Crosstalk between the HpArsRS two-component system and HpNikR is necessary for maximal activation of urease transcription

Beth M. Carpenter1†, Abby L. West2†, Hanan Gancz1, Stephanie L. Servetas1, Oscar Q. Pich1, Jeremy J. Gilbreath1, Daniel R. Hallinger3, Mark H. Forsyth3, D. Scott Merrell1* and Sarah L. J. Michel2*

1Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD, USA, 2Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland, USA, 3Department of Biology, The College of William and Mary, Williamsburg, VA, USA

*Correspondence:
D. Scott Merrell, Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA
smichel@rx.umaryland.edu
†These authors have contributed equally to this work.

Keywords: nikR, arsRS, pylori, urease, Helicobacter, regulation, pH

*Helicobacter pylori* NikR (HpNikR) is a nickel dependent transcription factor that directly regulates a number of genes in this important gastric pathogen. One key gene that is regulated by HpNikR is *ureA*, which encodes for the urease enzyme. *In vitro* DNA binding studies of HpNikR with the *ureA* promoter (*P*<sub>ureA</sub>) previously identified a recognition site that is required for high affinity protein/DNA binding. As a means to determine the *in vivo* significance of this recognition site and to identify the key DNA sequence determinants required for *ureA* transcription, herein, we have translated these *in vitro* results to analysis directly within *H. pylori*. Using a series of GFP reporter constructs in which the *P*<sub>ureA</sub> DNA target was altered, in combination with mutant *H. pylori* strains deficient in key regulatory proteins, we confirmed the importance of the previously identified HpNikR recognition sequence for HpNikR-dependent *ureA* transcription. Moreover, we identified a second factor, the HpArsRS two-component system that was required for maximum transcription of *ureA*. While HpArsRS is known to regulate *ureA* in response to acid shock, it was previously thought to function independently of HpNikR and to have no role at neutral pH. However, our qPCR analysis of *ureA* expression in wildtype, ΔnikR and ΔarsS single mutants as well as a ΔarsS/ΔnikR double mutant strain background showed reduced basal level expression of *ureA* when *arsS* was absent. Additionally, we determined that both HpNikR and HpArsRS were necessary for maximal expression of *ureA* under nickel, low pH and combined nickel and low pH stresses. *In vitro* studies of HpArsRS-P with the *P*<sub>ureA</sub> DNA target using florescence anisotropy confirmed a direct protein/DNA binding interaction. Together, these data support a model in which HpArsRS and HpNikR cooperatively interact to regulate *ureA* transcription under various environmental conditions. This is the first time that direct “cross-talk” between HpArsRS and HpNikR at neutral pH has been demonstrated.
H. pylori is a microaerophilic, Gram negative pathogen that infects over half of the world’s human population (Marshall and Warren, 1984; Loughlin, 2003). Colonization occurs in the highly acidic gastric mucosal layer as well as in the gastric epithelial surface of the human stomach. Prolonged H. pylori infection is associated with the development of gastritis, peptic ulcer disease, Mucosal-Associated Lymphoid Tissue (MALT) lymphoma and gastric adenocarcinoma (Marshall and Warren, 1984; Cover and Blaser, 1992; Sepulveda and Coelho, 2002; Loughlin, 2003; Kusters et al., 2006). Current therapies for H. pylori infection require antibiotic cocktails of two, three or four drugs that are often not well tolerated due to adverse side effects (Loughlin, 2003; Kusters et al., 2006). If H. pylori infection is left untreated, colonization will persist throughout a person’s lifetime (Marshall and Warren, 1984; Blaser, 1990; Cover and Blaser, 1992; Dunn et al., 1997; Sepulveda and Coelho, 2002; Loughlin, 2003). The propensity for chronic infection by H. pylori, coupled with the large rate of infection, manifests as a significant disease burden worldwide (Kusters et al., 2006). Thus, there is a great need to develop novel targeted anti-Helicobacter agents that are well tolerated (Loughlin, 2003).

While it displays optimal growth at neutral pH, H. pylori is one of a select number of bacteria that can survive under highly acidic conditions; this makes it ideally suited to life in the gastric mucosa. The cytosolic pH of H. pylori ranges from 5.3 to 7.5, and the organism can endure periodic acid shocks of pH < 2 (Wen et al., 2003, 2009; van Vliet et al., 2004a). One key feature that enables H. pylori to survive under such harsh conditions is its ability to convert host urea into ammonia, which serves to buffer the cytoplasmic/periplasmic pH, as well as to neutralize the immediate environment upon excretion from the bacterial cell (Wen et al., 2003; van Vliet et al., 2004a,b; Sachs et al., 2005, 2006; Scott et al., 2007). The majority of ammonia is produced by the nickel dependent urease enzyme, which converts urea to ammonia and bicarbonate. Urease accounts for approximately 10% of the total protein content of H. pylori (van Vliet et al., 2004a; Carter et al., 2009), and expression of the operon of genes that encode urease is known to be subject to environmental regulation.

The nickel dependent metalloregulatory protein, HpNikR, is known to be a key regulator of urease expression (van Vliet et al., 2002; Contreras et al., 2003; Ernst et al., 2005; Dosanjh and Michel, 2006; Maier et al., 2007). HpNikR functions as a tetrameric metalloregulatory protein. Each HpNikR monomer is comprised of two domains, named the N-terminal and C-terminal domains (West et al., 2010, 2012; Benini et al., 2011). To form the functional tetrameric protein, all four C-terminal domains form a central tetrameric metal binding domain (MBD), which serves as the site of nickel coordination. The MBD is then flanked on either side by two N-terminal domains that fold to form two dimeric DNA binding domains (DBD). The DBDs adopt a classic ribbon-helix-helix fold, which is commonly found in DNA binding proteins (Figure 1A) (Chivers and Sauer, 1999, 2000, 2002; Chivers and Tahirrov, 2005; Schreiter and Drennan, 2007; West et al., 2010).

HpNikR requires Ni(II) coordination to the MBD in order to recognize and bind to DNA via the DBD (Abraham et al., 2006; Benanti and Chivers, 2007; Dosanjh et al., 2007; Bahlawane et al., 2010). Crystallographic studies conducted by one of our laboratories have shown that Ni(II) coordinates to two distinct sites at the MBD of HpNikR: two Ni(II) ions coordinate at square planar sites that utilize a 3 His/1Cys ligand set and two Ni(II) ions coordinate to a square pyramidal/octahedral sites that utilize a 3 His/ 2–3 H2O ligand set (Figure 1A) (West et al., 2010, 2012). This heterogeneous nickel coordination controls the overall flexibility of the DBDs to favor high affinity DNA binding (West et al., 2012).

HpNikR is a global regulator of transcription in H. pylori (Contreras et al., 2003; van Vliet et al., 2004a; Ernst et al., 2005; Abraham et al., 2006). In addition to urease, HpNikR activates and represses the expression of at least forty genes in response to nickel availability (van Vliet et al., 2002; Contreras et al., 2003; Ernst et al., 2005, 2006; Abraham et al., 2006; Davis et al., 2006; Dosanjh and Michel, 2006; Benanti and Chivers, 2007, 2011; Dosanjh et al., 2007, 2009; Zambelli et al., 2008; Li and Zamble, 2009; Muller et al., 2011; Romagnoli et al., 2011; Evans and Michel, 2012; Jones et al., 2015). Genes which have been shown to be directly regulated by HpNikR encode for proteins that regulate nickel uptake (nixA, frpB4, fecA3, frpB2, ceuE), storage (hpn, hpn-like) and regulation (nikR), genes involved in iron uptake (exbB) and regulation (fur), genes involved in infection (hsP4), and genes that encode for outer membrane proteins (exbB/tonB) (van Vliet et al., 2002; Contreras et al., 2003; Delany et al., 2005; Ernst et al., 2005, 2006; Abraham et al., 2006; Davis et al., 2006; Benanti and Chivers, 2007; Dosanjh et al., 2007, 2009; Jones et al., 2015). The direct binding of HpNikR to the promoter sequences of these genes has been shown via DNase footprinting, electrophoretic mobility shift assays (EMSA), fluorescence anisotropy (FA) and/or isothermal titration calorimetry (ITC) (Contreras et al., 2003; Delany et al., 2005; Ernst et al., 2005, 2006; Abraham et al., 2006; Davis et al., 2006; Benanti and Chivers, 2007, 2011; Dosanjh et al., 2007, 2009; Zambelli et al., 2008; Li and Zamble, 2009; Muller et al., 2011; Romagnoli et al., 2011; Jones et al., 2015). In addition, Krezel and co-workers recently reported a direct role for the RNA polymerase alpha-subunit C-terminal domain in promoting HpNikR/ureA binding (Borin et al., 2014). From those studies, a general model for HpNikR gene regulation has been formulated. In this model, HpNikR activates transcription by binding upstream of the RNA polymerase binding site, which aids in the recruitment of RNA polymerase (i.e., for ureA). In contrast, HpNikR represses transcription by a simple steric hindrance mechanism in which HpNikR blocks the interaction of RNA polymerase with the promoter by binding at promoter sequences overlapping the −10 or −35 hexameric boxes (e.g., nikR, nixA, fur, frpB4, exbB, fecA3) (van Vliet et al., 2002; Delany et al., 2005; Ernst et al., 2006; Wolfram et al., 2006; Danielli et al., 2009; Dosanjh et al., 2009). In addition to the genes for which direct protein/DNA binding has been established, approximately 30 additional genes have been predicted to be regulated by HpNikR (Contreras et al., 2003; Delany et al., 2005; Ernst et al., 2005; Abraham et al., 2006; Davis et al., 2006; Ernst et al., 2006; Benanti and Chivers, 2007; Dosanjh et al., 2007; Zambelli et al., 2008;
FIGURE 1 | Structure of holo-HpNikR and architecture of the ureA promoter. (A) The structure of holo-HpNikR. Essential areas of the protein are highlighted as follows: the metal binding domain (MBD) in red, the DNA binding domain (DBD) in blue, 4-site (top left) and 5/6-site (bottom right). This image was constructed in pymol (accession number pdb 3LGH). (B) The recognition sites for HpNikR and HpArsR on the H. pylori ureA promoter. Highlighted in gray is the recognition site for HpNikR, while solid and dashed lines above the sequence designate the minimum and maximum protected regions from DNase protection assays for the two distinct binding sites of HpArsR as previously described (Pflock et al., 2005). (C) A cartoon demonstrating the operator overlap at the ureA promoter for HpNikR (pdb 3LGH) and HpArsR within the context of the biological role of HpArsR. The region colored purple represents the overlapping promoter sites.

Dosanjh et al., 2009; Li and Zamble, 2009; Benanti and Chivers, 2011; Muller et al., 2011; Romagnoli et al., 2011). Whether these additional genes are regulated directly or indirectly has yet to be established. These genes include components involved in motility (cheV, flaA, flaB) and stress response (hrcA, grpE, dnaK), as well as outer membrane proteins (omp11, omp31, omp32) (Contreras et al., 2003; Dosanjh et al., 2009).

The promoters for the genes that are directly regulated by HpNikR share common sequences, but are not identical. These DNA targets share a partially palindromic sequence composed of two sets of seven base pairs (half-sites), separated by eleven bases (Delany et al., 2005; Dosanjh et al., 2007, 2009; Stoof et al., 2010; Evans and Michel, 2012). HpNikR binds to a subset of the promoters with high affinity ($K_d \sim \text{nM}$) and a subset of the
promoters with low affinity \((K_d \sim \mu M)\) (Dosanjh et al., 2009). The promoter sequences for which high affinity recognition is measured are from genes that encode for nickel regulated proteins \((ureA, nixA, fbp4, fecA3)\), while the promoter sequences for which low affinity binding has been measured are from genes that encode for other proteins \((fur, nikR, cxbB)\) (Dosanjh et al., 2009). Thus, in vitro \(Hp\text{NikR}\) preferentially recognizes genes that encode for nickel-regulated proteins. The DNA target sequences for which high affinity DNA binding is observed have greater conservation of sequence at the second half-site (Evans and Michel, 2012). Studies using the \(ureA\) promoter in which the DNA sequences have been systematically altered have identified key bases within the second half-site that are essential for high-affinity protein/DNA binding (Delany et al., 2005; Dosanjh et al., 2009; Evans and Michel, 2012). In addition, the intact partial-palindrome is required for high affinity DNA binding by \(Hp\text{NikR}\) in vitro (Dosanjh et al., 2009). When either half of the partial palindrome was modified to all cytosines, the affinity of \(Hp\text{NikR}\) for the \(ureA\) promoter decreased from 8.0 \(\pm\) 1 nM to 1000 \(\pm\) 94 nM for WT/C (CCTCCAAGATATAACACTAATTCAATTTACCCCCCCATATTTAATTAAGTTAATGA) and 4900 \(\pm\) 780 nM for C/WT (CCTCCAAGATATAACACTAATTCAATTTACCCCCCCATATTTAATTAAGTTAATGA) (Dosanjh et al., 2009; West et al., 2012). When both halves of the palindrome were modified, DNA binding was fully abrogated (Dosanjh et al., 2009).

The \(Hp\text{ArsRS}\) two-component system has also been shown to regulate a wide variety of genes in \(H.\ pylori\). These genes include those that encode for proteins involved in acid resistance, (including urease), acetone metabolism (acetone carboxylase), resistance to oxidative stress (thioredoxin reductase), and quorum sensing (Pflock et al., 2005, 2006a,b; Wen et al., 2007; Goodwin et al., 2008; Loh et al., 2010). Within the regulatory pathway, \(Hp\text{ArsS}\) serves as a sensor protein that phosphorylates the \(Hp\text{ArsR}\) response regulator. Phosphorylation occurs via a two-step process: \(Hp\text{ArsS}\) autophosphorylates at histidine 214 and then transfers the phosphate to aspartic acid 52 on \(Hp\text{ArsR}\) to generate \(Hp\text{ArsR}-P\) (Schar et al., 2005; Pflock et al, 2006a; Joseph and Beier, 2007; Gupta et al., 2009; Muller et al., 2009). \(Hp\text{ArsR}\) appears essential for bacterial viability and binds to different promoter elements based on the phosphorylation state. Binding at \(P_{\text{ureA}}\) requires phosphorylation and the binding site of \(Hp\text{ArsR}-P\) overlaps with the binding site recognized by \(Hp\text{NikR}\) (Pflock et al., 2005) (Figures 1B,C). Given that \(Hp\text{ArsR}\) and \(Hp\text{NikR}\) respond to different environmental conditions, acid and nickel, respectively, these two regulators are believed to function independently of one another (Pflock et al., 2005). Most of the studies that have investigated binding of \(Hp\text{NikR}\) to target promoters have been conducted in vitro. However, it is clear that the cytoplasm of the bacterial cell is a much more complex environment than the one modeled in an in vitro experiment. There is one \(in\ vivo\) study of \(Hp\text{NikR}\) mediated nickel response in which quantitative real-time PCR was used to measure \(Hp\text{NikR}\) regulation of target genes (Muller et al., 2011). In this study, a complex relationship between Ni(II) availability and activation or repression of a series of genes was determined, suggesting that \(Hp\text{NikR}\) activity \(in\ vivo\) may be more complex than observed \(in\ vitro\). The goal of the studies described herein was to determine if the DNA sequences required for recognition of \(P_{\text{ureA}}\) by \(Hp\text{NikR}\) are the same \(in\ vitro\) and \(in\ vivo\). To this end, we constructed transcriptional reporters in which the wildtype \(ureA\) promoter or mutant versions of the promoter were fused to the gene encoding GFP. Reporter assays were then used to monitor \(ureA\) promoter activity directly in \(H.\ pylori\). The wildtype \(P_{\text{ureA}}\) sequence exhibited high levels of GFP expression that increased with increasing concentrations of nickel. Unexpectedly, mutation of the half-sites did not prevent basal level urease expression, but negated the Ni(II) dependence of \(ureA\) expression. These results suggested that another factor rescues \(P_{\text{ureA}}\) transcription when the DNA target sequence for \(Hp\text{NikR}\) is compromised. Herein, we show that the \(Hp\text{ArsRS}\) acid response regulatory system, which has previously been shown to regulate \(ureA\) expression in response to low pH, also affects \(ureA\) transcription at neutral pH. This is the first time that \(Hp\text{ArsR}\) has been shown to regulate urease expression in conjunction with \(Hp\text{NikR}\), and we propose that there is a cooperative interaction between these two regulators to control urease expression in \(H. pylori\).

### Materials and Methods

#### Bacterial Strains and Growth

Primer sequences are listed in Table 1 and bacterial strains and plasmids used in this study are listed in Table 2. The \(H.\ pylori\) strains used in this study are all derivatives of G27 (Covacci et al., 1993; Baltrus et al., 2009). \(H.\ pylori\) strains were maintained as frozen stocks at \(-80^\circ\)C in brain heart infusion broth (BD Biosciences) supplemented with 10% fetal bovine serum (Gibco) and 20% glycerol (EMD chemicals, Inc.). Bacterial strains were cultured essentially as previously described (Carpenter et al., 2007). Briefly, strains were grown on horse blood agar (HBA) plates that contained 4% Columbia agar base (Neogen Corporation), 5% defibrinated horse blood (HemoStat Laboratories, Dixon, CA), 0.2% β-cyclodextrin (Sigma), 10 μg/ml vancomycin (Amresco), 5 μg/ml cefsulodin (Sigma), 2.5 U/ml polymyxin B (Sigma), 5 μg/ml trimethoprim (Sigma), and 8 μg/ml amphotericin B (Amresco). Liquid cultures of \(H. pylori\) were grown in brucella broth (Neogen Corporation) supplemented with 10% fetal bovine serum and 10 μg/ml vancomycin at 37°C with shaking at 100 rpm. As noted in Table 2, where appropriate, cultures and plates were supplemented with 8 μg/ml chloramphenicol (Cm) (EMD Chemicals, Inc.) and/or 25 μg/ml kanamycin (Kan) (Gibco). In addition, where detailed below, some HBA plates contained 5% sucrose (Suc) (Sigma). Both liquid and plate cultures were grown under microaerobic conditions (5% O2, 10% CO2, and 85% N2) generated with an Anoxomat gas evacuation and replacement system (Spiral Biotech) in gas evacuation jars. Exponential phase cultures were grown in liquid culture for 20 h, while stationary phase cultures were grown for 44 h.

#### Construction of a \(\Delta\text{nikR::cat}\) \(H. pylori\) Strain

The \(H\text{pnikR}\) mutant strain was constructed using a strategy that resulted in replacement of the \(H\text{pnikR}\) sequence with the \(cat\)
TABLE 1 | List of oligonucleotides used in this study.

| Name                     | Sequence (5′–3′) site | References                        |
|--------------------------|-----------------------|-----------------------------------|
| SOE PRIMERS ureA PROMOTER MUTANTS |                       |                                   |
| Urea_F_PROM_KpnI         | GGTACCCTATGCGAAATTAA | KpnI                              |
| Urea_R_PROM_XbaI         | TCTAGAAGCTGTAGCTCTAT | XbaI                              |
| F1_urea_prom_switch      | AAATACAGTTATCTATCTCT | This study                        |
| R1_urea_prom_switch      | TAATTATTATTATTATCTTT | This study                        |
| F2_urea_prom_switch      | AAATACAGTTATCTATCTCT | This study                        |
| R2_urea_prom_switch      | TAATTATTATTATTATCTTT | This study                        |
| F3_urea_prom_switch      | AAATACAGTTATCTATCTCT | This study                        |
| R3_urea_prom_switch      | TAATTATTATTATTATCTTT | This study                        |
| SOE PRIMERS NikR DELETION |                       |                                   |
| U1338-F                  | CCAAGCAGCTGCAAAAACA   | This study                        |
| U1338-R                  | TCTAGATGCTGAAAGCTCTG | SmaI, XhoI                        |
| D1338-F                  | TGATCTTACAAAGGCTCTG  | SmaI, XhoI                        |
| D1338-R                  | GCTTACGAACTGGCTCTG   | This study                        |
| SOE PRIMERS ArsS DELETION |                       |                                   |
| HP0165_Up_F              | AAGTTGTGATGCGGCACTTC | This study                        |
| HP0165_Up_R              | ATCTCTGGATTCTGATTCCT | This study                        |
| HP0165_Down_F            | TGATCTTACAAAGGCTCTG  | This study                        |
| HP0165_Down_R            | GCTTACGAACTGGCTCTG   | This study                        |
| SOE PRIMERS ureA PROMOTER DELETION |                   |                                   |
| urea_Up_F_prom_KanSacB   | GCGTGTCTTCTGCTACATCC | This study                        |
| urea_Up_R_prom_KanSacB   | CTCTGTGTTTGGCTGGTATTC | SmaI, XhoI                       |
| urea_Down_F_prom_KanSacB | CAAACAAAAAATAGCTGCAAC | SmaI, XhoI                       |
| urea_Down_R_prom_KanSacB | AGTCCTCAAGGAAACATCG  | This study                        |
| urea_Up_r_prom_complementation | GCCAAAAATAGCAAAGGCTG | This study                        |
| urea_Down_r_prom_complementation | GCCAAAAATAGCAAAGGCTG | This study                        |
| qRT-PCR PRIMERS          |                       |                                   |
| urea_qPCR_F              | GAAGAAGCGAGAGCTGTAA  | This study                        |
| urea_qPCR_R              | AGATGATGATGAGATGAGAG | This study                        |
| G27_16SRT_F              | ATGGATGCTTGTGACCTG  | This study                        |
| G27_16SRT_R              | TAAACATCTGCTGCTGCTG  | This study                        |
| Hp ArsR-HIS PROTEIN EXPRESSION AND PURIFICATION |         |                                   |
| arsR Fwd.Bam             | CCCGGATCCATGATAGAGAAG | BamHI                             |
| arsR Rev.HindIII         | CCCAGCGTCTTCTATGTTTA | HindIII                           |
| Hp ArsR-HIS FLOURESENCE ANISOTROPY TARGETS |           |                                   |
| PureA-F                  | CTCTCAATCTCTCAATTCCT | b, c, d, e                       |
| PureA_Wt/C               | CTCTCAATCTCTCAATTCCT | c                                 |
| PureA_Wt/T               | CTCTCAATCTCTCAATTCCT | c                                 |
| PureA/C                  | CTCTCAATCTCTCAATTCCT | c                                 |

Bold print indicates the sequence within the WT ureA promoter that was chosen for mutation.

The underlined sequences correspond to the restriction sites for the enzymes listed in the same row.

gene, which encodes for chloramphenicol resistance. Briefly, a 968 bp fragment containing the region directly upstream of nikR was amplified with primers U1338-F and U1338-R, the latter of which was engineered to contain SmaI and XhoI restriction sites. This fragment was then fused via splicing by overlap extension (SOE) PCR to a 1098 bp fragment containing the region immediately downstream of nikR, which was amplified with primers D1338-F and D1338-R, the former of which contains SmaI and XhoI restriction sites. The SOE product was cloned into pGEM-T Easy to create pDSM923. Sequence analysis showed that only the SmaI site was preserved in the SOE fusion product. The cat gene (Carpenter et al., 2007), which had first been cloned into pGEM-T Easy as pDSM278, was liberated via restriction digestion with EcoRI New England Biolabs...
(NEB), Klenow (NEB) treated, and then ligated with the SmaI (NEB) digested and calf intestine phosphatase (NEB) treated, pDSM923; the resulting nikR deletion construct was named pDSM924. pDSM924 was subsequently transformed into G27, and transformants were selected for on HBA plates containing 8 μg/mL Cm. Resulting colonies were screened for differences in size in the nikR region via PCR with the U1338-F and D1338-R primers. For those colonies showing the expected change in size, the PCR product was next sequenced to verify deletion of nikR. One such colony showing a deletion insertion of nikR was named DSM975.

**Construction of a Markerless ΔarsS H. pylori Strain**

The arsS mutant strain was created using pDSM922, which is a suicide vector that contains a counter selectable marker (generous gift of Liz Marcus and David Scott, UCLA School of Medicine). Briefly, the plasmid contains a kan-sacB counter selectable cassette, previously described by Copass et al. (1997), that is flanked by the 600 and 400 bp immediately upstream and downstream, respectively, of HP0165 (arsS). These regions were originally amplified using strain 43,504 as the template (Marshall and Goodwin, 1987). pDSM922 was naturally transformed into

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**TABLE 2 | List of strains used in this study.**

| Plasmid or Strain | Description | References |
|-------------------|-------------|------------|
| **PLASMIDS**      |             |            |
| pDSM278           | pGEM T-easy::cat | This study |
| pDSM462           | pGEM T-easy::WT ureA promoter | This study |
| pDSM923           | pGEM T-easy::upstream region of nikR fused to downstream region | This study |
| pDSM924           | pGEM T-easy::ΔnikR::cat | This study |
| pDSM922           | pBluescript::ΔarsS::kan-sacB | This study |
| pDSM1070          | pGEM T-easy::upstream region of arsS fused to downstream region | This Study |
| pDSM199           | pTM117::promoterless | Carpenter et al., 2007 |
| pDSM463           | pTM117::WT ureA promoter | This study |
| pDSM697           | pTM117:: ureA promoter mutant C/C | This study |
| pDSM698           | pTM117:: ureA promoter mutant C/WT | This study |
| pDSM796           | pTM117:: ureA promoter mutant WT/C | This study |
| **STRAINS**       |             |            |
| H. pylori strains |             |            |
| G27               |             | Baltrus et al., 2009 |
| DSM215            | G27 (pTM117::promoterless), Kanr | This study |
| DSM464            | G27 (pTM117::WT ureA promoter), Kanr | This study |
| DSM763            | G27 (pTM117:: ureA promoter mutant C/C), Kanr | This study |
| DSM764            | G27 (pTM117:: ureA promoter mutant C/WT), Kanr | This study |
| DSM797            | G27 (pTM117:: ureA promoter mutant WT/C), Kanr | This study |
| DSM975            | G27 ΔnikR::cat, Cm r | This study |
| DSM980            | G27 ΔnikR::cat (pTM117::promoterless), Cm², Kanr | This study |
| DSM976            | G27 ΔnikR::cat (pTM117::WT ureA promoter), Cm², Kanr | This study |
| DSM977            | G27 ΔnikR::cat (pTM117:: ureA promoter mutant C/C), Cm², Kanr | This study |
| DSM978            | G27 ΔnikR::cat (pTM117:: ureA promoter mutant C/WT), Cm², Kanr | This study |
| DSM979            | G27 ΔnikR::cat (pTM117:: ureA promoter mutant WT/C), Cm², Kanr | This study |
| DSM983            | G27 ΔarsS markerless | This study |
| DSM1069           | G27 ΔarsS::kan-sacB, Kanr, Suc² | This study |
| DSM1071           | G27 ΔarsS/nikR::cat, Cm² | This study |
| DSM1398           | G27 ΔarsS markerless (pTM117::promoterless), Kanr | This study |
| DSM1399           | G27 ΔarsS markerless (pTM117::WT ureA promoter), Kanr | This study |
| DSM1400           | G27 ΔarsS markerless (pTM117:: ureA promoter mutant C/C), Kanr | This study |
| DSM1401           | G27 ΔarsS markerless (pTM117:: ureA promoter mutant C/WT), Kanr | This study |
| DSM1402           | G27 ΔarsS markerless (pTM117:: ureA promoter mutant WT/C), Kanr | This study |
| DSM1403           | G27 ΔarsS/nikR::cat (pTM117::promoterless), Cm², Kanr | This study |
| DSM1404           | G27 ΔarsS/nikR::cat (pTM117::WT ureA promoter), Cm², Kanr | This study |
| DSM1405           | G27 ΔarsS/nikR::cat (pTM117:: ureA promoter mutant C/C), Cm², Kanr | This study |
| DSM1406           | G27 ΔarsS/nikR::cat (pTM117:: ureA promoter mutant C/WT), Cm², Kanr | This study |
| DSM1407           | G27 ΔarsS/nikR::cat (pTM117:: ureA promoter mutant WT/C), Cm², Kanr | This study |
G27, and transformants were selected on HBA plates containing 25 µg/ml kanamycin. The resulting transformants were patched on HBA plates containing 5% sucrose to ensure sucrose sensitivity, and deletion insertion of arsS was then confirmed by PCR amplification of the region with HP0165_up_F and HP0165_down_R primers followed by sequencing with the same primers. One such ΔarsS mutant was named DSM1069, which then served as the background strain to create the markerless mutant.

To create the markerless deletion strain, the region immediately upstream and downstream of arsS were fused together via SOE PCR; the upstream region was amplified with the HP0165_up_F and HP0165_up_R primers, the downstream region was amplified with the HP0165_down_F and HP0165_down_R primers, and the products from these reactions were gel purified and fused together via SOE PCR using primers (HP0165_up_F and HP0165_down_R). The resulting product was gel purified, and naturally transformed into DSM1069. Transformants were selected on HBA plates containing 5% sucrose and then patched onto HBA plates containing 25 µg/ml kanamycin to ensure kanamycin sensitivity; double crossover homologous recombination resulted in the replacement of the kan-sacB cassette to create a markerless deletion of the arsS gene. Proper integration was confirmed by PCR and sequencing with the HP0165_up_F and HP0165_down_R primers. The resulting strain was named DSM983.

**Construction of a ΔarsS/nikR::cat H. pylori Strain**

The ΔarsS/nikR::cat mutant strain was created by naturally transforming pDSM924, the nikR::cat deletion construct, into H. pylori strain DSM983. Transformants were then screened on HBA plates supplemented with 8 µg/mL Cm to ensure chloramphenicol resistance. Proper integration was confirmed first by PCR with the U_1338_F and D_1338_R primers. For those colonies showing the expected change in size for the nikR gene, the PCR product was sequenced to verify deletion of nikR. To ensure that the ΔarsS markerless deletion was still intact for this strain, PCR with the HP0165_up_F and HP0165_down_R primers followed by sequencing of the PCR product was performed. The resulting strain was named DSM1071.

**Construction of ureA::GFP Transcriptional Fusions**

Transcriptional fusions to the promoterless gfpmut3 allele carried on pTM117 (Carpenter et al., 2007) were constructed to monitor ureA expression. Briefly, the wildtype ureA promoter was PCR amplified from G27 using the Urea_F_Prom_KpnI and Urea_R_Prom_XbaI primers; these primers incorporate KpnI and XbaI restriction sites, respectively. The resulting PCR fragment was subcloned into pGEM-T Easy (Promega) to create pDSM462. The ureA promoter was then removed via digestion with KpnI (NEB) and XbaI (NEB) and ligated into the appropriately digested pTM117 vector to create pDSM463. The proper fusion was confirmed by PCR amplification with the Urea_F_Prom_KpnI and Urea_R_Prom_XbaI primers and by sequencing with the same primers. pDSM463 was then naturally transformed into G27, DSM975, DSM983 and DSM1071, and transformants were selected on HBA plates containing 25 µg/ml Kan. The individual strains transformed with pDSM463 are described in Table 2.

**GFP Reporter Assays**

The ability of the ureA transcriptional fusions to drive expression of GFP was assessed visually utilizing an Olympus BX61 fluorescent microscope, as well as using flow cytometry as previously described (Carpenter et al., 2007). Briefly, strains containing the ureA promoter fusions were grown overnight in liquid cultures containing varying NiSO₄ concentrations (0, 0.5, 1.0, 10 µM) (Sigma). Following overnight growth, 0.5–1.5 ml of each culture was pelleted and resuspended in 1–2 ml of sterile 1× phosphate-buffered saline depending on the density of the culture. Bacterial clumps and culture debris were subsequently removed by passing the resuspended culture through a 1.2-µm Acrodisc PSF syringe filter ( Pall). Flow cytometry analysis for the ureA fusion constructs was performed using either a Beckman Coulter Epics XL-MCL flow cytometer with a laser setting of 750 V or a BD SLR II flow cytometer. 20,000 events were collected for each assay. WinList 3D, version 6.0 (Verity Software House) and FlowJo, version X (FLOWJO, LLC) were used to analyze the flow cytometry data. These experiments were performed 3–5 times for each strain-reporter plasmid combination.

**RNA Isolation, cDNA Synthesis and RT-PCR**

In addition to the GFP reporter assays, RT-PCR was utilized to measure ureA expression under normal, 10 µM nickel supplemented, low pH (pH 5.0), and combined nickel supplementation and low pH conditions. Bacterial liquid
cultures of DSM1 (WT G27), DSM975 (∆nikR), DSM983 (∆arsS), and DSM1071 (∆arsSmikR) were grown for 18 h, and then each culture was divided into four equal portions. The first portion was utilized for RNA isolation and represents the normal media sample. The remaining portions were pelleted and resuspended in one of the following supplemented liquid culture medias: 10 μM NiSO₄ (Sigma-Aldrich), pH 5.0 (achieved through the addition of HCl to the media), or pH 5.0 with 10 μM NiSO₄. These portions of each culture were then maintained for another 90 min prior to RNA isolation. RNA isolation was performed as previously described (Thompson et al., 2003). The integrity of the RNA was determined through visualization on agarose gels. Next, cDNA was generated as previously described (Gilbreath et al., 2012) using the Quantitect reverse transcriptase kit (Qiagen) according to the manufacturer’s protocol. Control reactions for each sample were also performed without the addition of reverse transcriptase (noRT) enzyme. Control cDNA synthesis, Quantitative real-time PCR (qPCR) for cDNA synthesis, quantitative real-time PCR (qPCR) for ΔarsR as well as the 16S internal reference gene was performed using the primers listed in Table 1. qPCR was conducted similar to the methods used by Gilbreath et al. (2012). Briefly, qPCR reaction mixtures composed of 1x Roto-Gene SYBR green RT-PCR master mix, 3 pmol each of forward and reverse primer pair, and 1 μL of either cDNA or noRT reaction for use as template were combined and brought to a total volume of 20 μL with water. The following 2-step cycling conditions were used: 5 min at 95°C (initial activation) followed by 35 cycles of 5 s at 95°C (denaturation) and 10 s at 50°C (annealing/extension); SYBR green fluorescence was measured at each extension step. Relative gene expression was calculated using the 2^−ΔΔCT method. Post-run melt curve analysis were performed to ensure specificity of amplification. Four biologically independent replicates of these experiments were conducted.

H. pylori HpArsR Cloning, Expression and Purification

PCR primers were designed to amplify the arsR gene from H. pylori J99 with BamH1 and HindIII restriction sites included. The arsR gene was ligated into a pQE3 vector (Qiagen), which includes an N-terminus hexa-histidine coding sequence. The identity of the cloned arsR gene was confirmed by DNA sequencing. For protein expression, arsR-pQE3 was transformed into E. coli M15-pREP4 cells, and grown in LB medium containing ampicillin (100 μg/mL) and kanamycin (20 μg/mL). When the culture reached an A600 of 0.6, protein expression was induced for 4 h with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG). Cells were collected by centrifugation at 12,000 × g for 10 min. All buffers utilized in the HpArsR studies contained 5 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP) to prevent oligomerization of the protein. Cell pellets were resuspended in 20 mM Tris-HCl, 500 mM NaCl, 5 mM TCEP, 1 mM phenylmethylsulfonyl fluoride (PMSF) pH 7.5 and then lyzed by sonication on ice. After sonication, the preparation was centrifuged at 31,000 × g for 20 min. The supernatant (~100 mL) was collected and applied to a 30-mL metal affinity chromatography column (His-Bind, Novagen) charged with Ni(II). The column was washed with a 50 mM imidazole gradient to remove proteins bound non-specifically to the column, and the protein of interest, including the hexa-histidine tag, was eluted with 250 mM imidazole over 45 mL. Five milliliter fractions corresponding to the elution were collected, and the purity of the proteins was visually assessed using 15% SDS-PAGE gels stained with coomassie. Fractions determined to be >95% pure were pooled, and buffer exchanged into 50 mM Tris-HCl, 5 mM MgCl₂, 50 mM KCl, 5 mM TCEP, pH 7.5 and concentrated to a volume of 6 mL using Amicon Ultra-15 centrifugal filters [5-kDa molecular weight cut-off (MWCO) membrane] for use in DNA binding assays.

In vitro Phosphorylation of HpArsR

HpArsR was phosphorylated (HpArsR-P) in vitro by incubating the protein in phosphorylation buffer (50 mM Tris-HCl, 5 mM MgCl₂, 50 mM KCl, 5 mM TCEP, pH 7.5) with 50 mM acetylphosphate for 60 min at 25°C (McCleary and Stock, 1994; Dietz et al., 2002). Phosphorylation yields were not independently measured. Post-phosphorylation, the KCl concentration was adjusted to 100 mM to be consistent with past salt concentrations used in the studies of HpNikR DNA recognition (Dosanjh et al., 2007, 2009; West et al., 2012).

Oligonucleotide Probes

HPLC-purified oligonucleotide probes were purchased from Integrated DNA Technologies (Coralville, IA) and were either labeled with fluorescein (F) or unlabeled as indicated in Table 1. Upon receipt, the probes were resuspended in DNase-free water and quantified. To obtain double stranded probes, each oligonucleotide probe was mixed with a probe with the complementary sequence such that there was a 1.25:1 ratio of unlabeled to labeled oligonucleotide probe in annealing buffer (10 mM Tris, 10 mM NaCl at pH 8.0). The annealing reaction mixtures were placed in a water bath set to a temperature 10°C higher than the melting temperatures (Tms) of the component oligonucleotides. The water bath was then immediately turned off, and the annealing reaction mixtures were allowed to cool overnight. Double-stranded oligonucleotides were quantified and stored at −20°C (Dosanjh et al., 2007, 2009; West et al., 2012).

Fluorescence Anisotropy Monitored Titrations of HpArsR-P and HpArsR with PureA

A fluorescence anisotropy (FA) assay was used to characterize the interaction of HpArsR-P and HpArsR with the ureA promoter and related mutants. Measurements were taken on an ISS PC-1 spectrofluorimeter configured in the L format with an excitation wavelength of 495 nm and an emission wavelength of 519 nm. The band-pass for excitation was 2 nm and 1 nm for emission. 15 nM of PureA in 50 mM Tris-HCl, 5 mM MgCl₂, 100 mM KCl, 5 mM TCEP, pH 7.5 was added to a cuvette that had been pretreated with 5 mM bovine serum albumin (BSA) to prevent adherence of either the protein or DNA to the cuvette walls.

Direct titrations: For direct titrations, either HpArsR-P or HpArsR was titrated into fluorescein labeled ureA (ureA-F) and the change in anisotropy as a function of added protein was measured. The data were analyzed by converting the anisotropy to fraction bound, Fbound (the fraction of HpArsR-P bound
to DNA at a given DNA concentration), using the equation (Lakowicz, 1999):

$$F_{\text{bound}} = \frac{r - r_{\text{free}}}{(r_{\text{bound}} - r)Q + (r - r_{\text{free}})}$$

Where $r_{\text{free}}$ is the anisotropy of the fluorescein-labeled oligonucleotide, $r_{\text{bound}}$ is the anisotropy of the DNA/protein complex at saturation, and $Q$, is the quantum yield ratio of the bound to the free form and is calculated from the fluorescence intensity changes that occur ($Q = I_{\text{bound}}/I_{\text{free}}$). The typical Q for HpArsR-P DNA binding experiments was $Q = 0.87$. $F_{\text{bound}}$ was plotted against the protein concentration and fit using a one-site binding model:

$$P + D \rightleftharpoons PD$$

$$K_d = \frac{[P][D]}{[PD]}$$

$$F_{\text{bound}} = \frac{P_{\text{total}} + D_{\text{total}} + K_d - \sqrt{(P_{\text{total}} + D_{\text{total}} + K_d)^2 - 4P_{\text{total}}D_{\text{total}}}}{2D_{\text{total}}}$$

Where P is the protein concentration and D is the DNA concentration. Each data point from the FA assay represents the average of 31 readings taken over a time course of 100 s. Each titration was carried out in triplicate.

**Competitive Titrations**

For competitive titrations, an unlabeled DNA oligomer was titrated into a solution containing 1500 nM HpArsR-P and 5 nM $P_{\text{ureA}}$-F and the decrease in anisotropy ($r$) as the unlabeled DNA oligomer competed with the labeled oligonucleotide was recorded. The resultant anisotropy values were converted to fraction bound. The competition experiments were performed with HpArsR-P concentrations at levels near saturation to minimize the amount of unlabeled DNA required to complete the titrations. Experiments were performed aerobically as no difference in binding was observed between experiments performed anaerobically and aerobically (data not shown).

Binding isotherms were fit using Mathematica (version 8 Wolfram Research) to a model that involved the mass action equations for the three competing equilibria:

$$P + D_1 \rightleftharpoons PD_1$$

$$P + D \rightleftharpoons PD$$

$$PD_1 + D \rightleftharpoons PD + D_1$$

Where P is the protein (HpArsR-P), $D_1$ is fluorescently labeled DNA, and D represents unlabeled DNA. The value for $K_1$ was determined from the forward titrations and thus used as a known parameter for the fit. Mathematica software was used to combine Equations (1)–(3) and to solve the resulting cubic equation in terms of $PD_1$ using non-linear, least squares analysis. All titrations were carried out in triplicate (Dosanjh et al., 2009).

**Statistical Analysis**

Statistical analysis on qRT-PCR and flow cytometry data was conducted using GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA). A One-Way ANOVA was used to compare basal levels of expression in $\Delta$nikR, $\Delta$arsS, and $\Delta$arsS/niR to WT. A Two-Way ANOVA with Tukey’s correction for multiple comparisons was used to analyze fold differences in expression between WT H. pylori, $\Delta$nikR, $\Delta$arsS, and $\Delta$arsS/niR. Similarly, mean fluorescent intensity (MFI) values obtained from flow cytometry were assessed using a two-way ANOVA with Tukey’s correction for multiple comparisons. A $p < 0.05$ was considered significant.

**Results**

**Regulation of $P_{\text{ureA}}$ by HpNikR**

HpNikR positively regulates the expression of urease by binding to a partially palindromic sequence located on the ureA promoter ($P_{\text{ureA}}$) (Dosanjh et al., 2007, 2009; Evans and Michel, 2012). The DNA sequence that HpNikR recognizes and binds to is AT rich and consists of seven base pairs separated by an eleven base pair linker sequence that is not thought to be directly involved in the protein/DNA recognition event (Figures 1B,C). When either half of the palindrome is mutated to all cytosines, the in vitro affinity of HpNikR for the DNA sequence is diminished by 3 orders of magnitude from a $K_d$ of 8.0 ± 1 nM to a $K_d$ of 1.0 ± 0.09 or 4.9 ± 0.8 μM for the WT/C or C/WT mutants, respectively (Dosanjh et al., 2009; West et al., 2012). When both halves of the palindrome are mutated to all cytosines, DNA binding is fully abrogated (Dosanjh et al., 2009). Thus, both sides of the recognition palindrome appear to be important for HpNikR/DNA binding.

To determine if these in vitro identified DNA sequence requirements are also observed in vivo, a series of Green Fluorescent Protein (GFP) reporter constructs in which the DNA recognition sequence within the ureA promoter was varied, to mirror the modifications that were studied in vitro, were created. These constructs, named WT/WT, WT/C, C/WT, and C/C, were unmodified or modified at each palindrome as indicated in Table 2. To measure the effect of these modifications on the nickel-dependent transcription of urease controlled by endogenous HpNikR, expression of GFP was monitored in H. pylori strain G27 and an isogenic $\Delta$nikR strain grown in the presence of increasing Ni(II) concentrations.

Visual inspection of the stains by fluorescence microscopy showed that fluorescence was only observed in strains carrying constructs in which the ureA promoter was fused to GFP; promoterless controls showed no fluorescence (data not shown). Quantitative analysis of GFP expression was achieved using flow cytometry, as described in the materials and methods. Maximum GFP expression was observed for the WT/WT reporter construct carried in the wildtype H. pylori strain. This was the only reporter construct for which Ni(II) dependence was observed; a statistically significant concentration-dependent increase in GFP expression was observed as the Ni(II) concentration was increased (Figure 2 and Tables 3, 4). In the wildtype H. pylori strain background, GFP expression levels for the WT/C and C/C
reporter constructs were similar to the GFP expression levels observed for the WT/WT reporter construct when the media was not supplemented with Ni(II) (Figure 2 and Table 4). In contrast, basal levels of ureA expression in the C/WT background were significantly lower than WT/WT in the wildtype H. pylori strain ($p < 0.0001$). At high Ni (II) concentrations, the reporter constructs containing mutation of the palindrome half sites (WT/C and C/WT) as well as full site (C/C) exhibited decreased GFP expression levels when compared to the unmodified WT/WT construct (Table 4). En masse, the fact that we observed high basal levels of expression of ureA with some of the mutant constructs was unexpected; our in vitro findings suggest that mutation of both sides of the palindrome completely abrogate HpNikR binding at the ureA promoter (Dosanjh et al., 2009). Given that nickel responsiveness was completely lost for each mutated promoter construct (Figure 2 and Table 4) and because increasing nickel concentrations are known to result in increased HpNikR activity, this finding suggested that another regulator may play a role in ureA expression under the in vivo conditions examined in these experiments.

To specifically examine the contribution of HpNikR to the observed levels of ureA expression, the same four promoter constructs were next examined in an isogenic ΔnikR strain. Interestingly, GFP expression was not entirely abrogated for the WT/WT promoter though expression was not nickel responsive (Figure 2 and Table 4). Once again, this suggests that the GFP expression observed in the wildtype strain is regulated by HpNikR as well as another regulatory factor (Figure 2). Mutating either side of the HpNikR binding sequence in the $P_{\text{ureA}}$ promoter abrogated the Ni (II) response and resulted in varying effects on basal levels of ureA expression. Mutation of the right half of the recognition sequence (WT/C) resulted in a significant decrease in expression ($p = 0.0021$) as compared to the WT/C construct in the wildtype strain. Likewise, disruption of both segments of the palindrome (C/C) resulted in a dramatic decrease in ureA expression in the ΔnikR mutant (Figure 2) that was similar to the decrease observed in the WT/C construct (Table 4). Interestingly, mutating the left half (C/WT) of the recognition sequence did not lead to a significant reduction in ureA expression ($p = 0.4408$) as compared to the C/WT construct in the wildtype strain. When considered together, these findings suggest that an additional regulatory factor is involved in urease transcription. Moreover, given that in vitro studies have clearly shown that HpNikR does not bind to the C/C mutant construct (Dosanjh et al., 2009), the demonstrated decrease in transcription when HpNikR is absent in the strain carrying the C/C mutant construct suggests that HpNikR has a hitherto unidentified indirect role in $P_{\text{ureA}}$ transcription.

**Regulation of $P_{\text{ureA}}$ by HpArsR**

The observation that $P_{\text{ureA}}$ transcription occurs for the WT/C and C/WT GFP reporter constructs in the presence and absence
of $HpNikR$ independent of nickel availability suggested that another factor regulates $ureA$ transcription in these conditions. The two-component system $HpArsRS$ has been shown to mediate pH-responsive urease expression (Pflock et al., 2005, 2006a,b; Wen et al., 2006, 2007). Furthermore, the $HpArsR$ binding site within the $ureA$ promoter partially overlaps the site recognized by $HpNikR$ (Figures 1B,C). To determine if $HpArsR$ was responsible for the observed urease expression, $arsS$ and $arsS/nikR$ deletion strains of $H. pylori$ were constructed and GFP fluorescence was measured for each of the $P_{ureA}$ constructs. Of note, this strategy was chosen since $HpArsR$ was previously shown to be essential and cannot be deleted (Beier and Frank, 2000; McDaniel et al., 2001); however, $HpArsR$ regulates urease in its phosphorylated form ($HpArsR$-P) (Pflock et al., 2005). Therefore, deletion of $HpArsS$ effectively inactivates $HpArsR$ independent regulation of urease since $HpArsS$ is required for $HpArsR$ phosphorylation (Pflock et al., 2005, 2006a).

To dissect the role of $HpArsR$ ($arsS$) in $ureA$ expression, transcription was measured for each of the $P_{ureA}$ constructs. As expected, because $HpNikR$ is present in the $arsS$ strain background, the WT/WT promoter showed Ni(II) dependent GFP expression. However, the relative amounts of GFP expression were significantly lower in this strain background as compared to the wildtype strain (Table 4): for example, a mean GFP fluorescence of 4688 fluorescence units was measured for the $arsS$ strain in 10 mM nickel as compared to 10,282 fluorescence units for wildtype under the same nickel concentrations (Figure 2 and Table 3). This finding suggests that under non-acidic conditions, $HpArsR$-P interacts cooperatively with $HpNikR$ to increase expression of the WT urease promoter. In the $arsS/nikR$ background, though a basal level of GFP expression is observed, this expression is nickel independent (Figure 2).

For the WT/C and C/C mutant promoters, the absence of endogenous $HpArsS$ (and thus, $HpArsR$-P) resulted in lower levels of GFP expression as compared to those observed in the wildtype strains ($p < 0.0001$ for both). In each case, GFP expression was unaffected by nickel concentration. Similar to what was seen in the $nikR$ background, while the WT/C and C/C promoters resulted in decreased expression, the decrease in $ureA$ expression in the CWT background was not significant as compared to the same construct carried in the wildtype strain (Table 4). Furthermore, basal levels of $ureA$ expression in $nikR$ and $arsS$ strains were similar regardless of which $ureA$ promoter was present (Table 4). In the $arsS/nikR$ background, the WT/C and C/WT mutant promoters showed levels of expression similar to those observed in the $nikR$ background. As was observed in the $nikR$ single mutant background, none of these promoter constructs was nickel responsive in the double mutant background (Figure 2 and Table 3). In the $arsS$ and $arsS/nikR$ backgrounds, the C/C promoter fusion produced low levels of GFP expression that were slightly less than the levels observed in the $nikR$ background. Significant GFP expression was only observed for this construct in the wildtype strain. Taken en masse, these findings are consistent with cooperative interaction of $HpNikR$ and $HpArsR$-P at the $ureA$ promoter to achieve maximal regulation of urease. Of note, the role of $HpArsR$-P in this regulation occurred in the absence of acidic pH, which is considered to be the major environmental signal controlling $HpArsR$ activity (Pflock et al., 2005, 2006a,b; Wen et al., 2006, 2007).

To further investigate the cooperative regulation of $ureA$ by $HpNikR$ and $HpArsR$-P and to confirm that the use of plasmid based transcriptional fusions was not artificially affecting our results, we next directly assessed $ureA$ expression directly from the chromosome via qPCR analysis on RNA extracted from WT, $nikR$, $arsS$, and $arsS/nikR$ $H. pylori$ strains. We assessed $ureA$ expression in strains that were exposed to normal growth media as well as to medias that were 1) supplemented with excess nickel (10 mM), 2) adjusted to acidic pH (pH 5.0), or 3) supplemented with excess nickel and adjusted to acidic pH (10 mM + pH 5.0). Comparison of basal levels of $ureA$ expression between the WT and mutant strains under normal growth conditions showed that there was little to no difference in $ureA$ expression between WT and $nikR$ ($p = 0.319$); this was expected based on previous data (Ernst et al., 2005). Conversely, a statistically significant decrease in basal $ureA$ expression was seen in the $arsS$ ($p = 0.009$) and $arsS/nikR$ ($p = 0.0116$) strains (Figure 3A). As with the data obtained with the GFP fusions (Figure 2), these data suggest that $HpArsRS$ is necessary for maximal expression of $ureA$ under normal growth conditions.

Following exposure to 10 mM Ni$^{2+}$, $ureA$ expression was strongly upregulated in WT $H. pylori$ (approximately six-fold). However, the extent of $ureA$ upregulation was significantly decreased in the $nikR$ strain ($p = 0.0159$). Although not significant, a decrease in Ni-dependent upregulation of $ureA$ was also observed in the $arsS$ strain background (three-fold increase compared to six-fold in WT) (Figure 3B). Additionally

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**Table 3** Mean GFP fluorescence in normal and 10 μM NiSO$_4$ supplemented media.

| Strain          | WT/WT | C/WT | WT/C | C/C |
|-----------------|-------|------|------|-----|
|                 | N. media* + Ni(II)† | Ni(II) dependent | N. media* + Ni(II)† | Ni(II) dependent | N. media* + Ni(II)† | Ni(II) dependent | N. media* + Ni(II)† | Ni(II) dependent |
| G27             | 3252 | 10282 | yes  | 3094 | 2671 | no   | 1498 | 1309 | no   | 3890 | 3538 | no   |
| $\Delta nikR$   | 1708 | 1959 | no   | 642  | 643  | no   | 1298 | 1259 | no   | 686  | 686  | no   |
| $\Delta arsS$   | 1399 | 4688 | yes  | 332  | 368  | no   | 782  | 785  | no   | 439  | 410  | no   |
| $arsS/nikR$     | 963  | 1110 | no   | 527  | 618  | no   | 1186 | 1266 | no   | 446  | 465  | no   |

* All values are represented as mean GFP fluorescence. † 10 μM NiSO$_4$ added to media.
in the ΔarsS/nikR double mutant there was no change in ureA expression upon exposure to nickel (Figure 3B). These data suggest that both HpNikR and HpArsRS are required for maximal expression of ureA upon nickel stress. This point is further supported by the fact that the observed difference in nickel dependent ureA expression between ΔnikR and ΔarsS was not statistically significant (p = 0.5529). Similarly, upon exposure to acidic pH, ureA expression was increased approximately eight-fold in the WT strain, six-fold in ΔnikR, five-fold in ΔarsS but only two-fold in the ΔarsS/nikR double mutant (Figure 3C). A statistically significant difference in ureA expression under low pH was only observed when comparing WT and the ΔarsS/nikR double mutant (p = 0.0467). Given that similar levels of ureA expression were observed in both the ΔnikR and ΔarsS strains, this suggests that both regulatory proteins are necessary for maximal expression in the low pH environment; thus, HpNikR appears to play a previously unknown role in the acid responsive regulation of ureA (Figure 3C). Lastly, changes in ureA expression were monitored following simultaneous exposure to excess nickel and low pH. Again, the largest increase in expression was observed in the WT strain background (nine-fold). Although not statistically significant, ureA expression was only moderately increased in the ΔnikR (four-fold) and ΔarsS (three-fold) strains (Figure 3D). Of note, under these conditions,
ΔnikR and ΔarsS were not significantly different from each other (p = 0.8729) or from the ΔarsS/ΔnikR double mutant (p = 0.1722 and p = 0.4847, respectively). However, given that no change in ureA expression was observed for the ΔarsS/ΔnikR double mutant, ureA expression in the ΔarsS/ΔnikR double mutant was significantly different from WT (p = 0.0059) (Figure 3D). En masses, these data support the notion that both HpNikR and HpArsRS are important for regulation of ureA expression under normal conditions as well as in low pH and nickel supplemented environments.

Fluorescence Anisotropy to Measure HpArsR-P/UREA Binding in vitro

Based on previous DNase footprinting studies, HpArsR-P binds to two regions of the P<sub>ureA</sub> promoter (Pflock et al., 2005). These two operators are made up of 41 and 57 base pairs, respectively, and the larger operator sequence includes bases recognized by HpNikR (Figures 1B,C). Based upon our in vivo results that suggest co-regulation of the ureA promoter by HpArsR and HpNikR, the in vitro DNA binding properties of HpArsR-P for the P<sub>ureA</sub> sequences recognized by HpNikR were measured using fluorescence anisotropy (FA), which is an approach that has been successfully used to measure HpNikR/P<sub>ureA</sub> binding (Dosanjh et al., 2007, 2009; Evans and Michel, 2012; West et al., 2012). The FA data for HpArsR-P with P<sub>ureA</sub> WT/WT, WT/C, C/WT, and C/C showed comparable binding isotherms (Figure 4), with K<sub>d</sub>s of 17 ± 2.0, 24 ± 0.5, 20 ± 0.6, and 23 ± 0.7 nM respectively. These findings show that HpArsR-P can bind to the same P<sub>ureA</sub> promoter sequence as HpNikR <em>in vitro</em>, and it can also bind to sequences in which the palindrome recognized by HpNikR is altered. HpArsR did not exhibit any DNA binding (data not shown).

Discussion

HpNikR regulates urease production by binding to and activating transcription of P<sub>ureA</sub> (van Vliet et al., 2002, 2004a; Abraham et al., 2006; Dosanjh et al., 2007). The details of the protein/DNA
binding interaction have been systematically studied in vitro and a key partially palindromic recognition sequence has been identified (van Vliet et al., 2002, 2004a; Abraham et al., 2006; Dosanjh et al., 2007). When either half-site of the recognition palindrome is modified to all cytosines, the affinity of HpNikR for the DNA is significantly reduced, while mutation of both half sites to all cytosines abrogates binding (Dosanjh et al., 2009). Those findings led us to conclude that these half-sites were the recognition elements required for HpNikR-mediated activation of ureA expression. Herein, we present studies designed to translate our previous in vitro findings to the in vivo conditions present within the H. pylori cell. These in vivo findings revealed that additional factors are involved in the regulation of urease. Specifically, maximal activation of ureA transcription required the HpArsRS two component system in addition to HpNikR.

The initial suggestion that a second factor may regulate ureA transcription came from GFP reporter assays of the PureA WT/C and C/WT half-site mutants. Some level of nickel-independent GFP expression was still observed for all PureA constructs; though, the levels of expression were only nickel dependent in WT/WT (Figure 2 and Table 4). Interestingly, while PureA transcription was significantly decreased for the C/WT half site mutation, WT/C and C/C promoter mutations yielded similar levels of GFP expression to WT/WT in the wildtype H. pylori strain background (Figure 2 and Table 4). These high levels of expression suggested that a second factor was involved in regulating ureA transcription in vivo. This hypothesis was further supported by the levels of ureA expression observed in the ΔnikR strain background. For the PureA half-site mutant, C/WT, the level of ureA transcription was similar to that observed in the wildtype strain while for the WT/C mutant, the level of ureA transcription was approximately five-times less than in the wildtype strain. Additionally, expression of the C/C mutant construct was greatly diminished (approximately six times), indicating that the high level of expression seen for this construct in the wildtype strain background was HpNikR dependent. However, in vitro protein/DNA binding data has clearly demonstrated that HpNikR is unable to physically interact with this altered C/C DNA target. Thus, the role that HpNikR plays in regulating the C/C target appears to be indirect.

Given these data, we sought to identify the other factor required for proper regulation of ureA transcription at neutral pH (pH 7.5). The HpArsRS two-component system is also known to control urease expression in H. pylori. However, this system, which is responsive to pH, is thought to function primarily under acidic conditions (Marcus et al., 2012). The data presented in this study, demonstrate that HpArsRS functions with HpNikR to properly regulate urease expression at neutral pH. Within the HpArsRS two-component system, HpArsS functions as the histidine kinase, and HpArsR functions as the cognate response regulator. Upon sensing acidic pH, HpArsS phosphorylates HpArsR, which results in activation of the regulator (Pflock et al., 2006a; Joseph and Beier, 2007; Gupta et al., 2009; Muller et al., 2009). Interestingly, HpArsS is not essential for H. pylori survival, but HpArsR is essential (Schar et al., 2005). This suggests that the non-phosphorylated form of Hp ArsRS regulates some essential component within the H. pylori cell. HpArsR has been shown to bind some target promoters in its non-phosphorylated form, while it only binds other promoters in its phosphorylated form (Wen et al., 2006). The ureA promoter is known to be bound and regulated by the phosphorylated form of HpArsR (Pflock et al., 2005). Thus, deletion of arsS from G27 results in an inactive HpArsR response regulator in terms of regulation of the ureA promoter. Using this strain we found that although expression of the WT/WT fusion was still nickel-dependent, the expression levels were significantly lower at all Ni (II) concentrations tested than in the G27 background (Figure 2 and Table 4), suggesting a role for HpArsR-P in maximal expression from this promoter. Similarly, the WT/C half-site mutant, showed considerable reduction in PureA::gfp expression in the absence arsS; once again indicating a role for HpArsR-P. Interestingly the C/WT half-site mutant appeared to lock the level of expression of ureA at a basal level regardless of the strain background examined. Conversely, the C/C mutation affected ureA transcription in all of the tested mutant strain backgrounds, suggesting concomitant regulation by both HpNikR and HpArsRS. Additionally, qPCR analysis revealed that basal levels of ureA expression were significantly reduced in the absence of arsS (p = 0.009) but not nikR (p = 0.319) (Figure 3A). Further support for this idea comes from the analysis of ureA expression following exposure to nickel, low pH or both stressors combined. Regardless of the stressor, the highest levels of ureA expression were observed in the WT strain background, with each single deletion showing only moderate levels of ureA and the least amount of expression occurring in the double ΔarsS/ΔnikR strain (Figure 3).
Taken together, these data support a model of $ureA$ transcription that involves “cross-talk” between $Hp$NikR and $Hp$ArsRS to maximize induction of urease even under neutral conditions. Prior to this study, these two regulators were believed to function independently as $Hp$NikR is responsive to nickel levels and $Hp$ArsRS is responsive to acidic shock (Pflock et al., 2005). Therefore, the observation that $ureA$ transcription is 2–2.5 times lower in the $\Delta$arsS background is particularly compelling since, under these conditions, $Hp$NikR is present and functional. Surprisingly, the data presented here also indicate a role for $Hp$NikR in the response to acid stress; previous reports suggested that the acid-induced increase in $ureA$ expression was independent of NikR (Pflock et al., 2005). Using qualitative primer extension, Pflock et al. showed that there were similar increases in $ureA$ expression upon exposure to pH 5.0 in strains with and without nikR (Pflock et al., 2005). However, in this work, we observed that the increase in $ureA$ expression was less than that of wildtype in the $\Delta$nikR mutant strain. The differences in our data and the previously published work could be due to differences in assay sensitivities (primer extension vs. qPCR) as well as differences in exposure to stress conditions. In the previous work, the bacteria were exposed to low pH for 60 min as compared to our 90 min exposure. Perhaps, a longer observation period following exposure to low pH allows for better measurement of the transcriptional changes in response to the stressor. Our data fully support the model that both $Hp$NikR and $Hp$ArsRS are necessary for maximal levels of $ureA$ expression in response to low pH regardless of nickel concentration.

Although, the discovery of cross-talk between $Hp$NikR and $Hp$ArsR is unexpected, crossstalk, involving $Hp$NikR, $Hp$ArsR, or other $H. pylori$ regulatory proteins in general, is not unique. For example, $Hp$NikR and $Hp$Fur co-regulate fur transcription (Delany et al., 2001, 2005) and $Hp$ArsS and $Hp$FlgS work in concert to recruit and activate urease (Marcus et al., 2012). In addition, regulatory crossstalk among transcription factors has been observed within $E. coli$. Specifically, the transcription factors MarA and Rob of $E. coli$, which are involved in the response to chemical stressors consequently enabling antibiotic resistance, are co-regulated through transcriptional cross-talk with each other (Miller et al., 1994; Martin et al., 1996; Martin and Rosner, 1997; Michan et al., 2002; Schneider and Levy, 2006; McMurry and Levy, 2010; Warner and Levy, 2010).

Though the in vivo transcriptional assays revealed a role of $Hp$ArsRS in regulation of $ureA$ transcription, they did not demonstrate whether this role was a direct protein/DNA binding interaction, or an indirect effect via another, yet to be identified, factor. As previous studies have shown a requirement of phosphorylation for DNA binding at the $ureA$ promoter (Pflock et al., 2005), a direct effect would likely involve $Hp$ArsR-P (Dietz et al., 2002). Using FA, we examined whether $Hp$ArsR-P directly bound to the four DNA targets (WT/WT, WT/C, C/WT, and C/C) by titrating $Hp$ArsR-P with fluorescently tagged DNA targets. A change in FA, indicative of binding, was observed for all four DNA targets when $Hp$ArsR-P was titrated. No DNA binding was observed when the control non-phosphorylated $Hp$ArsR was studied. These data indicate that $Hp$ArsRS directly regulates $ureA$ by binding to a 48-mer promoter sequence.

Together, the in vitro and in vivo data obtained for $Hp$ArsR-P provide valuable insight into the role of $Hp$ArsR from a biophysical and a biological perspective. The in vitro results that we obtained for $Hp$ArsR-P binding to $ureA$ (and related mutants) teach us that $Hp$ArsR-P binds to the $ureA$ promoter in a very different way than $Hp$NikR. $Hp$NikR requires a specific sequence (the pseudo-palindrome) for high affinity DNA binding (Dosanjh et al., 2009; Evans and Michel, 2012). In contrast, $Hp$ArsR-P does not require this specific pseudo-palindromic sequence for binding (i.e., there is equivalent binding when the pseudo-palindromic sequence is modified).

Two factors are often important when proteins bind to DNA: sequence and shape (Rohs et al., 2010; Parker and Tullius, 2011). For $Hp$NikR evidence indicates that sequence - the pseudo-palindromic sequence found within the $ureA$ promoter - is important for binding; when the sequence is modified, binding is abrogated (Dosanjh et al., 2009; Evans and Michel, 2012). In contrast, for $Hp$ArsR-P the data indicate that the pseudo-palindromic sequence is not important; when the sequence is modified, binding is not affected (vide supra). This may mean that $Hp$ArsR-P/$ureA$ binding is driven by shape (overall conformation/structure of the DNA), rather than sequence, or that the sequence recognized by $Hp$ArsR-P contains additional oligonucleotides than the sequence recognized by $Hp$NikR.

The in vivo data, for which the entire promoter is present (rather than the short stretch of DNA utilized in the in vitro binding studies), revealed that when the $ureA$ sequence was modified to the C/WT sequence and $Hp$ArsR-P driven expression was measured, the expression decreased. In contrast, $Hp$ArsR-P driven expression for all of the other $ureA$ sequences was not dramatically affected. This finding indicates that there must be another factor (or factors), beyond the direct recognition of $Hp$ArsR-P with the 48-mer $ureA$ target sequences in vivo, that is important for $Hp$ArsR-P regulation of $ureA$ in vivo.

The combination of in vitro and in vivo data presented here allows us to learn both about (i) the very specific binding event between $Hp$ArsR-P and $ureA$ (48 mer), which informs on the fundamental biophysical basis of binding, and (ii) the overall regulation by $Hp$ArsR-P at the cellular level, which informs on the biological mechanism. We initiated these studies to determine how the in vitro $Hp$NikR/$ureA$ binding that we had previously measured translated in an in vivo setting. The data indicate that the in vivo regulation is more complex than the in vitro protein/DNA binding. Furthermore, we identified $Hp$ArsR-P as another key player in this regulation. By then looking at $Hp$ArsR-P both in vivo and in vitro, we can draw the same conclusion regarding $Hp$ArsR-P function: that its role in regulation in vivo is more complex than its in vitro protein/DNA binding.

Prior to the work presented here, $Hp$NikR and $Hp$ArsR were thought to function as independent regulators of transcription, with $Hp$NikR involved in regulation of urease in response to intracellular nickel availability and $Hp$ArsRS involved in urease regulation in response to intracellular acid shock (Pflock et al., 2005). Strikingly, the studies presented here reveal that both $Hp$NikR and $Hp$ArsR-P are necessary for maximum Ni(II) dependent regulation of urease in vivo as well as the maximal
response to acid shock. The two proteins are not independent regulators but, instead, work cooperatively to regulate ureA transcription. This is the first time that “cross-talk” between HpNiR and HpArsRS has been demonstrated, and further studies will be required to tease out the interactions that promote this cooperative effect.

Author Contributions

Conceived and designed the experiments: AW, BC, DM, SM. Strain Construction: AW, BC, HG, JG. Performed the experiments: AW, HG, BC, DH, OP, SS. Contributed reagents/materials/analysis tools: MF, DH. Wrote the paper: AW, BC, DM, SM.

Acknowledgments

SLJM thanks NSF CHE1306208 for support of these studies. We are grateful to the members of the Merrell lab for their invaluable microbiology expertise. Prof. Edwin Pozharski (UMB) for assistance with PyMol, Prof. David Scott and Liz Marcus (UCLA School of Medicine) for the AsaS::kan-sacB deletion strain of H. pylori and Mohsin Khan (UMB) for editing Figure 1A. DSM thanks Prof. Andrew Snow and Sasha Larsen for their assistance with flow cytometry technique and analysis. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the NIH, the NSF, the Department of Defense, or the Uniformed Services University of the Health Sciences.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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