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Growth Phase and Metal-Dependent Transcriptional Regulation of the *fecA* 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 cofactors 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 *f*erric *u*ptake *r*egulator 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-fecI* 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, when 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 Ni^{2+}-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

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description[a]	Source or reference
E. coli strains		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*β	18
BL21(DE3)	*hsdS gal* (λ*cIts*857 *ind1 Sam7 nin5 lacUV5*-T7 gene 1)	28
H. pylori strains		
G27	Clinical isolate; wild type	32
G27 *fur* (Δ*fur*::Km)	G27 derivative with *fur* gene mutation; Km^r	8
G27 *nikR* (Δ*nikR*::Km)	G27 derivative with *nikR* gene mutation; Km^r	23
G27 *fur nikR* (Δ*fur*::Km Δ*nikR*::*cat*)	G27 derivative with *fur* and *nikR* gene mutations; Km^r Cp^r	23
G27 *vac*::P_{fecA3}-*lacZ*	G27 derivative containing the wild-type P_{fecA3} promoter-*lacZ* fusion in the *vacA* locus; Km^r	This study
G27 *vac*::OP-I mutant P_{fecA3}-*lacZ*	G27 derivative containing the OP-I mutant P_{fecA3} promoter-*lacZ* fusion in the *vacA* locus; Km^r	This study
G27 *vac*::OP-II mutant P_{fecA3}-*lacZ*	G27 derivative containing the OP-II mutant P_{fecA3} promoter-*lacZ* fusion in the *vacA* locus; Km^r	This study
Plasmids		
pGEM-T Easy	General cloning vector; Amp^r	Promega
pGEMT-P_{fecA1}	pGEM-T Easy derivative containing 402 bp of *fecA1* promoter region, amplified by PCR with primers A1F and A1R	This study
pGEMT-P_{fecA2}	pGEM-T Easy derivative containing 380 bp of *fecA2* promoter region, amplified by PCR with primers A2F and A2R	This study
pGEMT-P_{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^r	Stratagene
pBS-P_{fecA3}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_{fecA3}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_{fecA3}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
pVac::Km	pGEMZ derivative containing a kanamycin cassette	10
pVac::P_{A3}lacZ	pVac::Km derivative containing the transcriptional fusion P_{fecA3}-*lacZ*; Km^r	This study
pVac::$P_{A3MuOpI}$lacZ	pVac::Km derivative containing the transcriptional fusion OP-I mutant P_{fecA3}-*lacZ*; Km^r	This study
pVac::$P_{A3MuOpII}$lacZ	pVac::Km derivative containing the transcriptional fusion OP-II mutant P_{fecA3}-*lacZ*; Km^r	This study
pET15b	IPTG-inducible vector overexpressing N-terminally His_6-tagged recombinant protein; Amp^r	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

[a] IPTG, isopropyl-β-D-thiogalactopyranoside.

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% cyclodextrin, and Dent's or Skirrow's antibiotic supplement or on *Brucella* agar plates with 5% fetal calf serum (Oxoid) and Dent's or Skirrow's antibiotic supplement at 37°C under microaerophilic conditions (9% CO_2, 91% air atmosphere, and 95% humidity) in a water-jacketed thermal incubator. Liquid cultures were grown in modified *Brucella* broth supplemented with 5% fetal calf serum (Oxoid) and Dent's or Skirrow's antibiotic supplement at 37°C with constant agitation (125 rpm). To measure the metal-dependent transcriptional response, master cultures (40 ml) of the wild-type and mutant strains were grown to mid-log phase (optical density at 600 nm [OD_{600}], 0.5 to 0.6), divided into four equal-volume subcultures, and treated for 15 min with freshly made 1 mM $FeSO_4$, 1 mM $NiSO_4$, or 100 μM 2,2-dipyridyl prior to RNA extraction. Similarly, to monitor the expression of the *fecA* genes over time, aliquots of 10 ml from a 100-ml culture were obtained at different time points, harvested by centrifugation in the presence of phenol-ethanol stop solution, and frozen for RNA extraction. *E. coli* strains DH5α and BL21(DE3) were grown in Luria-Bertani broth. Ampicillin (100 μg/ml), kanamycin (25 μg/ml), and chloramphenicol (30 μg/ml) were added when required.

DNA manipulation and cloning of the promoter regions. Standard molecular biology techniques were carried out for DNA purification, PCR analyses, restriction digestion, and cloning (24). All restriction and modification enzymes were used according to the instructions of the manufacturer (New England Biolabs). The promoter regions of interest were amplified from genomic DNA by PCR using the primer pairs listed in Table 2 (A1F-A1R for *fecA1*, A2F-A2R for *fecA2*, A3F-A3R for *fecA3*, A3.1-A3.4 for *fecA3* promoter fragment Rx1, A3.1-A3.2 for *fecA3* promoter fragment Rx2, and A3.3-A3.4 for *fecA3* promoter fragment Rx3) and cloned into the pGEM-T Easy (Promega) or pBlueScript (Stratagene) vector to generate plasmids pGemT-P_{fecA1}, pGemT-P_{fecA2}, pGemT-P_{fecA3}, pBS-

TABLE 2. Primers used for PCR amplification of the promoter regions and for primer extension reactions

Oligonucleotide name	Sequence (5′→3′)[a]
A1F	CCTT*GGATCC*ATTATGAGTTTTGACAGCAT
A1R	GGGA*GAATTC*AAGTTTGTAGCGTATTTATT
A2F	TTTA*GGATCC*TTTTGCTCAGTGGTTGTCAC
A2R	TATA*GAATTC*GACGCTGGTTTTGCGATAGC
A3Fnew	ATTT*GGATCC*AGCGTCAAAGAATGTCTTGT
A3Rnew	TAAT*GAATTC*TTTCAAGTAGAATCACG
A3.1	*GGAATTC*ACGAACGCCTAT
A3.2	CG*GGATCC*TATGATAAAATT
A3.3	*GGAATTC*AATTCGCAGAAT
A3.4	CG*GGATCC*AAAAGATTTTCA
A3.6	TCC*CCCGGG*AATAAAATTTTA
A3.7	TCC*CCCGGG*CCCATGAAAATC
A3.8	TCC*CCCGGG*TTAATAGGCGTT
A3.9	TCC*CCCGGG*AAAATAAAAAAA
PEfecA1	CCAAAACAGCCAAAGAGACTA
PEfecA2	ATTTTTGCTCAGTGGTTGTCA
5PEfecA3	ATTCTTTCAAGTAGAATCACGC
A3Z1	GTATCGATAAGCTTGATATC
5′kat	CACATCTTTATTAACCAT

[a] Bases in italics correspond to exogenous restriction sites.

P_{fecA3}Rx1, pBS-P_{fecA3}Rx2, and pBS-P_{fecA3}Rx3. Appropriate restriction sites were introduced at the primer 5′ end to facilitate the subsequent cloning. Qiagen gel extraction and PCR purification kits were used according to the manufacturer's instructions.

Mutagenesis of the promoter of *fecA3*. The promoter region of *fecA3* was mutagenized by introducing an SmaI site within the putative NikR consensus sites identified by sequence analysis in order to replace the hemioperators partially or completely. The primer pairs A3Fnew-A3.7 and A3Rnew-A3.6 and the primer pairs A3Fnew-A3.9 and A3Rnew-A3.8 were used to mutate the operator II (OP-II) and operator I (OP-I) sites, respectively. The regions of interest were amplified from pGemT-P_{fecA3}, subcloned into pBlueScript, and subsequently ligated via the SmaI site to generate the plasmids pBS-PA3MuOpII and pBS-PA3MuOpI. The mutations were confirmed by sequence analyses.

Construction of *lacZ* transcriptional fusions and integration into the *vacA* locus of *H. pylori*. The wild-type and mutant *fecA3* promoters were cloned via the restriction sites BamHI and EcoRI into pBlueScript SK so as to have in-frame transcriptional fusions with the *lacZ* 3′ region occurring on the vector. These constructs were recovered by PvuII-BamHI double digestion, blunted, and cloned into the pVac::Km transformation vector (10) by exploiting a HincII site. The transcriptional fusions were inserted into the *vacA* locus on the chromosome of *H. pylori* by homologous recombination; positive colonies on agar plates were selected according to the antibiotic resistance phenotype. The integrations were confirmed by PCR amplification with primers A3Z1 and A3Fnew.

RNA isolation and primer extension analyses. Total RNA was extracted by a hot-phenol procedure as described previously (6); RNA integrity and purity were ensured by electrophoresis on 1% agarose gels. Primer extension analyses were performed with 15 μg of total RNA and 10 pmol of 5′-end-labeled primers as described previously (11). The oligonucleotides used for primer extension reactions are listed in Table 2. The same set of primers was utilized to map the transcription start site of the corresponding cloned promoter region in a sequencing reaction using a T7 sequencing kit (USB Corp.). The quantification of the *fecA* mRNA extension products was performed by using ImageQuant software, after image acquisition by a Storm PhosphorImager (Molecular Dynamics).

Overexpression and purification of recombinant His_6-Fur and His_6-NikR. Recombinant His_6-Fur (11) and His_6-NikR (7) were overexpressed and purified under native conditions as described previously. Thrombin protease (10 U/mg) was used to remove the N-terminal histidine tag according to the instructions of the manufacturer (Amersham GE Healthcare). The purified, untagged proteins are hereinafter referred to as Fur and NikR. Proteins were stored in phosphate-buffered saline at −80°C and dialyzed overnight against the footprinting buffer (50 mM Tris-Cl, pH 7.85, 50 mM KCl, 10 mM $MgCl_2$, 0.01% Igepal CA-630, 10% glycerol, 1 mM dithiothreitol) or 20 mM HEPES buffer (pH 7.85) prior to the DNA binding experiments. A Bradford colorimetric assay kit (Bio-Rad) was used to quantify the protein fractions with bovine serum albumin as the standard.

Probe preparation and DNase I footprinting. The vectors pGEMT-P_{fecA1}, pGEMT-P_{fecA2}, and pGEMT-P_{fecA3} were linearized with BamHI (for pGEMT-P_{fecA1} and pGEMT-P_{fecA2}) or EcoRI (for pGEMT-P_{fecA3}), dephosphorylated with calf intestinal phosphatase, and labeled at the 5′ ends with [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham GE Healthcare) by using T4 polynucleotide kinase. The labeled DNA probes were further digested with EcoRI (for pGEMT-P_{fecA1} and pGEMT-P_{fecA2}) or BamHI (for pGEMT-P_{fecA3}), and the products were separated by native polyacrylamide gel electrophoresis and purified as described previously (7). The binding reactions between approximately 20 fmol of labeled probe and increasing concentrations of NikR (expressed per NikR tetramer) and Fur (expressed per Fur dimer) were carried out in the footprinting buffer (50 mM Tris-Cl, pH 7.85, 50 mM KCl, 10 mM $MgCl_2$, 0.01% Igepal CA-630, 10% glycerol) at room temperature for 15 min using 1 μg of salmon sperm DNA (Invitrogen) as a nonspecific competitor in a final volume of 50 μl. An excess of $NiSO_4$ (100 μM) or $FeSO_4$ (100 μM) was added where indicated. Afterwards, DNase I (0.04 U), diluted in footprinting buffer containing 5 mM $CaCl_2$, was added to the reaction mixture and digestion was allowed to occur for 90 s. The reaction was then stopped, and the samples were treated as described previously (11). Samples were resuspended in 6 μl of formamide loading buffer, denatured at 95°C for 3 min, separated on 8 M urea–6% acrylamide sequencing gels, and autoradiographed. A modified G+A sequencing ladder protocol (21) was employed to map the binding sites.

DNA electrophoretic mobility shift assay. A DNA gel retardation assay of discrete regions of the *fecA3* promoter was performed. Three fragments of approximately 130, 60, and 60 bp were amplified from pGEMT-P_{fecA3} by using the primer pairs listed in Table 2 to generate the plasmids pBS-P_{fecA3}Rx1, pBS-P_{fecA3}Rx2, and pBS-P_{fecA3}Rx3, respectively. Following double restriction digestion to isolate the fragment of interest, the DNA was dephosphorylated with calf intestinal phosphatase and labeled at both ends with T4 polynucleotide kinase and [γ-^{32}P]ATP; unincorporated radiolabeled nucleotide was removed with a G-50 microspin column (GE Healthcare). The binding reaction was carried out for 15 min in a mixture of 20 mM HEPES-OH (pH 7.85), 50 mM KCl, 10% glycerol, 0.02% Igepal CA-630, and 0.1 mM dithiothreitol with 200 ng of salmon sperm DNA as a nonspecific competitor. Twenty femtomoles of radiolabeled target DNA and increasing concentrations of NikR were used in a final volume of 15 μl in the presence of 100 μM $NiSO_4$. Reaction mixtures were resolved on a native gel (6% polyacrylamide [19:1], 20 mM MOPS [morpholinepropanesulfonic acid], 5 mM sodium acetate, pH 7.0), and the gels were prerun at 50 V for 30 min prior to loading and then run at 170 V for 2 h at room temperature.

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 *feoB*, 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 36 and 40 bases 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 *E. coli* promoter consensus sequence and less conserved −35 regions (Fig. 1D). Notably, the −10 sequence of P_{fecA3} 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

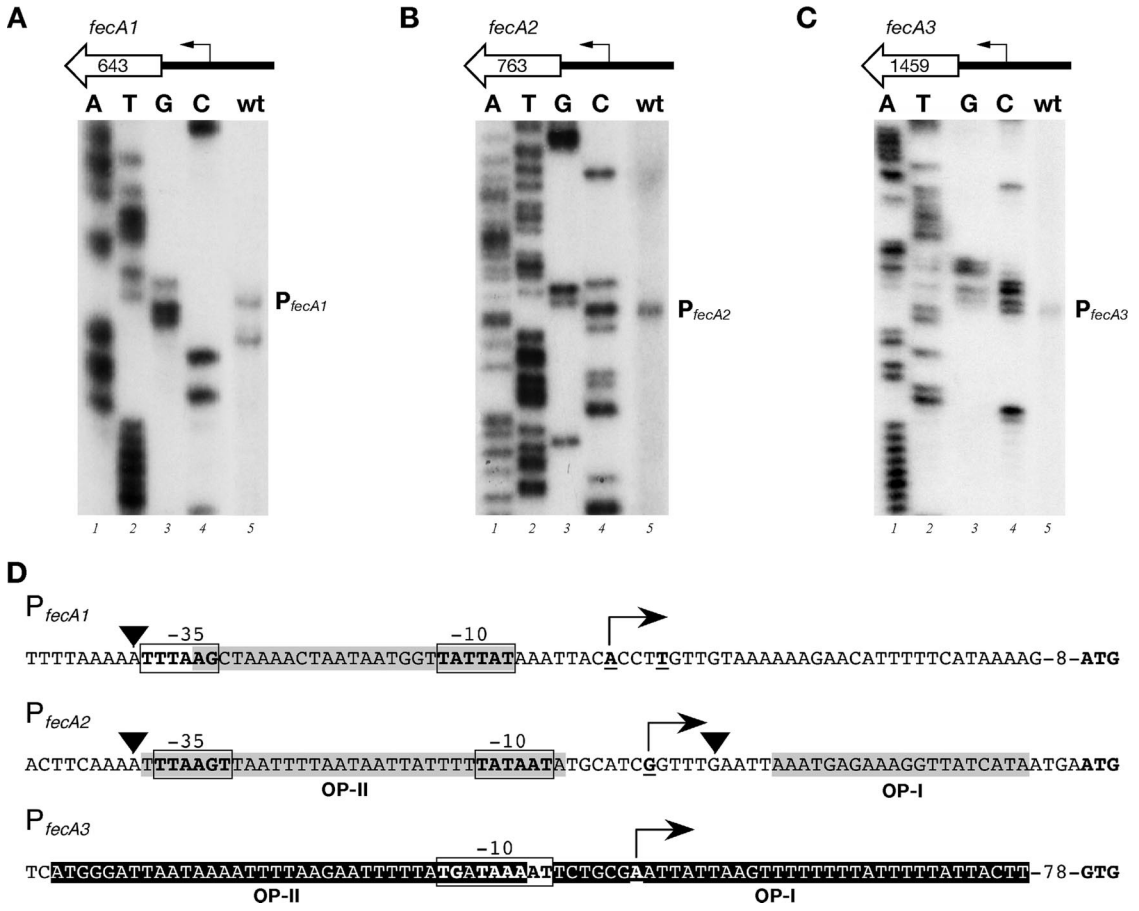

FIG. 1. Identification of the *H. pylori fecA* promoter regions. (A to C) The 5′ ends of the *fecA1* (A), *fecA2* (B), and *fecA3* (C) transcripts were identified by primer extension analyses. A schematic representation of the *fecA* gene and its promoter in *H. pylori* strain G27 is shown at the top of each panel. *fecA1*, *fecA2*, and *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 *fecA* transcripts are indicated. Sequence reactions (A, T, G, and C) were performed with the same primers and with plasmids pGEMT-P_{fecA1} (A), pGEMT-P_{fecA2} (B), and pGEMT-P_{fecA3} (C) as templates. (D) Summary of relevant features within the nucleotide sequences of the *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 *H. pylori fecA* genes encode polypeptides with less than 30% amino acid sequence identity and approximately 50% sequence similarity to *E. coli* FecA.

of *H. pylori* are transcribed by the vegetative σ^{80}-containing RNA polymerase.

Growth phase-dependent activity of the *fecA* promoters. To begin the functional characterization of the *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 P_{fecA1} promoter showed no significant variations in the amount of mRNA throughout growth (Fig. 2A). In contrast, transcription from the P_{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 P_{fecA3} promoter decreased over time (Fig. 2C). We conclude that while transcription from the P_{fecA1} promoter remains unchanged during growth, transcription from the P_{fecA2} and P_{fecA3} promoters appears to be inversely regulated, with the maximum expression of *fecA2* and *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.

P_{fecA1} and P_{fecA2} promoters are repressed by Fur. To study the transcriptional regulation of the *fecA* genes, primer extension analyses of RNAs extracted from the wild-type, Δ*nikR*, Δ*fur*, and double mutant Δ*nikR* Δ*fur* 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 P_{fecA1} promoter was detected under conditions of no treatment, whereas no transcription from P_{fecA2} was detected (Fig. 3A and B, lanes 1). P_{fecA1} was repressed after the addition of 1 mM $FeSO_4$ to the medium (Fig. 3A, lane 2) and slightly repressed by 1 mM $NiSO_4$ (Fig. 3A, lane 3), and both P_{fecA1} and P_{fecA2} were in-

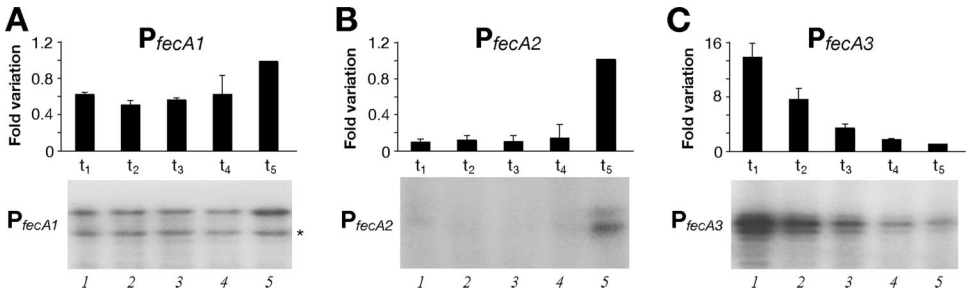

FIG. 2. Growth phase-dependent transcription of P_{fecA1} (A), P_{fecA2} (B), and P_{fecA3} (C) promoters. The wild-type strain was grown to an OD_{600} of 1.0, starting from an overnight stationary-phase preinoculum freshly diluted to an OD_{600} of 0.1. Total RNAs were extracted from equal volumes 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-μg 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.

duced by the addition of the iron chelator (Fig. 3A and B, lanes 4). Transcription levels for both promoters in the Δ*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 Δ*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 Δ*nikR* mutant strain (Fig. 3C, lanes 9 to 12), suggesting that NikR is responsible for the nickel-

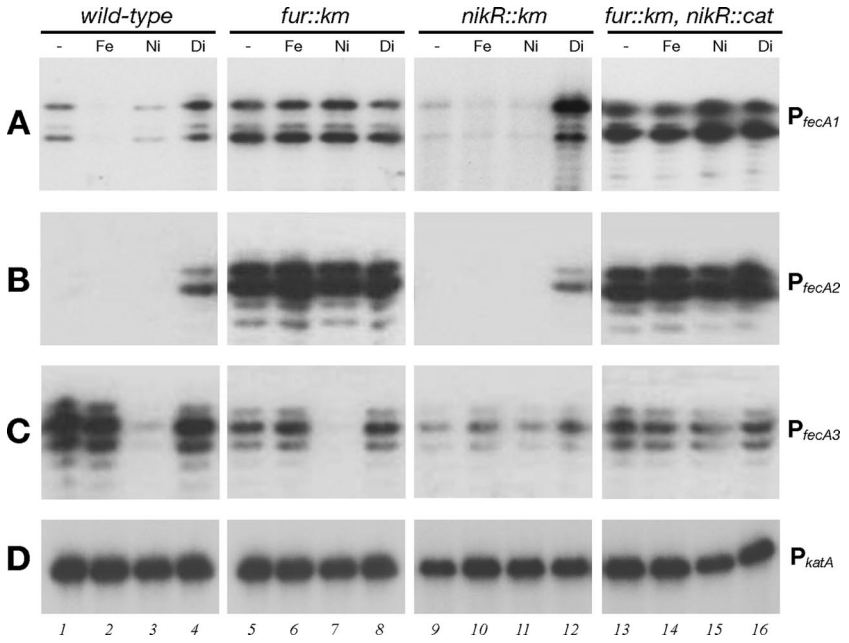

FIG. 3. Fur- and NikR-mediated metal regulation of the P_{fecA} promoters. (A to C) Quantitative primer extension analyses of the responses of P_{fecA1} (A), P_{fecA2} (B), and P_{fecA3} (C) promoters to metal addition or chelation. Total RNAs were extracted from cultures of the *H. pylori* G27 wild type, *H. pylori* G27 *nikR*::Km (Δ*nikR*) and G27 *fur*::Km (Δ*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 μM 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} [8]) transcript was carried out.

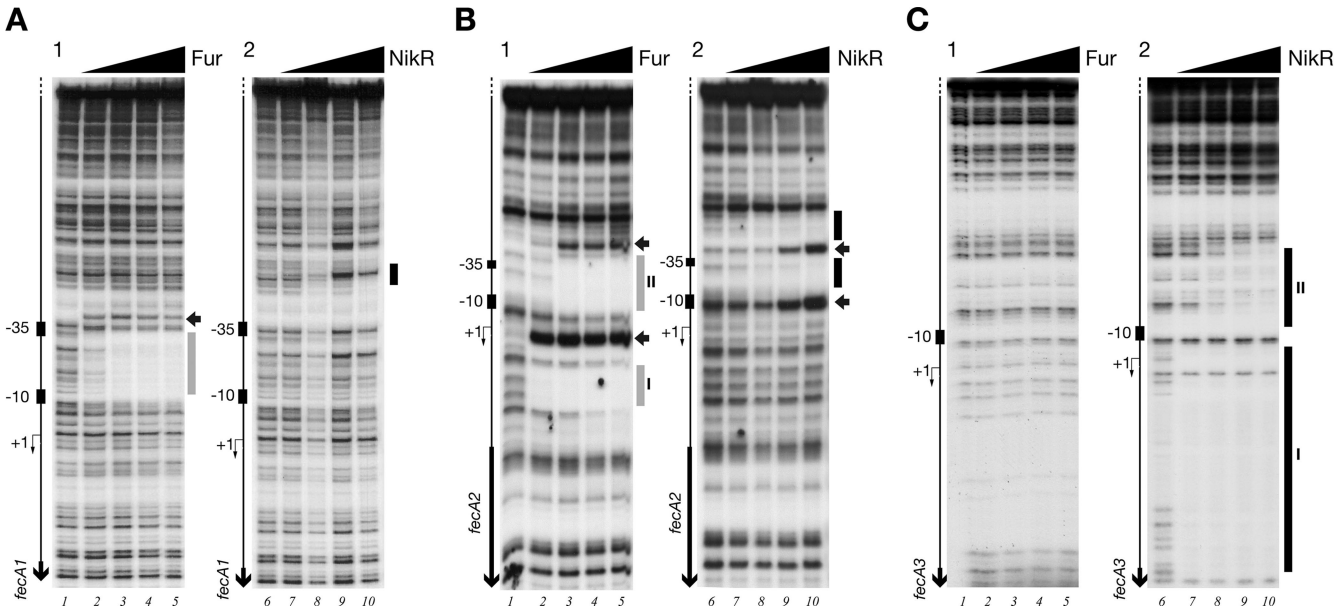

FIG. 4. In vitro binding of Fur and NikR to the P_{fecA1} (A), P_{fecA2} (B), and P_{fecA3} (C) promoter regions of *H. pylori* G27. Divalent iron and nickel (100 μM) were added as cofactors in the binding buffers of Fur and NikR, respectively. Samples of approximately 20 fmol of 5′-end-labeled probes were incubated with increasing concentrations of Fur dimer, 0 nM (lanes 1), 6 nM (lanes 2), 18 nM (lanes 3), 60 nM (lanes 4), and 180 nM (lanes 5), or NikR tetramer, 0 nM (lanes 6), 14.8 nM (lanes 7), 29.6 nM (lanes 8), 74 nM (lanes 9), and 148 nM (lanes 10). A G+A sequence reaction ladder for each promoter probe was run in parallel (data not shown) to map the binding sites with respect to the transcriptional start site (+1), indicated by a bent arrow to the left of each gel. The relative positions of the putative −10 and −35 elements are symbolized by small rectangles, and vertical black arrows indicate open reading frames. The vertical bar(s) to the right of each gel indicates the region(s) protected from DNase I digestion at increasing concentrations of protein (gray, Fur; black, NikR); horizontal black arrows represent bands of hypersensitivity.

dependent repression of P_{fecA3}. It is worth noting that there was a general reduction in the level of transcription from P_{fecA3} in the NikR mutant (Fig. 3C, lanes 9 to 12) and also, to a lesser extent, in the Δ*fur* mutant (Fig. 3C, lanes 5 to 8) and in the Δ*fur* Δ*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 Δ*fur* mutant strain (Fig. 3C, lane 7), suggesting that Fur is not directly involved in the transcriptional regulation of P_{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_{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_{fecA1}, P_{fecA2}, and P_{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_{fecA1}, P_{fecA2}, and P_{fecA3} promoter probes.

Figure 4A, B, and C show the patterns of protection of Fur and NikR on the P_{fecA1}, P_{fecA2}, and P_{fecA3} promoter probes. Fur binds to the P_{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_{fecA1} promoter (Fig. 4A2, lanes 9 and 10).

The P_{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_{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_{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_{fecA3} promoter by NikR in response to nickel requires two adjacent binding sites.

The high-affinity binding of Fur to P_{fecA1} and P_{fecA2} and the high-affinity binding of NikR to P_{fecA3} are consistent with the transcriptional analyses in Fig. 3 and support the idea of the re-

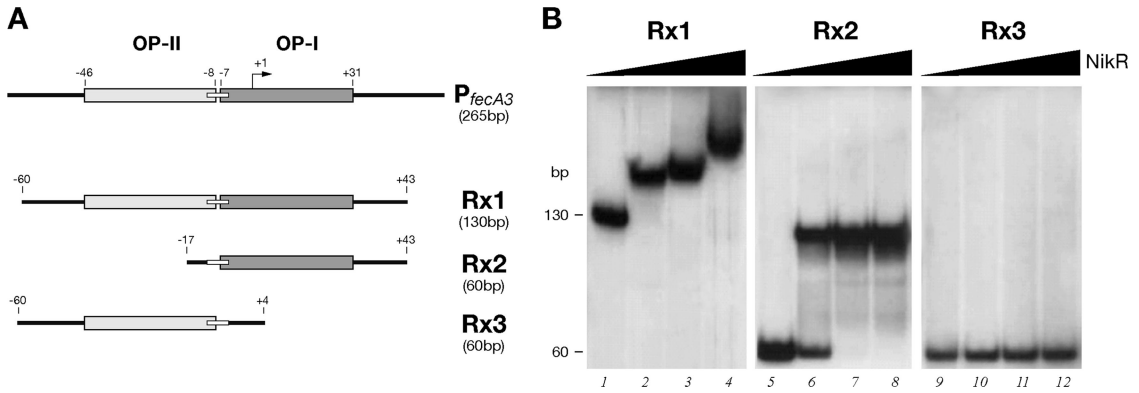

FIG. 5. Schematic representation of key features of the NikR operators on the P_{fecA3} promoter and DNA segments used as probes (A) in DNA binding assays with the NikR protein (B). DNA retardation was induced by the addition of NikR (100 μM $NiSO_4$ was present in the binding buffer). NikR was added at the following concentrations: lanes 1, 5, and 9, 0 nM; lanes 2, 6, and 10, 9.8 nM; lanes 3, 7, and 11, 98 nM; and lanes 4, 8, and 12, 490 nM.

pressive role of Fur at the P_{fecA1} and P_{fecA2} promoters and the repressive role of NikR at the P_{fecA3} promoter in response to iron and nickel, respectively.

Cooperative binding of NikR to the OP-I and OP-II sites of the P_{fecA3} operator. The presence of two adjacent binding sites for NikR on the P_{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 μM $NiSO_4$ (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_{fecA3}. In order to better understand the roles of the two NikR binding sites in the transcriptional regulation of P_{fecA3}, we constructed two mutant promoters, with mutations in either the OP-I or the OP-II site, fused to a truncated *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 *vacA* locus of the *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 *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 *H. pylori*, they are important determinants for the colonization of the stomach epithelium, survival in the gastric mucosa, and virulence. *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 *fur* and *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

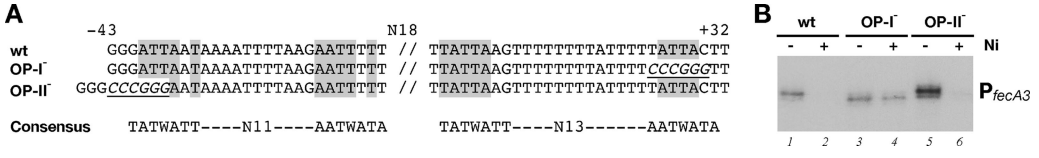

FIG. 6. (A) Alignment of the wild-type (wt) and mutated operators of the P_{fecA3} promoter. The inverted repeats within the operator sites corresponding to the NikR consensus region are highlighted, and the SmaI restriction site is underlined and represented in italics. (B) Primer extension with mid-log-phase total RNA extracted from a recombinant G27 strain harboring the P_{fecA3}-*lacZ* transcriptional fusions with wild-type and mutant OP-I and OP-II sites in response to a pulse of 100 μM $NiSO_4$. +, present; −, absent.

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 over time, 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 Δ*nikR* strain. However, the constitutive level of transcription of *fecA3* in the Δ*nikR* mutant and, to a lesser extent, also that in the Δ*fur* 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, transcriptome analyses conducted in exponential and advanced growth phases suggested that *fecA3* transcript levels are derepressed in the Δ*fur* 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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