Growth Phase and Metal-Dependent Transcriptional Regulation of the 
feceA Genes in Helicobacter pylori

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Balancing metal uptake is essential for maintaining a proper intracellular metal concentration. Here, we report the transcriptional control exerted by the two metal-responsive regulators of Helicobacter pylori, Fur (iron-dependent ferric uptake regulator) and NikR (nickel-responsive regulator), on the three copies of the fecA genes present in this species. By monitoring the patterns of transcription throughout growth and in response to nickel, iron, and a metal chelator, we found that the expression of the three fecA genes is temporally regulated, responds to metals in different ways, and is selectively controlled by either one of the two regulators. fecA1 is expressed at a constant level throughout growth, and its expression is iron sensitive; the expression of fecA2 is mainly off, with minor expression coming up in late exponential phase. In contrast, the expression of fecA3 is maximal in early exponential phase, gradually decreases with time, and is repressed by nickel. The direct roles of Fur and NikR were studied both in vitro, by mapping the binding sites of each regulator on the promoter regions via DNase I footprinting analysis, and in vivo, by using primer extension analyses of the fecA transcripts in fur and nikR deletion strains. Overall, the results show that the expression of each fecA gene is finely tuned in response to metal availability, as well as during the bacterial growth phase, suggesting specific and dedicated functions for the three distinct FecA homologues.

Metals are important for many biological functions as co-factors of essential metalloproteins and enzymes. At the same time, metal overload may be lethal. Therefore, several mechanisms control the intracellular metal concentration so that uptake, availability, and storage are tuned to the physiological needs and possible toxic effects are limited (16, 17). In the human pathogen Helicobacter pylori, the causative agent of several gastric pathologies (2), metal homeostasis is maintained principally through the transcriptional regulation of genes coding for metal uptake and storage proteins (5, 6, 13, 25). The ability to acquire iron as well as nickel plays a central role in the successful colonization of the gastric niche and has been shown to be a prerequisite for infection in a mouse model (27, 31). To date, two pleiotropic regulators are known to be involved in the concerted control of iron- and nickel-responsive gene expression: a member of the ferric uptake regulator family (Fur) (9) and a homologue of the NikR protein (5, 33). Several open reading frames, annotated as putative metal transport systems, appear to be under the control of either one or both regulators, as revealed by analyses of transcript abundance in wild-type and mutant strains cultivated in medium enriched with or depleted of metal (12, 14, 15, 30). However, few such metal transport systems have been studied in some detail.

Herein, we report the study of the transcriptional regulation of three fecA genes of H. pylori encoding putative outer membrane proteins that in Escherichia coli are involved in ferric dicitrate transport (19). In gram-negative bacteria, the FecA system appears to be regulated at multiple levels: by metal-dependent repressors such as Fur or by iron starvation sigma factors (fecR-fecl system) whose expression is in turn regulated by a feedback loop involving the N-terminal domain of TonB (22). The genome of H. pylori (1, 3, 29) includes three genes annotated as fecA homologues. Two, namely, fecA1 and fecA2, have been shown previously via chromatin immunoprecipitation-on-chip enrichment (6) and/or transcriptional studies (12, 30) to be part of the Fur regulon. The regulation of fecA3 is less straightforward. Although it appears to belong to the Fur regulon, its transcription appears to be affected by fur mutation in the advanced growth phase only, while intracellular Fur protein concentrations increase in the wild-type strain (6). Others have reported it to be iron regulated but in a Fur-independent fashion (12). The fecA3 gene was also proposed to be indirectly repressed by NikR (5). In contrast, Ernst and coworkers (15) showed that the transcription of the fecA3 gene is repressed by NikR only in the presence of nickel and that in vitro a recombinant NikR protein appears to bind a specific region of the fecA3 promoter (P fecA3) in a nickel-dependent manner, substantiating the hypothesis of its Ni2+-dependent regulation (15).

To gain a better understanding of the regulation of the fecA genes in H. pylori, we studied their transcriptional regulation during growth and after enrichment with metal or chelation and analyzed the binding of Fur and NikR to their promoter regions. We demonstrated that the three fecA genes are differentially transcribed during the growth phase, that Fur directly controls the
iron-dependent transcriptional responses of *fecA1* and *fecA2*, and that NikR brings about the metal-dependent regulation of *fecA3* by binding cooperatively to two adjacent operator sites.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The *H. pylori* strains used in this study are listed in Table 1. All strains were recovered from -80°C glycerol stocks and grown on Columbia agar plates containing 5% horse blood (Oxoid), 0.2% cycloextrin, and Dent's or Skirrow's antibiotic supplement or on grown on Columbia agar plates containing 5% horse blood (Oxoid), 0.2% cy-

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description*</th>
<th>Source or reference</th>
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<tr>
<td><strong>E. coli</strong> strains</td>
<td></td>
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<tr>
<td>DH5α</td>
<td><em>supE44 ΔlacU169 (Δ880 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1B</em></td>
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<tr>
<td>BL21(DE3)</td>
<td><em>hsdS gal (xets857 ind1 Sam7 nin5 lacU5ΔT7 gene 1)</em></td>
<td>28</td>
</tr>
<tr>
<td><strong>H. pylori</strong> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G27</td>
<td>Clinical isolate; wild type</td>
<td></td>
</tr>
<tr>
<td>G27 <em>fur</em> (∆fur::Km)</td>
<td>G27 derivative with <em>fur</em> gene mutation; Km'</td>
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<tr>
<td>G27 <em>nikR</em> (∆nikR::Km)</td>
<td>G27 derivative with <em>nikR</em> gene mutation; Km'</td>
<td>23</td>
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<tr>
<td>G27 <em>fur nikR</em> (∆fur::Km ∆nikR::cat)</td>
<td>G27 derivative with <em>fur</em> and <em>nikR</em> gene mutations; Km' Cpr'</td>
<td>23</td>
</tr>
<tr>
<td>G27 vac::P<em>fecA3</em>lacZ</td>
<td>G27 derivative containing the wild-type <em>P</em>&lt;sup&gt;fecA3&lt;/sup&gt; promoter-lacZ fusion in the vacA locus; Km'</td>
<td>This study</td>
</tr>
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<td>G27 vac::OP-I mutant <em>P</em>&lt;sup&gt;fecA3&lt;/sup&gt;lacZ</td>
<td>G27 derivative containing the OP-I mutant <em>P</em>&lt;sup&gt;fecA3&lt;/sup&gt; promoter-lacZ fusion in the vacA locus; Km'</td>
<td>This study</td>
</tr>
<tr>
<td>G27 vac::OP-II mutant <em>P</em>&lt;sup&gt;fecA3&lt;/sup&gt;lacZ</td>
<td>G27 derivative containing the OP-II mutant <em>P</em>&lt;sup&gt;fecA3&lt;/sup&gt; promoter-lacZ fusion in the vacA locus; Km'</td>
<td>This study</td>
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**Plasmids**

- pGEM-T Easy General cloning vector; Amp' | Promega |
- pGEMT-*fecA1* pGEM-T Easy derivative containing 402 bp of *fecA1* promoter region, amplified by PCR with primers A1F and A1R | This study |
- pGEMT-*fecA2* pGEM-T Easy derivative containing 380 bp of *fecA2* promoter region, amplified by PCR with primers A2F and A2R | This study |
- pGEMT-*fecA3* pGEM-T Easy derivative containing 265 bp of *fecA3* promoter region, amplified by PCR with primers A3F new and A3R new | This study |
- pBlueScript (pBS) General cloning vector; Amp' | Stratagene |
- pBS-*P*<sup>fecA3</sup>Rx1 pBlueScript derivative containing a 130-bp fragment of *fecA3* promoter region, amplified by PCR with primers A3.1-A3.4 | This study |
- pBS-*P*<sup>fecA3</sup>Rx2 pBlueScript derivative containing a 60-bp fragment of *fecA3* promoter region, amplified by PCR with primers A3.1-A3.2 | This study |
- pBS-*P*<sup>fecA3</sup>Rx3 pBlueScript derivative containing a 60-bp fragment of *fecA3* promoter region, amplified by PCR with primers A3.3-A3.4 | This study |
- pBS-PA3MuOpI pBlueScript derivative containing the 265-bp *fecA3* promoter region with a SmaI site introduced into OP-I by PCR with the primers A3Fnew-A3.9 and A3Rnew-A3.8 | This study |
- pBS-PA3MuOpII pBlueScript derivative containing the 265-bp *fecA3* promoter region with a SmaI site introduced into OP-II by PCR with the primers A3Fnew-A3.7 and A3Rnew-A3.6 | This study |
- pBS-*P*<sup>fecA3</sup>Rx4 pBlueScript derivative containing a 60-bp fragment of *fecA3* promoter region, amplified by PCR with primers A3.3-A3.4 | This study |
- pVacc:*Km* pGEMZ derivative containing a kanamycin cassette | 10 |
- pVacc:*P*<sup>fecA1</sup>lacZ pVacc:*Km derivative containing the transcriptional fusion *P*<sup>fecA1</sup>-lacZ; Km' | This study |
- pVacc:*P*<sup>fecA2</sup>lacZ pVacc:*Km derivative containing the transcriptional fusion OP-I mutant *P*<sup>fecA2</sup>-lacZ; Km' | This study |
- pVacc:*P*<sup>fecA3</sup>lacZ pVacc:*Km derivative containing the transcriptional fusion OP-II mutant *P*<sup>fecA3</sup>-lacZ; Km' | This study |
- pET15b IPTG-inducible vector overexpressing N-terminally His<sub>6</sub>-tagged recombinant protein; Amp' | Novagen |
- pET15b-nikR pET15b derivative containing the nikR coding sequence cloned in frame within the NdeI/BamHI restriction sites | 7 |
- pET15b-fur pET15b derivative containing the fur coding sequence cloned in frame within the NdeI/XhoI restriction sites | 8 |

* IPTG, isopropyl-β-D-thiogalactopyranoside.
Table 2. Primers used for PCR amplification of the promoter regions and for primer extension reactions

<table>
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<tr>
<th>Oligonucleotide name</th>
<th>Sequence (5′→3′)*</th>
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<tbody>
<tr>
<td>A1F</td>
<td>CTTGAGGCTTCTGAAGGAGGAAT</td>
</tr>
<tr>
<td>A1R</td>
<td>GGAAATTGTTGAAGGAAT</td>
</tr>
<tr>
<td>A2F</td>
<td>TTTAGGATCTTCGATGTTGTCATC</td>
</tr>
<tr>
<td>A2R</td>
<td>TATGGCATGTCGAGGTGTCG</td>
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<tr>
<td>A3Fnew</td>
<td>ATTTGCGGATCTGAAGGAGGTA</td>
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<tr>
<td>A3Rnew</td>
<td>TAAGATGTTCTCAGAAGATCAG</td>
</tr>
<tr>
<td>A3.1</td>
<td>CCGGGTCGCCACACCCCTT</td>
</tr>
<tr>
<td>A3.2</td>
<td>CGGAGCTTCCATGATAAAAT</td>
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<tr>
<td>A3.3</td>
<td>GGAAATTGTTGAAGGAAT</td>
</tr>
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<td>A3.4</td>
<td>CGGAGCTTCCAAAAGATTTC</td>
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<td>A3.6</td>
<td>TCCCCCCGGTTTAAAGGTT</td>
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<td>A3.9</td>
<td>TCCCCCCGGTAAATGAAAAAA</td>
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<td>PefecA1</td>
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<td>PefecA2</td>
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<td>A3Z1</td>
<td>TAGCTAGATCCTGATAT</td>
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<tr>
<td>5′kat</td>
<td>CACAGCTTGATATACACAT</td>
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*Bases in italics correspond to unwanted extension sites.

$P_{fecA1}$, $P_{fecA2}$ and $P_{fecA3}$ are DNA templates used to quantify the protein fractions with bovine serum albumin as the standard.

**Results**

Genome and promoter organization of the genes encoding the outer membrane FecA homologue proteins in *H. pylori*. In the sequenced *H. pylori* genomes, three homologues of the E. coli fecA gene, which encodes an iron dicitrate transporter belonging to the family of TonB-dependent transporters, are annotated (1, 3, 29). In strain G27, the fecA1 gene maps 248 bp downstream of the iron transporter gene fecB, the fecA2 gene maps 194 bp downstream of a hypothetical polycistronic group of genes involved in fatty acid and phospholipid metabolism, and the fecA3 gene maps 360 bp downstream of the rocF gene, encoding an arginase.

To map the fecA transcriptional initiation sites, primer extension experiments were performed with total RNAs extracted from *H. pylori* G27 cultures, and results are shown in Fig. 1. The fecA1 start site showed as two bands mapping 33 and 36 bp upstream of the annotated start codon (Fig. 1A). The transcriptional start site of fecA2 mapped 33 bp upstream of the translational start site (Fig. 1B), while the fecA3 transcriptional start site mapped 112 bp upstream of the annotated GTG start codon (Fig. 1C). All upstream sequences showed −10 regions with homology to the canonical TATAAT and −35 regions (Fig. 1D). Notably, the −10 sequence of fecA1 is preceded by a TG motif (20), indicative of a −10 extended promoter. Based on the presence of conserved sequence elements upstream of the transcriptional start sites, we conclude that the fecA genes
of \textit{H. pylori} are transcribed by the vegetative \textit{n}^{\text{90}}-containing RNA polymerase.

**Growth phase-dependent activity of the \textit{fecA} promoters.** To begin the functional characterization of the \textit{fecA} genes, we monitored their transcription during bacterial growth. Aliquots of bacterial cultures were sampled at different time points of growth, and the extracted RNAs were assayed by quantitative primer extension analyses, with the results shown in Fig. 2.

Transcription from the \textit{P}_{\text{fecA1}} promoter showed no significant variations in the amount of mRNA throughout growth (Fig. 2A). In contrast, transcription from the \textit{P}_{\text{fecA2}} promoter was detectable only at the end of the time course experiment (when the cultures had an OD of 1.0) (Fig. 2B), while transcription from the \textit{P}_{\text{fecA3}} promoter decreased over time (Fig. 2C). We conclude that while transcription from the \textit{P}_{\text{fecA1}} promoter remains unchanged during growth, transcription from the \textit{P}_{\text{fecA2}} and \textit{P}_{\text{fecA3}} promoters appears to be inversely regulated, with the maximum expression of \textit{fecA2} and \textit{fecA3} in late and early log phase, respectively. It is likely that this temporal transcriptional regulation reflects distinct roles for FecA2 and FecA3 during growth.

\textit{P}_{\text{fecA1}} and \textit{P}_{\text{fecA2}} promoters are repressed by Fur. To study the transcriptional regulation of the \textit{fecA} genes, primer extension analyses of RNAs extracted from the wild-type, \textit{n}^{\text{90}}\textit{ikR}, \textit{n}^{\text{90}}\textit{ur}, and double mutant \textit{dn}^{\text{90}}\textit{ikR} \textit{dn}^{\text{90}}\textit{ur} strains, grown to mid-log phase and treated in parallel for 15 min with iron, nickel, or iron chelator, were carried out. Representative results are shown in Fig. 3.

As expected, transcription from the \textit{P}_{\text{fecA1}} promoter was detected under conditions of no treatment, whereas no transcription from \textit{P}_{\text{fecA2}} was detected (Fig. 3A and B, lanes 1). \textit{P}_{\text{fecA1}} was repressed after the addition of 1 mM FeSO\textsubscript{4} to the medium (Fig. 3A, lane 2) and slightly repressed by 1 mM NiSO\textsubscript{4} (Fig. 3A, lane 3), and both \textit{P}_{\text{fecA1}} and \textit{P}_{\text{fecA2}} were in-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Identification of the \textit{H. pylori} \textit{fecA} promoter regions. (A to C) The 5' ends of the \textit{fecA1} (A), \textit{fecA2} (B), and \textit{fecA3} (C) transcripts were identified by primer extension analyses. A schematic representation of the \textit{fecA} gene and its promoter in \textit{H. pylori} strain G27 is shown at the top of each panel. \textit{fecA1}, \textit{fecA2}, and \textit{fecA3} are preceded by intergenic regions of 248, 194, and 360 bp, respectively. Fifteen micrograms of total RNA extracted from cultures of the wild-type (wt) strain were hybridized with specific primers (Table 2) and elongated with reverse transcriptase. The elongated primer bands mapping the 5' ends of the \textit{fecA} transcripts are indicated. Sequence reactions (A, T, G, and C) were performed with the same primers and with plasmids pGEMT-\textit{P}_{\text{fecA1}} (A), pGEMT-\textit{P}_{\text{fecA2}} (B), and pGEMT-\textit{P}_{\text{fecA3}} (C) as templates. (D) Summary of relevant features within the nucleotide sequences of the \textit{fecA} promoter regions. The mapped transcriptional start site (+1) of each promoter is represented by a bent arrow; hexamers corresponding to the putative −10 and −35 regions are boxed and shown in boldface; gray boxes indicate the regions protected by Fur in DNase I footprinting assays; black boxes indicate NikR binding regions; arrowheads identify nucleotides hypersensitive to DNase I digestion. The bases in the start codons for the FecA1, FecA2, and FecA3 proteins are indicated in bold. The three \textit{H. pylori} \textit{fecA} genes encode polypeptides with less than 30% amino acid sequence identity and approximately 50% sequence similarity to \textit{E. coli} FecA.
}\end{figure}
duced by the addition of the iron chelator (Fig. 3A and B, lanes 4). Transcription levels for both promoters in the \( H_9004 \) fur mutant were high under all conditions tested (Fig. 3A and B, lanes 5 to 8), suggesting that \( P_{fecA1} \) and \( P_{fecA2} \) are Fur and iron repressed, and the moderate response to nickel at the \( P_{fecA1} \) promoter also appears to be Fur mediated. In the \( H_9004 \) nikR mutant, while transcription from \( P_{fecA1} \) was completely derepressed by iron chelation, it appeared to be repressed under all other conditions. The general trend of \( P_{fecA1} \) and \( P_{fecA2} \) transcription observed in the nikR mutant was comparable to that in the wild-type strain (Fig. 3A and B, lanes 9 to 12). As expected, both promoters were derepressed in the double mutant (Fig. 3A and B, lanes 13 to 16). We conclude that transcription from the \( P_{fecA1} \) and \( P_{fecA2} \) promoters is iron regulated in a Fur-dependent manner.

**Nickel-dependent repression of \( P_{fecA3} \) is mediated by NikR.** The levels of \( fecA3 \) transcripts in RNA samples from wild-type cultures after the addition of iron or iron chelator were comparable to those from the untreated cultures (Fig. 3C, lanes 1, 2, and 4), indicating that \( fecA3 \) is not iron regulated. In contrast, upon the addition of 1 mM NiSO\(_4\) to growing cells, a significant reduction in the transcript level was detected (Fig. 3C, lane 3), suggesting that the addition of nickel strongly downregulates the \( fecA3 \) promoter. The negative effect exerted by nickel was lost in the \( H_9004 \) nikR mutant strain (Fig. 3C, lanes 9 to 12), suggesting that NikR is responsible for the nickel-

![FIG. 2. Growth phase-dependent transcription of \( P_{fecA1} \) (A), \( P_{fecA2} \) (B), and \( P_{fecA3} \) (C) promoters. The wild-type strain was grown to an OD\(_{600}\) of 1.0, starting from an overnight stationary-phase preinoculum freshly diluted to an OD\(_{600}\) of 0.1. Total RNAs were extracted from equal volumes of culture at time points t1, t2, t3, t4, and t5, corresponding to OD\(_{600}\)s of 0.2, 0.4, 0.6, 0.8, and 1.0. Primer extensions were performed in triplicate with promoter-specific primers and 15-\( \mu \)g RNA samples extracted from independent cultures. Results from representative time course experiments are shown in the bottom panels. The fast-migrating band in panel A (*) was taken as an internal control for the RNA samples. The intensity of the bands at each time point for the \( P_{fecA1} \) transcript (A), the \( P_{fecA2} \) transcript (B), and the \( P_{fecA3} \) transcript (C) is reported as the change (\( n \)-fold) from the signal obtained at time t5. Error bars indicate standard deviations of results for three independent replicates.](http://jb.asm.org/)

![FIG. 3. Fur- and NikR-mediated metal regulation of the \( P_{fecA} \) promoters. (A to C) Quantitative primer extension analyses of the responses of \( P_{fecA1} \) (A), \( P_{fecA2} \) (B), and \( P_{fecA3} \) (C) promoters to metal addition or chelation. Total RNAs were extracted from cultures of the \( H. pylori \) G27 wild type, \( H. pylori \) G27 nikR::Km (\( \Delta \)nikR) and G27 fur::Km (\( \Delta \)fur), and the G27 double mutant (nikR::cat fur::Km) grown to exponential phase and treated for 15 min with 1 mM FeSO\(_4\) (Fe), 1 mM NiSO\(_4\) (Ni), or 100 \( \mu \)M 2,2-dipyridyl (Di). A control reaction (−, no treatment) is also shown. (D) As a negative control, primer extension of the catalase gene (\( P_{katA} \)) transcript was carried out.](http://jb.asm.org/)
dependent repression of $P_{\text{fecA3}}$. It is worth noting that there was a general reduction in the level of transcription from $P_{\text{fecA3}}$ in the NikR mutant (Fig. 3C, lanes 9 to 12) and also, to a lesser extent, in the $\Delta\text{fur}$ mutant (Fig. 3C, lanes 5 to 8) and in the $\Delta\text{fur}\Delta\text{nikR}$ double mutant (Fig. 3C, lanes 13 to 16) compared to that in the wild-type strain. However, the downregulation in response to Ni$^{2+}$ was not affected in the $\Delta\text{fur}$ mutant strain (Fig. 3C, lane 7), suggesting that Fur is not directly involved in the transcriptional regulation of $P_{\text{fecA3}}$. As expected, the response to nickel was lost in the double mutant (Fig. 3C, lane 15). These data suggest that the repressive effect of Ni$^{2+}$ on $P_{\text{fecA3}}$ is mediated by NikR and that the deletion of fur and/or nikR may affect the steady-state level of transcription.

**Differential patterns of Fur and NikR binding to the $P_{\text{fecA1}}$, $P_{\text{fecA2}}$, and $P_{\text{fecA3}}$ promoter regions.** In order to understand whether the transcriptional responses revealed by RNA analyses were due to the direct binding of the metal-responsive transcriptional regulators Fur and NikR to specific operator sites, we performed footprinting assays of recombinant purified proteins with $P_{\text{fecA1}}$, $P_{\text{fecA2}}$, and $P_{\text{fecA3}}$ promoter probes.

Figure 4A, B, and C show the patterns of protection of Fur and NikR on the $P_{\text{fecA1}}$, $P_{\text{fecA2}}$, and $P_{\text{fecA3}}$ promoter probes. Fur binds to the $P_{\text{fecA1}}$ promoter, protecting a region of 27 bp spanning positions $-8$ to $-34$, overlapping with the $-10$ and partially with the $-35$ promoter elements, and at a concentration of 18 nM, fully preventing DNase I digestion (Fig. 4A1, lane 3). A DNase I-hypersensitive site is apparent upstream of the binding region. The addition of NikR at high concentrations (74 to 148 nM) to the same promoter probe resulted in a faint area of protection spanning positions $-54$ to $-65$ of the $P_{\text{fecA1}}$ promoter (Fig. 4A2, lanes 9 and 10).

The $P_{\text{fecA2}}$ promoter in the presence of Fur presented two regions of altered DNase I digestion (Fig. 4B1, lanes 2 to 5), one extending from nucleotide $+10$ to nucleotide $+29$ (region I) and the other spanning nucleotides $-7$ to $-39$ (region II). In addition, Fur appears to have differential affinities for the two operators, binding at 6 nM (Fig. 4B1, lane 2) to OP-I and at 18 nM to OP-II (Fig. 4B1, lane 3). NikR, at high concentrations (Fig. 4B2), also protected a region of approximately 60 bp of the $P_{\text{fecA2}}$ promoter, from nucleotide $-7$ to nucleotide $-66$, resulting in a DNase I-hypersensitive site at position $-40$ and overlapping with the lower-affinity OP-II binding site for Fur (Fig. 4B2, lanes 8 to 10).

No Fur-dependent protection on $P_{\text{fecA3}}$ was observed (Fig. 4C, lanes 1 to 5). However, at high Fur concentrations, weak protection spanning positions $-6$ to $-35$ could be detected (data not shown). NikR demonstrates a high-affinity binding site, OP-I (Fig. 4C), completely saturated at 14.8 nM NikR (Fig. 4C, lanes 7 to 10) and corresponding to the previously reported operator region (15). Surprisingly, a lower-affinity site, OP-II (Fig. 4C, lanes 8 to 10), spanning nucleotides $-8$ to $-44$ and comprising the $-10$ region, was detectable at 74 nM protein. This may indicate that effective regulation of the $P_{\text{fecA3}}$ promoter by NikR in response to nickel requires two adjacent binding sites.

The high-affinity binding of Fur to $P_{\text{fecA1}}$ and $P_{\text{fecA2}}$ and the high-affinity binding of NikR to $P_{\text{fecA3}}$ are consistent with the transcriptional analyses in Fig. 3 and support the idea of the re-
pressive role of Fur at the P\textsubscript{fecA1} and P\textsubscript{fecA2} promoters and the repressive role of NikR at the P\textsubscript{fecA3} promoter in response to iron and nickel, respectively.

Cooperative binding of NikR to the OP-I and OP-II sites of the P\textsubscript{fecA3} operator. The presence of two adjacent binding sites for NikR on the P\textsubscript{fecA3} promoter raised the question of their interdependence. Therefore, we decided to subclone discrete fragments of this promoter: Rx1, containing the whole region recognized by NikR in the footprinting assay, and Rx2 and Rx3, containing the proximal (OP-I) and distal (OP-II) binding sites, respectively (Fig. 5A). We tested these distinct regions in gel shift assays with increasing amounts of NikR in the presence of 100 \( \mu \)M NiSO\(_4\) (Fig. 5B).

The addition of 9.8 nM NikR to the Rx1 probe (Fig. 5B, lane 2) resulted in the complete sequestration of the free probe to a slow-migrating band, indicative of high-affinity NikR-DNA probe complex formation. Interestingly, upon the addition of 98 and 490 nM NikR (Fig. 5B, lanes 3 and 4, respectively), the migration of the complex was further retarded, indicative of additional NikR bindings. In contrast, similar amounts of NikR led to the formation of a unique complex with OP-I (Fig. 5B, lanes 6 to 8) and no complexes with OP-II (Fig. 5B, lanes 10 to 12). This finding suggests that the distal site (OP-II) requires the occupancy of the proximal one to bind NikR in a cooperative manner.

In vivo role of the two NikR operators at P\textsubscript{fecA3}. In order to better understand the roles of the two NikR binding sites in the transcriptional regulation of P\textsubscript{fecA3}, we constructed two mutant promoters, with mutations in either the OP-I or the OP-II site, fused to a truncated \textit{lacZ} reporter gene. Based on the proposed NikR consensus motif (7), a SmaI site was inserted in order to disrupt one NikR hemioperator site, that of OP-I or OP-II, by the replacement of the nucleotides likely to be important for protein-DNA interaction (Fig. 6A). The wild-type and mutated transcriptional fusions were inserted into the \text{vacA} locus of the \textit{H. pylori} genome, and their activities were monitored by primer extension analyses (Fig. 6B). The wild-type construct gave a product whose transcription was nickel sensitive (Fig. 6B, lanes 1 and 2). The OP-I-mutated fusion resulted in nickel-insensitive transcription (Fig. 6B, lanes 3 and 4). The OP-II-mutated fusion produced a more intense band and maintained the nickel-dependent repression (Fig. 6B, lanes 5 and 6). These results suggest that the wild-type levels of the \textit{fecA3} transcript result from the balanced activities of the two operators, with OP-I controlling the response to nickel while OP-II controls the total level of transcripts.

**DISCUSSION**

Nickel and iron are essential cofactors for the activity of several enzymes. In \textit{H. pylori}, they are important determinants for the colonization of the stomach epithelium, survival in the gastric mucosa, and virulence. \textit{H. pylori} codes for two metal-responsive transcriptional regulators, Fur and NikR, which control the expression of many genes important for infection and are indispensable for pathogenesis, as both \textit{fur} and \textit{nikR} deletion strains are attenuated in the mouse model (4). Several lines of experimental evidence show that their regulons are overlapping and interconnected (4–7). In addition, their expression is interdependent, as mutual downregulation of the
promoters of fur and nikR has been shown to occur (7). Moreover, both regulators have been implicated in the control of transcription of the fecA genes.

In H. pylori, the three fecA genes are independent monocistronic genes, transcribed by the vegetative sigma factor and selectively regulated by Fur and NikR. Based on results from transcriptional analyses (Fig. 3) and in vitro studies (Fig. 4), we can conclude that Fur represses fecA1 and fecA2 in response to iron. Fur shows high affinity for the P_{fecA2} promoter in vitro, and in vivo transcription from P_{fecA2} is repressed fully. The Fur-dependent transcription from P_{fecA1}, for which Fur shows lower affinity in vitro, can be stimulated by the addition of iron to the cell and, to a lesser extent, also by the addition of nickel. The iron-dependent transcription of fecA1 and fecA2 is in accordance with previously reported analyses (12, 30). The expression patterns of these two genes throughout growth are different, with fecA1 showing a basal, constitutive level of expression, whereas fecA2 is repressed in the early phase of growth (Fig. 2). Although no study to date has examined the actual physiological roles of FecA1 and FecA2 in H. pylori or their binding activities toward siderophores or iron-associated molecules, it is legitimate to envisage that the different patterns of expression may reflect distinct roles for the two iron transporters. For instance, FecA1 may be a low-affinity iron transporter, ensuring the iron supply under unstimulated conditions, whereas FecA2 may be a high-affinity iron transporter whose expression needs to be tightly controlled. The low-affinity nickel-dependent binding of NikR to P_{fecA1} and P_{fecA2} in vitro seems to have a minor physiological role under the conditions tested, as there is no clear NikR-dependent response. We speculate that the observed lower level of transcription of P_{fecA1} in the NikR mutant than in the wild type may be an indirect effect of Fur-mediated repression, as Fur is overexpressed in the NikR mutant (7). However, we cannot exclude the possibility that the low-affinity binding of NikR on P_{fecA1}, upstream of the putative -35 promoter element, observed in vitro may exert a positive influence on transcription. In contrast, an explanation of NikR binding to P_{fecA2} could not be assigned, since there is no apparent nickel-sensitive response or nikR-dependent modulation of this promoter under the conditions tested. In view of the NikR- and nickel-responsive regulation of fecA3, we tentatively speculate that FecA3 plays a role in Ni^{2+} uptake, as has recently been shown for another, similarly regulated protein, FrpB4, which was annotated as an iron transporter (26), although this role has not been addressed to date.

Recently, Ernst and coworkers (15) showed that NikR represses the transcription of the fecA3 gene by binding to a specific region of P_{fecA3} in a nickel-dependent manner. Herein, we have shown that the expression of fecA3 is temporally regulated during growth (Fig. 2) and that NikR binds cooperatively, with different affinities, to two distinct sites within the P_{fecA3} promoter (Fig. 4 and 5). Our data indicate that the nickel-dependent NikR regulation of fecA3 seems to be less straightforward than expected. In fact, transcriptional analyses suggest a direct and an indirect role for NikR in the regulation of fecA3 (Fig. 3). P_{fecA3} in the wild-type strain seems to have a high level of transcription, under conditions of no treatment as well as in the presence of iron or an iron chelator. The addition of NiSO_{4} results in NikR-dependent downregulation of fecA3, as indicated by the loss of the response to nickel in the ΔnikR strain. However, the constitutive level of transcription of fecA3 in the ΔnikR mutant and, to a lesser extent, also that in the Δfur mutant are considerably lower than that in the wild type, implying indirect effects of the nikR and fur regulatory genes on the basal transcription of P_{fecA3}. These results appear to be in contradiction with the finding of Ernst and coworkers (15), who reported that Northern blot analysis showed apparently similar amounts of fecA3 mRNA in wild-type and nikR mutant strains. A possible explanation may lie in the fact that while Northern blots show all mRNA species accumulated in the cell, quantitative primer extension analyses reveal specific variations in the amounts of 5′ ends of the fecA3 mRNA, as substantiated by the results of the control experiment shown in Fig. 3 (panels C and D, lanes 9 to 12 versus 1 to 4). The repression of P_{fecA3} transcription in response to nickel occurs by the binding of NikR to a high-affinity primary binding site (OP-I) (Fig. 5) corresponding to a previously reported operator site (15), followed by cooperative binding to a repressing upstream site (OP-II) (Fig. 5).

P_{fecA3} transcription was shown to be substantially unaffected by iron treatment, in agreement with the data in previous reports (30). Nonetheless, transcription analyses conducted in exponential and advanced growth phases suggested that fecA3 transcript levels are derepressed in the Δfur strain only in the advanced growth phase, while chromatin immunoprecipitation-on-chip analysis identified fecA3 as a direct target of Fur in vivo (6). In vitro, Fur binds to P_{fecA3} with very low affinity (data not shown). These observations may tentatively point to a role of Fur in modulating the basal levels of fecA3 transcription in advanced growth phase, when the levels of Fur increase sufficiently to compete with NikR for binding to P_{fecA3}.

In conclusion, our analyses suggest that the transcription of the fecA genes is regulated in response to Fe^{2+} and Ni^{2+} concentrations via the specific interactions of the Fur and NikR regulatory proteins with the fecA gene promoters and possibly through direct and/or indirect feedback regulation of the Fur-NikR regulatory circuit. The degree of complexity of the overlapping regulatory circuits of these two proteins, required to guarantee metal homeostasis, is becoming more evident.

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REFERENCES


