reduced expression levels of *arsS*, *fleS* and *crdS* were observed following exposure to NO for 0.5 h compared with the levels in untreated control *H. pylori*. In contrast, the expression of *crdS* was significantly increased (approximately twofold; $P < 0.05$) following a 4 h NO treatment compared with the expression levels in untreated *H. pylori*.

Several histidine kinase knockout strains were then constructed (Δ*crdS*, Δ*crdRS*, Δ*arsS* and Δ*fleS*) (Fig. 2) to explore the possible role of *crdS* in the response to nitrosative stress. Both the wild-type and knockout strains showed comparable growth profiles when cultured in Brucella broth supplemented with 10% fetal bovine serum in a microaerophilic environment (Lai *et al*., 2006), suggesting that the wild-type and knockout strains exhibited similar viability under the culture conditions we used (Fig. 1C). To further assess whether TCSs play a crucial role in bacterial survival under nitrosative stress, the viability of the kinase knockout strains (Δ*crdS*, Δ*crdRS*, Δ*arsS* and Δ*fleS*) was tested. As shown in Fig. 1D, under NO-induced stress, survival was approximately 45% for the Δ*crdS* and Δ*crdRS* strains and 90% for wild-type compared with the survival of wild-type without NO. A similar trend was shown in *H. pylori* treatment with SNOP (Fig. S2). Additionally, the inhibition of survival for Δ*crdS* and Δ*crdRS H. pylori* by NO was in a time-dependent manner (Fig. S3). In contrast, the Δ*arsS* and Δ*fleS* strains showed viability that was comparable with that of wild-type *H. pylori* in the absence of NO (Fig. 1D). We then generated a *crdS* knockin Δ*crdS* strain (Δ*crdS*-in) to evaluate the compensation effects. Our data showed that the survival rate of the Δ*crdS*-in strain was approximately 80% of that for the wild-type strain when exposed to 40 μM NO (Fig. 1D). Taken together, these results suggest that

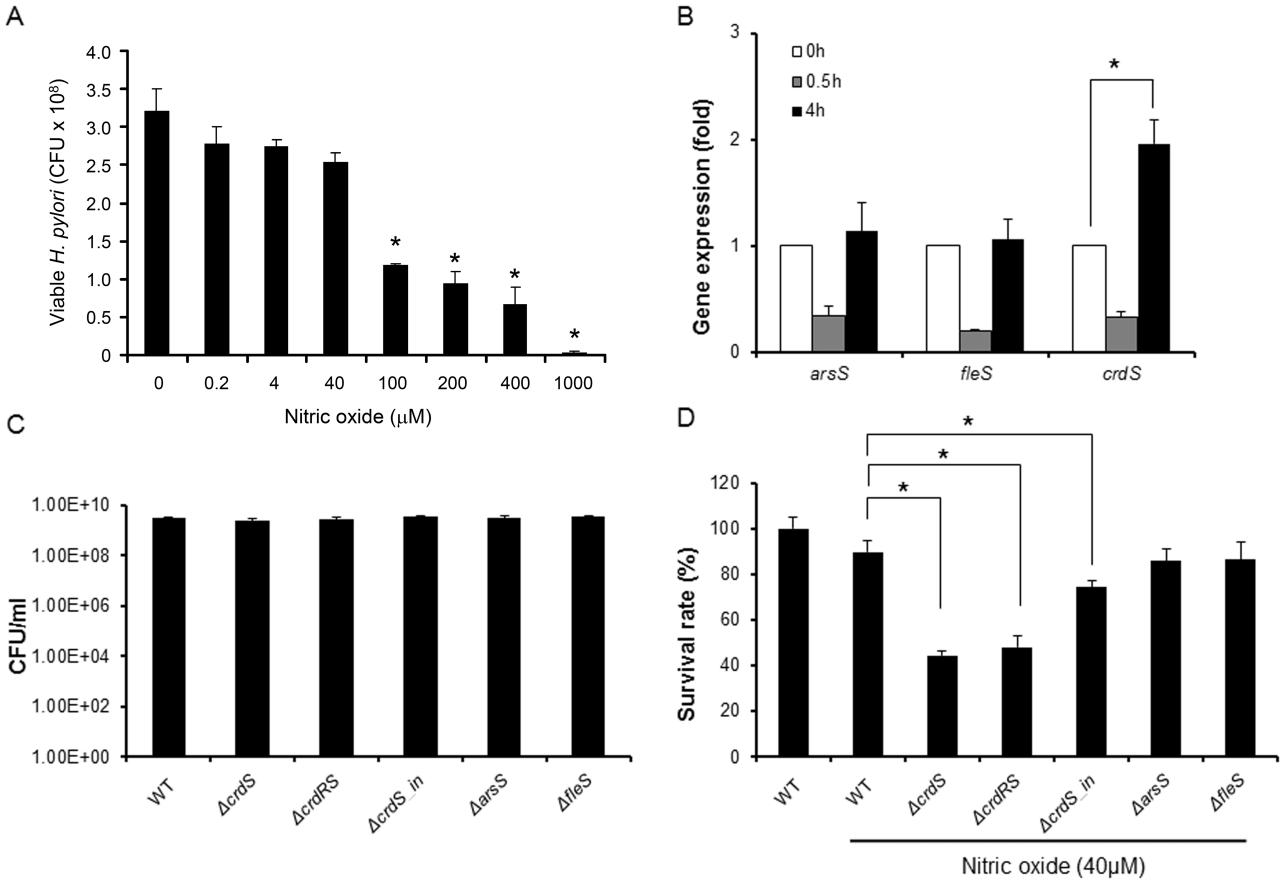



**Fig. 1.** Identification of *H. pylori* genes involved in nitrosative stress.
A. Bacterial survival assay of wild-type (WT) *H. pylori* cultivated in the presence of nitric oxide (NO; 0–1000 μM). The critical concentration of 40 μM NO was selected for subsequent experiments.
B. Gene expression levels in wild-type (WT) *H. pylori* treated with 40 μM NO for 0 , 0.5 and 4 h.
C. Bacterial viability test of WT and knockout (Δ*crdS*, Δ*crdRS*, Δ*arsS*, Δ*fleS* and Δ*crdS*-in) *H. pylori* strains.
D. Survival of WT *H. pylori* and knockout mutant strains in the presence of 40 μM NO. WT *H. pylori* treated with NO was used as a reference. The results presented are the mean and standard deviation of three independent experiments (*$P < 0.05$).

among the TCSs of *H. pylori*, *crdS* is the most important nitrosative stress-responsive histidine kinase.

### *Analysis of the global expression profiles of wild-type and ΔcrdS strains under nitrosative stress*

Transcriptome analysis using a genome-wide microarray containing 385,000 probes derived from the genome sequence of *H. pylori* 26695, possessing 60 base pairs in length, targeting 1576 genes were randomly spotted in five technical replicates. The experiment was performed to examine the NO-response genes controlled by *crdS*. Two conditions were tested in the experimental design: (i) wild-type *vs.* Δ*crdS*, and (ii) with or without nitrosative stress (+NO *vs.* –NO). Two high-throughput means were utilized: (1) cDNA microarray was used to measure the changes of gene expressions from Δ*crdS*, and (2) ChIP-on-chip tiling array was used to measure the binding affinity of CrdR at each probe in the whole genome of *H. pylori* 26695. The difference in the wild-type response to +NO or –NO was first tested using Student's *t*-test for each gene (each with five replicates). The fold changes in expression levels with respect to their significance (*P* values) are plotted in Fig. 3. NO-response genes in the wild-type strain were selected based on the following criteria: (i) a *P* value < 0.0000317, using Bonferroni adjustment to control for the familywise error rate at 0.05; and (ii) a > 1.8-fold change, which is equivalent to an expression log2 signal ≥ 0.85 (Table S1). A total of 145 genes (9.2% of the genome) showing differential expression were selected based on these criteria. The products of these genes were related to the cell envelope ($n = 17$); transport and binding proteins ($n = 13$); cellular processes ($n = 11$); DNA metabolism ($n = 11$); energy metabolism ($n = 5$); amino acid biosynthesis ($n = 4$); biosynthesis of cofactors, prosthetic groups and carriers ($n = 4$); protein synthesis

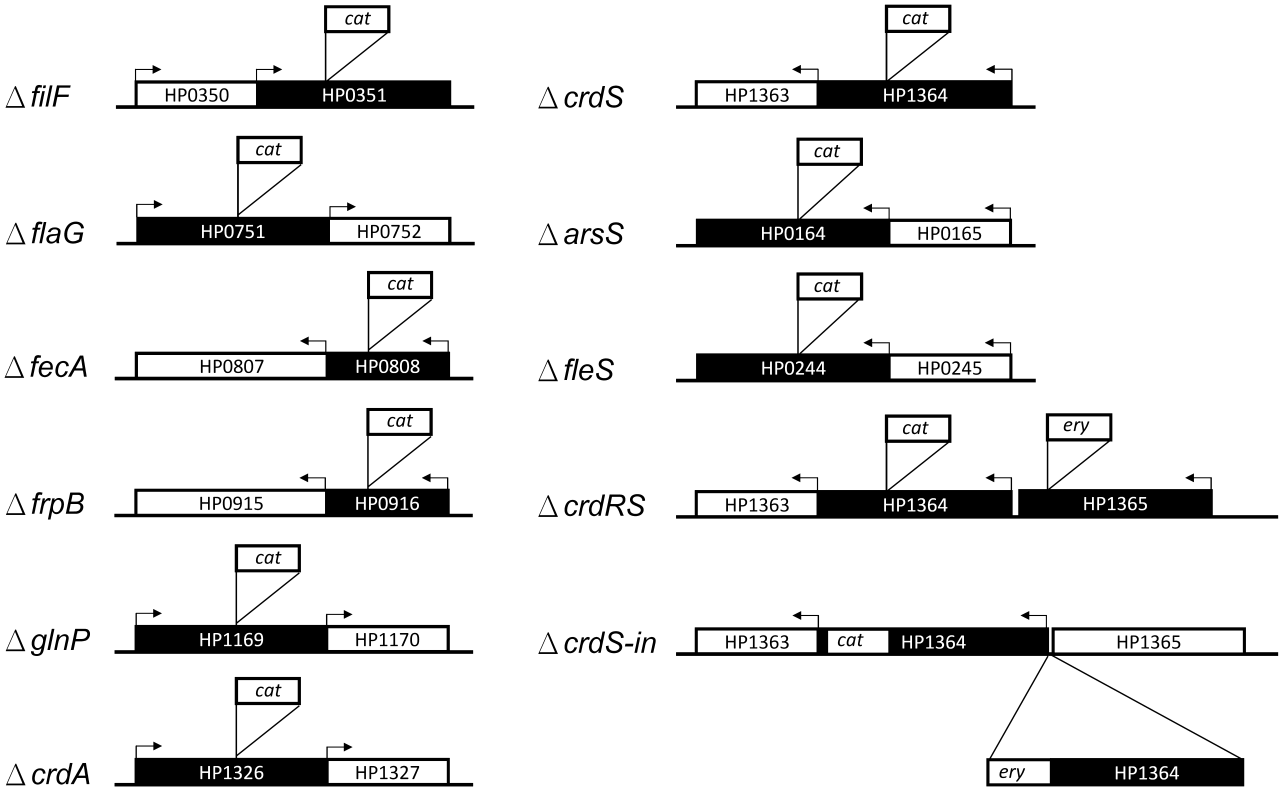



**Fig. 2.** Construction of various *H. pylori* mutants. Schematic representation of the *H. pylori* knockout and knockin (Δ*crdS-in*) mutants. The chloramphenicol resistance gene cassette (chloramphenicol acetyltransferase gene, *cat*) and erythromycin resistance gene cassette (*Ery*) were inserted into the target genes.

($n = 2$); general functions ($n = 4$); protein fate ($n = 3$); purines, pyrimidines, nucleosides and nucleotides ($n = 3$); regulatory functions ($n = 3$); fatty acid and phospholipid metabolism ($n = 2$); and central intermediary metabolism ($n = 1$). The remaining genes were hypothetical ($n = 62$).

We next investigated the differentially expressed genes between wild-type (+NO/–NO) and Δ*crdS* (+NO/–NO) *H. pylori*. Fig. 4 shows a scatter plot of log2 (+NO/–NO) expression in the wild-type strain relative to that in the Δ*crdS* strain. Of the 145 NO-responsive genes, 101

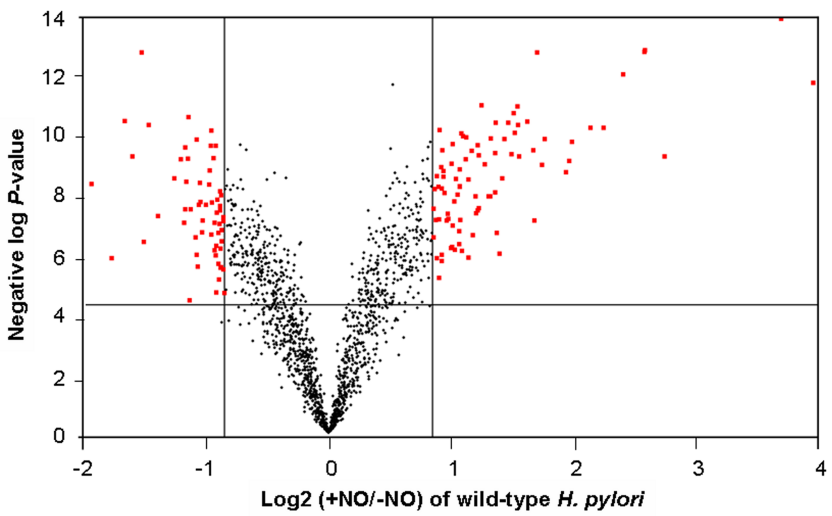



**Fig. 3.** Volcano plots to test the significance between NO-treated and untreated wild-type *H. pylori*. The *x*-axis shows the log 2-fold change in NO-treated (+NO) bacteria compared with untreated (–NO) bacteria. The *y*-axis shows the negative log10 *P* values.

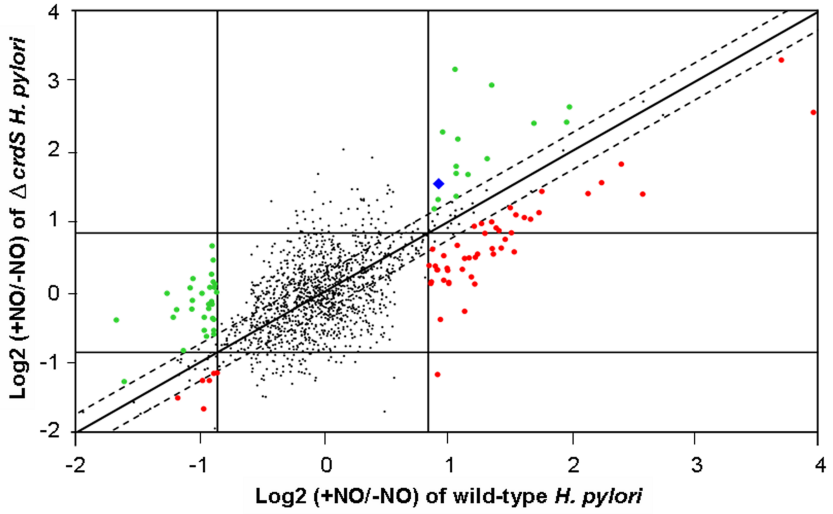



**Fig. 4.** Genome-wide expression profile of wild-type and Δ*crdS H. pylori* strains in response to nitrosative stress. The log2 (+NO/–NO) values of the 145 responsive genes identified in Fig. 3 were compared between the wild-type and Δ*crdS H. pylori* strains. The red dots indicate genes with higher (> 0.263) log2 (+NO/–NO) values in the wild-type strain than in the Δ*crdS* strain. The green dots indicate genes with higher (> 0.263) log2 (+NO/–NO) values in the wild-type strain than in the Δ*crdS* strain. The blue rhombus represents the HP1326 gene (*crdA*).

genes were laid at some distance from the diagonal zone (± 0.263). We carefully considered genes with a value ≥ 0.263 as putative response genes ($n = 56$) that are positively regulated by *crdS*, and those with a value of ≤ –0.263 ($n = 45$) were considered to be negatively regulated by *crdS*. These 101 genes were involved in amino acid biosynthesis ($n = 3$); biosynthesis of cofactors, prosthetic groups and carriers ($n = 2$); cell envelope formation ($n = 10$); cellular processes ($n = 7$); central intermediary metabolism ($n = 1$); DNA metabolism ($n = 4$); energy metabolism ($n = 3$); fatty acid and phospholipid metabolism ($n = 1$); general ($n = 3$); protein fate ($n = 3$); protein synthesis ($n = 2$); purines, pyrimidines, nucleosides and nucleotides ($n = 1$); regulatory functions ($n = 3$); and transport and binding proteins ($n = 10$). The remaining genes were hypothetical ($n = 48$) (Table S2). Despite the large number of hypothetical genes, two major classes of genes were identified that might be regulated by *crdS* under nitrosative stress conditions: genes encoding transport and binding proteins and proteins related to the cell envelope. Of note, one *crdRS*-regulated gene, copper resistance determinant (HP1326, *crdA*), which contributes to the homeostasis of intracellular copper metabolism in *H. pylori* (Waidner *et al.*, 2005), was also found to be induced by NO treatment, and is likely to be regulated by *crdS*.

### *qRT-PCR and survival analyses of putative CrdRS-controlled genes under nitrosative stress*

We then evaluated whether these genes showed differential expression upon exposure to nitrosative stress; particularly those encoding proteins involved in metal transport and homeostasis, such as iron(III) dicitrate (HP0686 and HP0807, *fecA*), iron-regulated outer membrane protein (HP0916, *frpB*), ABC transporter-permease protein (HP1169–HP1170, *glnP*), ABC transporter (HP1172, *glnH*), histidine and glutamine-rich protein (HP 1432), and copper resistance determinant (HP1326, *crdA*) (Table 1). Genes whose functions were associated with the cell envelope, including the flagellar MS-ring protein (HP0351, *fliF*; which is thought to be the first flagellar protein embedded in the membrane), the flagellar protein FlaG (HP0751, *flaG*) and a cellular process protein (HP1560, cell division protein, *ftsW*), all of which are downregulated under acidic conditions (Bury-Mone *et al.*, 2004), were also selected for further analysis. For all the genes examined, the patterns of differential expression shown by the microarray and qRT-PCR results correlated well, confirming that the expression of these genes showed true differences under nitrosative stress.

To further evaluate whether the NO-responsive genes influenced the sensitivity of *H. pylori* to nitrosative stress, knockout strains were constructed for HP0351 (*fliF*), HP0751 (*flaG*), HP0807 (*fecA*), HP0916 (*frpB*), HP1169 (*glnP*) and HP1326 (*crdA*) (Fig. 2). We performed survival assays after treating wild-type and knockout strains with NO. As shown in Fig. 5A, the survival rates were significantly lower for all the knockout strains tested except for ΔHP0807. ΔHP0351 (Δ*fliF*) and ΔHP0751 (Δ*flaG*), two strains with knockouts in genes that encode proteins that function in flagella biogenesis, had the lowest survival rates. ΔHP0916 (Δ*frpB*) and ΔHP1326 (Δ*crdA*) showed approximately 50% survival rates, which was similar to that of Δ*crdS* and Δ*crdRS* (Fig. 1D). In the absence of nitrosative stress, all tested strains showed comparable viability (Fig. 5B). These results suggest that *crdS*-regulated NO-response genes are mainly involved in metal transport or homeostasis and cell envelope structure and functions.

**Table 1.** Validation of potential NO-response genes by qRT-PCR.

| | Microarray (+NO/–NO) | | | qRT-PCR (+NO/–NO)[d] | | |
|---|---|---|---|---|---|---|
| ORF | WT[a] | Δ*crdS*[b] | index[c] | WT | Δ*crdS* | Description |
| Transport and binding proteins | | | | | | |
| HP0686 | 1.30 | 0.84 | 0.46 | 2.31 | 0.56 | Iron(III) dicitrate transport protein (*fecA*) |
| HP0807 | 3.69 | 3.31 | 0.38 | 1.78 | –1.85 | Iron(III) dicitrate transport protein (*fecA*) |
| HP0916 | 1.07 | 1.37 | –0.30 | 0.82 | 1.31 | Iron-regulated outer membrane protein (*frpB*) |
| HP1169 | 2.57 | 1.40 | 1.17 | 4.34 | 2.26 | ABC transporter, permease protein (*glnP*) |
| HP1172 | 1.09 | 2.18 | –1.09 | 0.61 | 2.01 | ABC transporter (*glnH*) |
| HP1432 | 1.54 | 0.58 | 0.96 | 1.90 | 1.45 | Histidine and glutamine-rich protein |
| HP1561 | –0.89 | –0.16 | –0.73 | –1.76 | –0.11 | Iron(III) ABC transporter, periplasmic iron-binding protein (*ceuE*) |
| Cellular processes | | | | | | |
| HP1560 | –0.88 | –1.14 | 0.27 | –1.181 | –8.181 | Cell division protein (*ftsW*) |
| General | | | | | | |
| HP1326 | 0.94 | 1.54 | –0.60 | 1.82 | 4.75 | Copper resistance determinant (*crdA*) |
| Cell envelope | | | | | | |
| HP0351 | 1.51 | 1.21 | 0.30 | 1.16 | 0.60 | Flagellar MS-ring protein (*fliF*) |
| HP0751 | 1.01 | 0.14 | 0.88 | 1.98 | 0.38 | Flagellar protein (*flaG*) |

**a.** Expression ratio of genes from the wild-type *H. pylori* strain treated with NO.
**b.** Expression ratio of genes from the Δ*crdS* mutant *H. pylori* treated with NO.
**c.** An index value was calculated for each gene by dividing the +NO/–NO expression ratio for the wild-type *H. pylori* strain and by the +NO/–NO ratio for the indicated Δ*crdS* mutant strain. The gene expression ratio (+NO/–NO) was represented as the value of log2 (+NO/–NO).
**d.** Relative quantification of gene expression (with or without NO treatment) was determined using the $2^{-\Delta\Delta Ct}$ method by Livak and Schmittgen as described in the Materials and Methods (Livak and Schmittgen, 2001).

*Evaluation of the CrdR DNA binding motifs in* H. pylori

We next sought to identify the CrdR binding motifs by using two powerful transcription factor binding site (TFBS) prediction algorithms, TFBSfinder (Tsai *et al.*, 2006) and Magiic-tfbs2 (Chen *et al.*, 2008). As shown in Table 2, the CrdR binding motifs predicted by TFBSfinder are WAAAAYAMW and TTTTACACWAW, and those predicted by Magiic-tfbs2 are AADCNHWAAWT and TTTTWWDN CAAA. Of note, the mapped *crdA* promoter region is located at –110 to –60 bp, and this region includes a CrdR binding segment that was previously identified by Waidner

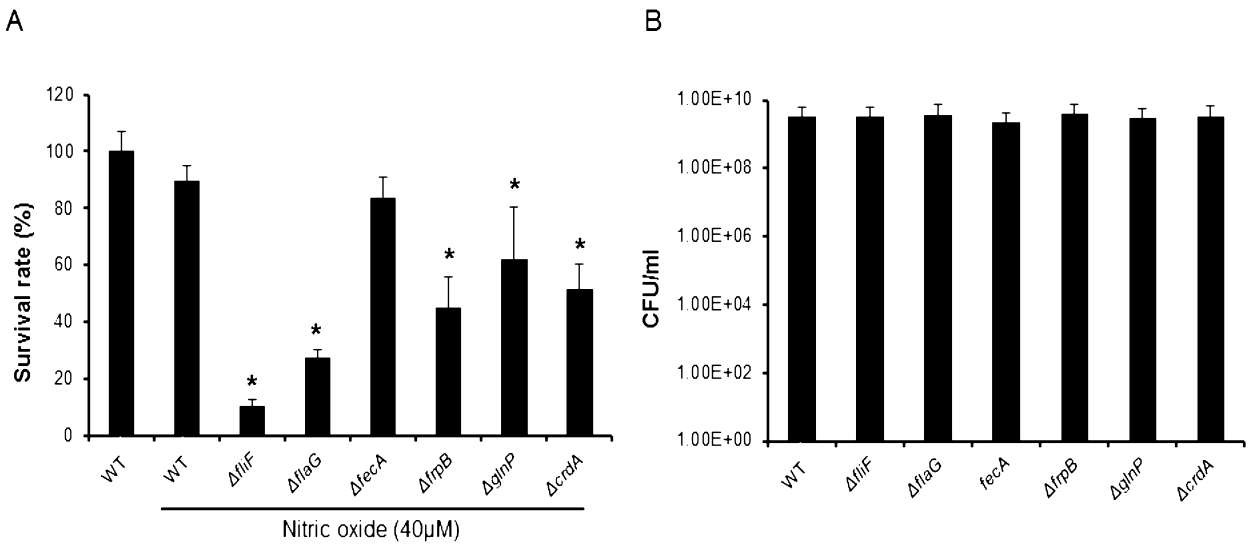
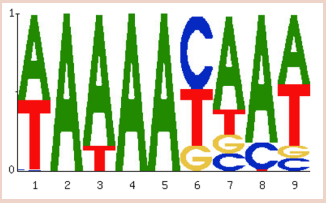
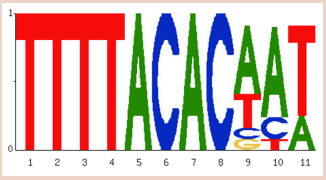



**Fig. 5.** Survival of *H. pylori* strains under nitrosative stress.
A. Bacterial survival assay of wild-type (WT) and histidine kinase knockout strains. WT and knockout *H. pylori* strains were treated with 40 μM NO. Percent survival was calculated as the CFUs of NO-treated *H. pylori* divided by the CFUs of untreated *H. pylori*.
B. The viability of WT and target gene knockout *H. pylori* strains in the absence of NO. **P* < 0.05 *vs.* wild-type *H. pylori* treated with NO, as determined by Student's *t*-test.

**Table 2.** Comparison of predicted CrdR binding motifs.

| Approaches | Predicted motif | Consensus[a] | Length |
|---|---|---|---|
| TFBSfinder (Tsai *et al*., 2006) |  | WAAAAYAMW | 9 |
| |  | TTTTACACWAW | 11 |
| Magiic-tfbs2 (Chen *et al*., 2008) |  | AADCNHWAAWT | 11 |
| |  | TTTTWWDNCAAA | 12 |
| Identified by (Waidner *et al*., 2005) | | AACACCATTTCCACAATTTTT | 21 |

**a.** Underlines indicate the consensus sequence of AAACNC.

*et al*. (2005) (Fig. 6). The consensus motifs derived from the predicted sequences are AAACNC and CACAA (Table 2). To test whether the predicted sequence AAACNC is critical for CrdR binding, nine probes were designed based on the *crdA* promoter sequence (–55 to –121 bp; Table 3; Waidner *et al*., 2005) and the EMSA assay was utilized to characterize the importance of tandem AC-rich region for CrdR binding. As expected, in the EMSA assay, recombinant CrdR exhibited significant binding to probe 1 (which contains the reported 21 bp *crdA* sequence from –71 to –91; AACACC-ATTT-CCACAA) (Fig. 7). Among the other tested probes (2–6), CrdR bound

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-121                                                                    -55
...CTTCTACTTAAAAACCCTAATTTTTTAAACACCATTTCCACAATTTTTACACAAAGAGGGTTA... crdA
----------------------------AACACCATTTCCACAATTTTT-------------------- Waidner et al.
-----------AAAAACCCTAATTTTT---------------------------------------- TFBSfinder, WAAAYAAW
-------------------------------------------TTTTACACAAA------------ TFBSfinder, TTTTACACWAW
-------------AAACCCTAATT---------------------------------------- Magiic-tfbs2, AADCNWWAAWT
---------------------------------------------TTTTTACACAAA------------ Magiic-tfbs2, TTTTWWDNCAAA
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**Fig. 6.** Identification of CrdR binding motifs in the *crdA* promoter.
TFBSfinder (Tsai *et al*., 2006) and Magiic-tfbs2 (Chen *et al*., 2008) are algorithms that were employed to predict the transcription factor binding sites in *crdA*. The promoter region of *crdA*, identified by Waidner *et al*. (2005), was compared with the mapping sites detected by TFBSfinder and Magiic-tfbs2.

**Table 3.** The diagram for positions of EMSA probes.

| No. of probes | Sequence[a] |
|---|---|
| Position | –121 –55 |
| *crdA* | ATACCTTCTACTTAA AAACCC TAATTTTTT AAACAC CATTTCCACAATTTTTACACAAAAGAGGGTT |
| 1 | ---------- AAACAC CATTTCCACAATTTT---------- |
| 1A | ---------- TTTGTG CATTTCCACAATTTT---------- |
| 2 | ---------- CACAA TTTTTACACAAAAGAG------ |
| 3 | ---------- AAACCC TAATTTTTT AAACAC ---------- |
| 3A | ---------- AAACCC TAATTTTTT TTTGTG ---------- |
| 3B | ---------- TTTGGG TAATTTTTT AAACAC ---------- |
| 4 | ----------CTTAA AAACCC TAATTTTTT A---------- |
| 5 | ATACCTTCTACTTAA AAACCC---------- |
| 6 | ----------TT AAACAC CATTTC CACAA TT---------- |

**a.** The putative binding motifs (AC rich) of each probe were denoted in rectangle boxes. Motifs in red of probes 1A, 3A and 3B were mutants derived from probes 1 and 3, respectively. Dashed lines indicate aligning with sequence the *crdA*.

to probes 3 and 6, and these two probes share an AAAC(A/C)C sequence (corresponding to –75 to –106 bp). Notably, two AC-rich motifs at the 3′ (AAACCC) and 5′ (AAACAC) ends were found in probe 3 (AAACCCTAATTTTTTAAACAC; corresponding to –85 to –106 bp). Replacement with complementary nucleotides in either motif (probe 3A: AAACCCTAATTTTTT*TTTGTG* and probe 3B: *TTTGGG*TAATTTTTTAAACAC) diminished CrdR binding, revealing the importance of both AC-rich motifs for CrdR–*crdA* interaction. However, No CrdR binding signal was detected for probe 5 (ATACCTTCTACTTAAAAACCC, corresponding to –101 to –121), which contained only one motif (AAACCC, corresponding to –101 to –106 bp). These results suggest that the two AC-rich regions (AAACCC or AAACAC near the 3′ end and AAACAC or CACAA near the 5′ end) are required for binding to CrdR. These data also demonstrate that the specific sequence AAACCC present in probes 3 and 6 is crucial for CrdR binding.

## Discussion

In the human stomach, *H. pylori* encounters a constantly changing, hostile environment. This pathogen has naturally developed a delicate strategy to swiftly respond to extracellular stimuli, including acid and innate immune responses (Gobert *et al.*, 2001; Schwartz and Allen, 2006;

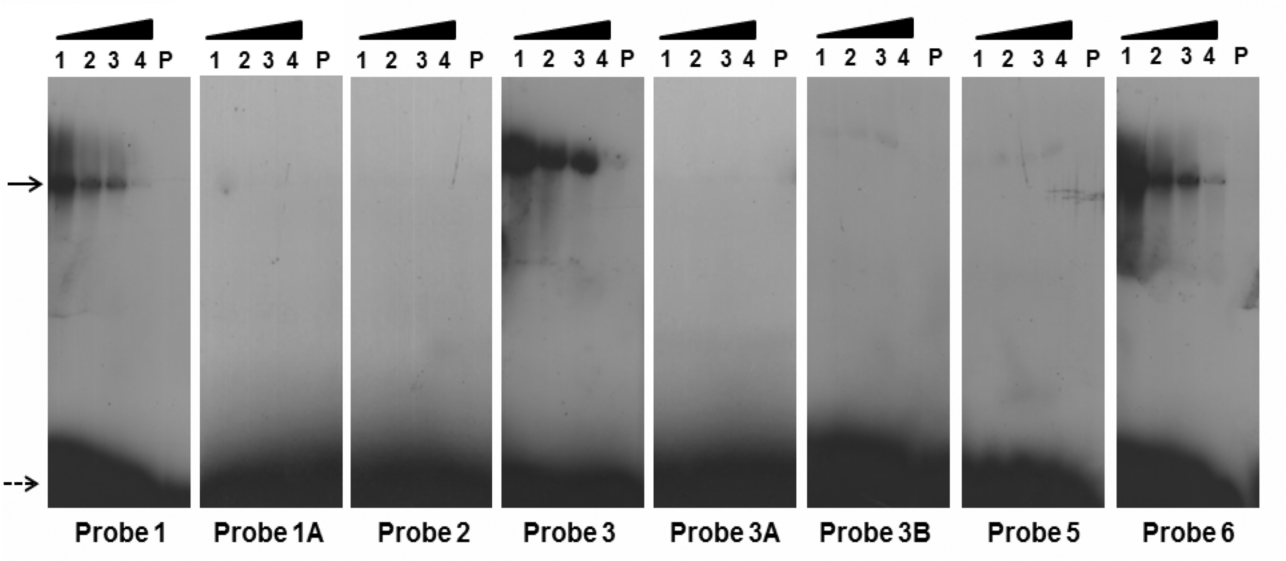


**Fig. 7.** Binding of CrdR to the promoter region of *crdA*. EMSA of CrdR binding to the radiolabeled probes indicated at the bottom. For each probe, cold chase experiments were performed with increasing concentrations of unlabeled probe (0-fold, 1-fold, 10-fold and 100-fold excess; lanes 1–4). P, unlabeled free probe. The protein/DNA complex signals are shown by the solid arrow; the free probe is shown by the dotted arrow.

Lu *et al*., 2012; Lai *et al*., 2013). The ArsRS TCS is an important regulator of acid responses (Pflock *et al*., 2005; 2006; Loh and Cover, 2006). The sensor FleS was recently shown to be involved in adaptation to low pH (Wen *et al*., 2009), in addition to its role (along with FleR) in flagella biosynthesis (Niehus *et al*., 2004). CrdS is thought to be a sensor for environmental copper ions as it induces the expression of *crdA* in the presence of copper (Waidner *et al*., 2005). CrdS was also shown to act as an acid sensor in *H. pylori* J99 (Loh and Cover, 2006), but not in 26695 and G27 (Muller *et al*., 2007). The results of the present study show that *crdS*, but not *fleS* nor *arsS*, was significantly upregulated in wild-type *H. pylori* in the presence of NO stress (Fig. 1). The findings that Δ*crdS* and Δ*crdRS*, but not the other histidine kinase knockout strains Δ*arsS* and Δ*fleS*, showed significantly reduced viability after exposure to nitrosative stress, suggesting that CrdS is a sensor for nitrosative stress and that the CrdRS TCS responds to this challenge.

CrdRS has been shown to be required for *H. pylori* colonization in the stomach in a murine infection model; however, its target genes have not been well investigated (Panthel *et al*., 2003). Global transcriptome analysis of wild-type *H. pylori* with and without NO treatment identified 145 differentially expressed genes. Apart from the 62 hypothetical genes, the responsive genes included those encoding proteins that function in the cell envelope, transport and binding, cellular processes and DNA metabolism (DNA modification and repair). Of note, *crdA* (HP1326), which is induced by copper ions, also showed greater than twofold higher expression under nitrosative stress (Fig. 4). Under nitrosative stress, HP0807, HP0051 and HP1169, which encode a hypothetical protein, an iron(III) dicitrate transport protein (*fecA*), cytosine-specific DNA methyltransferase and an ABC transporter-permease protein (*glnP*), respectively, had the highest levels of expression.

To evaluate whether these NO-responsive genes were regulated by CrdRS, the normalized expression values [log2 (+NO/–NO)] for wild-type and Δ*crdS* were compared, and 101/145 genes (69.7%) showing an index $\geq 0.263$ or $\leq -0.263$ were considered to be regulated by CrdS. Forty-eight were hypothetical genes, but the others fell into several major classes, including (i) transport and binding proteins for iron(III) dicitrate, iron(II), glutamine and nickel(II); (ii) proteins involved in the flagellar system; (iii) proteins involved in electron transfer; (iv) DNA repair enzymes; and (v) proteins that modify the cell envelope lipid composition. The qRT-PCR results for selected genes encoding transport and binding proteins (HP0686, HP0807, HP0916, HP1169, HP1172, HP1326, HP1432 and HP1561), and proteins involved in cellular processes (HP1560) and the cell envelope (HP0351 and HP0751) showed good agreement with the microarray data (Table 1).

Acquisition of sufficient amounts of iron is essential for the multiplication of bacterial pathogens within the host (Ratledge and Dover, 2000). Intriguingly, CrdS regulates the expression of a number of NO-responsive genes that encode metal transport and binding proteins including iron ions such as *fecA* that encodes iron(III) dicitrate transport protein, and proteins with cell envelope functions (Danielli and Scarlato, 2010; Pich and Merrell, 2013). One plausible scenario is that regulation of these genes may enable *H. pylori* to acquire a more efficient system to chelate metals, particularly iron, and/or NONOate/NO to reduce the levels of NO-mediated toxicity. Therefore, in addition to its role as a copper-stress TCS, the CrdRS system plays additional roles in chelating iron for its multiplication as well as NO in order to respond to the challenge posed by innate immunity. Interestingly, Fur, the ferric uptake regulator crucial for *H. pylori* colonization (van Vliet *et al*., 2002; Carpenter *et al*., 2009), is also shown to regulate acid acclimation, resistance to oxygen reactive species as well as nitrogen metabolism, revealing the dual roles of Fur in iron homeostasis and adaptation to stress (Ernst *et al*., 2005; Gancz *et al*., 2006). These findings suggest that *H. pylori* with small genome size fully utilizes metal-homeostasis regulators, including CrdRS and Fur, to acquire iron and simultaneously respond to host stresses for survival within this unique ecological niche.

We also tested whether these genes indeed were important for viability under nitrosative stress. Of the knockout strains examined [HP0351 (*fliF*), HP0751 (*flaG*), HP0807 (*fecA*), HP0916 (*frpB*), HP1169 (*glnP*) and HP1326 (*crdA*)], the most susceptible mutants were ΔHP0351 (Δ*fliF*) and ΔHP0751 (Δ*flaG*) (Fig. 5A), which are knockout strains of genes that encode the flagellar MS-ring protein and the flagellar protein FlaG, respectively. It is likely that the absence of either gene might lead to reduced mobility or altered membrane dynamics, rendering the bacteria more susceptible to NO attack (Niehus *et al*., 2004). Null mutants of genes encoding proteins involved in metal homeostasis [HP0916 (*frpB*) and HP1326 (*crdA*)] also showed reduced viability, underlying the crucial link between the response to nitrosative stress and metal metabolism in this gastric pathogen.

The CrdR binding region in the *crdA* promoter was previously identified (Waidner *et al*., 2005). In this study, we employed transcription factor binding site prediction algorithms and a global microarray method to identify the crucial DNA binding motif (Table 2). A CrdR–nucleotide complex model was subsequently built based on the structure of the PhoB–DNA complex. Interestingly, the apo structures of two response regulators have been solved, an orphan response regulator (Hong *et al*., 2007) and the ArsR-DBD (Gupta *et al*., 2009), which are dimeric. Competitive EMSA showed that two AC-rich regions (AAACCC or AAACAC near the 3′ end and AAACAC or

CCACAA near the 5′ end) spanning the *crdA* promoter region (–55 to –121) are required for CrdR binding (Table 3, Fig. 7), suggesting that a pair of CrdR proteins bind to the *crdA* promoter. In summary, our results demonstrate the importance of the CrdRS system in the regulation of *H. pylori* survival under NO stress. These findings also support the notion that iron homeostasis and defense against oxidative stress in *H. pylori* are intimately connected (Thompson *et al*., 2003). CrdRS is known to control copper-mediated induction of the copper resistance determinant. Our study further defines a new function for CrdRS in the nitrosative stress response. These findings have implications for the multiple roles of CrdRS owing to the small number of TCSs in *H. pylori* to respond to a diverse array of stimuli.

## Experimental procedures

### *Bacterial strains and culture conditions*

*Helicobacter pylori* 26695 (ATCC 700392; $CagA^+VacA^+$) was used as the reference strain. Isolation, identification and storage of the *H. pylori* 26695 strains were performed as previously described (Lai *et al*., 2008). Primary plate cultures of *H. pylori* 26695 were grown from glycerol stocks on blood agar for 2–3 days in a microaerobic environment (5% $O_2$, 10% $CO_2$ and 85% $N_2$) at 37°C. The bacteria were routinely cultured on Brucella agar plates (Becton Dickinson, Franklin Lakes, NJ) containing 10% sheep blood. Liquid cultures were grown in Brucella broth and 10% fetal bovine serum (HyClone, Logan, UT). Antibiotics were added at the following concentrations: ampicillin, 100 μg $ml^{-1}$; kanamycin, 50 μg $ml^{-1}$; and chloramphenicol, 34 μg $ml^{-1}$. *Escherichia coli* strains DH5 and JM109 were used for the cloning experiments and grown in Luria–Bertani (LB) medium containing ampicillin (100 μg $ml^{-1}$).

### *Construction of knockout* H. pylori

The isogenic mutant *H. pylori crdS*::*cat* was generated by insertion of the chloramphenicol resistance cassette ($Cm^r$) from pUOA20 (Wang *et al*., 1993) into the *crdS* of *H. pylori* through allelic replacement and selection of chloramphenicol-resistant clones (Lai *et al*., 2008). The *crdS* gene fragment was amplified from *H. pylori* 26695 strain chromosome by PCR and was cloned into the plasmid pGEMT (Promega, Madison, WI), yielding plasmid pGEMT-*crdS*. The forward and reverse oligonucleotide primers designed for PCR amplification of the *cat* sequence were cat_NheI_F (GGAAGATG CGCTAGCTGATCCTTCAAC) and cat_NheI_R (CCCGTC AAGCTAGCGTAATGCTCTGC), respectively. These primers were introduced via *Nhe*I sites at the 5′ and 3′ ends. The amplified product was digested with *Nhe*I and ligated into the *Nhe*I site of pGEMT-*crdS* to obtain plasmid, pGEMT-*crdS-cat*, with a *cat* gene inserted into *crdS* (*crdS*::*cat*). A *crdS*::*cat* mutant was generated by transforming pGEMT-*crdS-cat* into the parental strain, *H. pylori* 26695, by allelic replacement and selecting chloramphenicol-resistant clones using the natural transformation method described previously by Wang *et al*. (1993).

To generate the *crdS crdR* (ΔcrdRS), *arsS* (ΔarsS), *fleS* (ΔfleS), *crdA* (ΔcrdA), *filF* (ΔfilF), *flaG* (ΔflaG), *frpB* (ΔfrpB) and *glnP* (ΔglnP) knockout strains, similar procedures were performed using the *cat* cassette, the chloramphenicol resistance cassette ($Cm^r$) from pUOA20 (Wang *et al*., 1993) and the erythromycin resistance cassette ($Ery^r$) was obtained from pE194 (Gryczan *et al*., 1980). The primers used for PCR amplification of $Cm^r$ from pUOA20 were introduced a *Nhe*I site. The primers used for PCR amplification of erythromycin resistant gene (*ery*$^r$) from pE194 introduced an *Mfe*I site were: forward primer ery_MfeI_F (CAATAACAATTGCC GATTGCAGTATAA) and reverse primer ery_MfeI_R (ACA TAATCGACAATTGAAAAATAGGCACACG), respectively. These primers were introduced via *Mfe*I sites at the 5′ and 3′ ends. Correct integration of the antibiotic resistance cassettes into the target genes of the chromosome was verified by PCR (Table S3).

### *Generation of crdS knockin* H. pylori

A knockin *crdS* mutant (Δ*crdS*-in) was prepared based on Δ*crdS*. A full-length *crdS* was inserted into position between *HP1364* and *HP1365* inΔ*crdS*. The construct was designed to consist of homology segments flanking *ery*$^r$ and the full length of *crdS*, i.e. the homology 174 bp *HP1364* segment (N-HP1364), the erythromycin resistant gene (*ery*$^r$), the full-length of *HP1364* and the homology 255 bp fragment of *HP1365* (C-HP1365). In brief, two-step construction was done to prepare pGEMT-HP1364N-HP1364FL-HP1365C by employing SacI, AgeI and NcoI with the following PCR primers: HP1364N_SacI_F (GAGCTCGATCTTAGAAGCCA TTTTG) and HP1364N_AgeI_R (ACCGGTTTGGCTATCG CTTTAAC) for PCR amplification of N-HP1364; HP1364FL-HP1365C_AgeI_F (ACCGGTTTATCCTTGAAATTGAACGC) and HP1364FL-HP1365C_R (ATGATCCCATAGAAATCATG-CCTAACATTT) for PCR amplification of HP1364FL-C-HP1365. Finally, the *ery* gene was introduced into the AgeI site with the PCR amplified product by the paired primers ery-_AgeI_F (CAATAATCGACCGGTATTGCAGTATAA) and ery-_AgeI_R (GACATAATCGACCGGTAAAAAATAGGCACA) to obtain plasmid, pGEMT-*crdS-in.* Correct integrations of the antibiotic resistant cassettes into the target genes were verified by PCR. The knockin Δ*crdS*-in mutant was then generated by the natural transformation method as described earlier.

### *Bacterial viability assay in the absence or presence of NO*

*Helicobacter pylori* wild-type strain 26695 and the Δ*crdS*, Δ*arsS*, Δ*fleS* and Δ*crdS*-in *H. pylori* were grown on Brucella broth agar plates with appropriate antibiotics. The compound spermine NONOate (Calbiochem, San Diego, CA) was used as an NO donor. The concentrations of NO were determined from final NONOate concentrations which were based on the generation of 2 mol of NO from 1 mol of spermine NONOate. A 100 mM NONOate solution was freshly prepared for each experiment. The NO donor was dissolved in 1 × PBS and the solution was stored at 4°C until use. Two-day bacterial cultures

were prepared at $1.2 \times 10^9$ ml$^{-1}$ in fresh Brucella broth and incubated with 0.2 μM–2 mM NONOate at 37°C for 4 h. After incubation, the bacterial broth was serially diluted in PBS, plated onto Brucella blood agar plates and cultured for 3–4 days, after which the CFUs were counted. The critical concentration (20 μM NONOate) was employed and then determined for the follow-up *H. pylori* survival assays. All assays were performed independently in triplicate.

### *Construction of His-tagged CrdR expression plasmid and purification of CrdR*

The gene encoding the response regulator (*crdR*) was amplified from chromosomal DNA in *H. pylori* 26695 by PCR using CombiZyme DNA polymerase (Invitek GmbH, Germany) and inserted into the pQE30 expression vector (QIAGEN, Valencia, CA) to generate pQE30-CrdR. Primers HP1365-F (**5′**-AGCGCTACAATGGATCCATGCAAAAAAAGA-**3′**) and **HP1365-R** (**5′**-AGAGTTTAGTCGACTTTTTTTCATAGTGGG-**3′**), containing *Bam*HI and *Sal*I sites, respectively, were designed based on the nucleotide sequence of the reported *crdR* gene from *H. pylori* 26695 (accession number: NC_000915) (Tomb *et al.*, 1997). pQE30-CrdR was transformed into *E. coli* JM109 cells and expression of CrdR was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside at 28°C. Bacterial pellets were fractionated and the soluble proteins in the cytosolic fractions were collected. The expressed His-tagged CrdR fusion proteins were purified using immobilized cobalt-ion chromatography (GE Healthcare Bio-Sciences, Sweden) and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis to verify purity. The protein concentration was assayed according to the Bradford method using bovine serum albumin as the standard.

### *Microarray techniques and data analysis*

*Helicobacter pylori* 26695 RNA and Δ*crdS* RNA were isolated from bacteria grown to logarithmic phase in liquid broth using an RNeasy mini kit (QIAGEN) according to the manufacturer's protocol. For NONOate treatment, bacteria grown to logarithmic phase in liquid broth were diluted to $1.2 \times 10^9$ ml$^{-1}$ in fresh Brucella broth and incubated with 40 μM NO at 37°C. RNA preparations used for global transcriptional profiling were further purified using the RNeasy mini kit (QIAGEN). Preparation of cDNA was performed by following the manufacturer's protocol (Invitrogen, Carlsbad, CA). The final quality of cDNA was determined by spectrophotometric analysis.

A customer-made whole-genome microarray containing 385,000 probes covering the entire *H. pylori* 26695 genome was used for transcriptome analysis. The array was designed with 1576 genes, and each was spotted with 20 probes (apart from two genes). Each probe had an average length of 60 bp and was randomly spotted in five technical replicates. In addition, 35,983 random probes were used for the purpose of quality control. cDNA labeling and hybridization of Cy3-labeled DNA was performed according to the Nimblegen protocol (http://www.nimblegen.com/products/chip/custom/385k/index.html). The raw microarray data were analyzed using Nimblegen's software and exported into Microsoft Excel. The Cy3 signal values for the Δ*crdS* samples were normalized against the *H. pylori* 26695 reference control.

For the data analysis, 35,983 random probes were removed. Loss normalization was then used to provide probe-level data for the four arrays. Five replicates of the same probe were randomly split into five columns to create five hypothetical replicated samples. Each of the four combinations (WT/+NO, WT/–NO, Δ*crdS*/+NO, Δ*crdS*/–NO) was analyzed. The gene expression levels were summarized as the median of the 20 probes within each hypothetical sample because some genes show dramatically different expression levels across the probes. The analysis was based on 1576 expression summaries from the five replicates for each of the four combinations. Microarray data can be found at the NCBI Gene Expression Omnibus Website (http://www.ncbi.nlm.nih.gov/geo/; GEO accession: GSE69848).

### *qRT-PCR*

RNA was isolated from *H. pylori* 26695, either treated or untreated with 20 μM NONOate (37°C for 1 h) using an RNeasy mini kit (QIAGEN). Possible genomic DNA contamination was removed using DNaseI (New England BioLabs, Ipswich, MA). Primers were designed using Primer Express 3 (Applied Biosystems, Carlsbad, CA), and shown in Table S4. The primers were synthesized by Mission Biotech (Taipei, Taiwan) and were tested to determine amplification, specificity, efficiency and for linearity of amplification with RNA concentration. A typical 25 μl reaction contained 12.5 μl of SYBR Green Master Mix (Applied Biosystems), 250 nM of each primer and 1 ng of cDNA. All samples were run in an ABI Prism 7500 Sequence Detector (Applied Biosystems) for 40 cycles (30 s at 94°C, 30 s at 55°C, and 35 s at 72°C) in triplicate. The qRT-PCR products were checked by running on 2% agarose gels to ensure that there was only one product. Quantification of each target transcript after treatment with NONOate was performed in triplicate and normalized to 16s rRNA levels in the same sample. Gene expression was determined using the $2^{-\Delta\Delta Ct}$ method by Livak and Schmittgen, where $\Delta\Delta C_t = (C_{t,Target} - C_{t,16s})_{NONOate} - (C_{t,Target} - C_{t,16s})_{Control}$ (Livak and Schmittgen, 2001).

### *Inferring CrdR binding motifs and putative target genes*

Two transcription factor binding site prediction algorithms, TFBSfinder (Tsai *et al.*, 2006) and magiic-tfbs2 (Chen *et al.*, 2008), were utilized to fetch the putative binding motifs of *crdA* in *H. pylori*. The number and organization of promoters and operons located at an upstream intergenic region (≤ 200 bp) of an ORF(s) based on the *H. pylori* 26695 genome were predicted by using the approach which described by Price *et al.* (2005). Alignment of multiple promoter sequences of orthologous genes to derive the lists of potential target and non-target genes were performed based on the four genomes: *H. pylori* 26695, *H. pylori* J99, *H. pylori* HPAG1 and *H. pylori* G27 (Schneider *et al.*, 2006).

Two high-throughput experimental data were utilized: (1) cDNA microarray was used to measure the changes of gene

expressions from Δ*crdS* (GEO accession: GSE69848), and (2) ChIP-on-chip tiling array was used to measure the binding affinity of CrdR at each probe in the whole genome of *H. pylori* 26695. CrdR ChIP-on-chip data can be found at the NCBI Gene Expression Omnibus Website (GEO accession: GSE69848). Genes with greater than or equal to twofold expression change were considered as potential *crdR* regulatory target genes. Two criteria are considered to select a set of reliable target (CrdR-binding) genes and a set of non-target genes (as a control set) to find overrepresented and conserved motifs: (i) a binding score of $\geq 0.56$ that corresponds to false discovery rate $< 0.01$ for at least one probe within the promoter region in the tiling array data; and (ii) greater than or equal to twofold expression change in the microarray data. We defined those genes that have both features as the 'target' gene set, while those that show binding scores of $< 0$ across the promoter region in the tiling array data and less than twofold expression change in the microarray data are considered as the 'non-target' gene set. Sequences of $\geq 0.56$ binding score from target gene sets and those of $< 0$ binding score in the promoter region were then extracted for motif finding by TFBSfinder (Tsai *et al*., 2006) and magiic-tfbs2 (Chen *et al*., 2008).

### *EMSA*

The oligonucleotide probes were designed and synthesized primarily based on the *crdA* promoter sequence that consists of the predicted binding motifs from this study or the identified binding region (Waidner *et al*., 2005). The probes were radioactively labeled at the 5′-end by using T4 polynucleotide kinase (New England BioLabs) with [$\gamma$-$^{32P}$] ATP (50 μCi). Binding of the recombinant CrdR or CrdR-phosphorylated protein to DNA was carried out in a 30 μl reaction mixture containing [$\gamma$-$^{32P}$]-labeled DNA, 1 μg poly(dI-dC) (Sigma-Aldrich), 170 mM HEPES–KOH (pH 7.9), 4.2 mM EDTA, 3 mM DTT, 100 mM KCl, 20% glycerol and 62.5 mM $MgCl_2$. The mixture was incubated at room temperature for 1 h. Binding of the recombinant CrdR or CrdR-phosphorylated protein to DNA was carried out in a 30 μl reaction mixture containing [$\gamma$-$^{32P}$]-labeled DNA, 1 μg poly(dI-dC) (Sigma-Aldrich), 170 mM HEPES–KOH (pH 7.9), 4.2 mM EDTA, 3 mM DTT, 100 mM KCl, 20% glycerol and 62.5 mM $MgCl_2$. Cold competitor chase experiments were performed by adding 1-, 10- and 100-fold excess of a given unlabeled probe respectively into the mixture. After incubation at room temperature for 1 h, samples were then loaded directly onto a non-denaturing 6% polyacrylamide gel. Electrophoresis was carried out for 4 h at 150 V, the gel was then dried and analyzed by autoradiography.

### *Statistical analysis*

Student's *t*-test was used to calculate the statistical significance of experimental differences between two groups. Multiple testing results were corrected using the Bonferroni correction. The difference was considered significant when the *P* value was less than 0.05. Statistical analyses were carried out using the SPSS program (version 11.0; SPSS Inc., Chicago, IL).

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## Competing interests

We have no conflicts of interest to declare for this work.

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