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Positive Regulation of *fur* Gene Expression via Direct Interaction of Fur in a Pathogenic Bacterium, *Vibrio vulnificus*▽

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In pathogenic bacteria, the ability to acquire iron, which is mainly regulated by the ferric uptake regulator (Fur), is essential to maintain growth as well as its virulence. In *Vibrio vulnificus*, a human pathogen causing gastroenteritis and septicemia, *fur* gene expression is positively regulated by Fur when the iron concentration is limited (H.-J. Lee et al., J. Bacteriol. 185:5891–5896, 2003). Footprinting analysis revealed that an upstream region of the *fur* gene was protected by the Fur protein from DNase I under iron-depleted conditions. The protected region, from −142 to −106 relative to the transcription start site of the *fur* gene, contains distinct AT-rich repeats. Mutagenesis of this repeated sequence resulted in abolishment of binding by Fur. To confirm the role of this *cis*-acting element in Fur-mediated control of its own gene in vivo, *fur* expression was monitored in *V. vulnificus* strains using a transcriptional fusion containing the mutagenized Fur-binding site (*fur*$_{mt}$::*luxAB*). Expression of *fur*$_{mt}$::*luxAB* showed that it was not regulated by Fur and was not influenced by iron concentration. Therefore, this study demonstrates that *V. vulnificus* Fur acts as a positive regulator under iron-limited conditions by direct interaction with the *fur* upstream region.

Vibrio vulnificus is a halophilic marine microorganism which causes gastroenteritis and septicemia in immunocompromised humans (33). One of the important factors determining the survival of *V. vulnificus* under diverse environmental conditions is the ability to obtain iron (38). Syntheses of many toxins and virulence determinants are also regulated by intracellular iron concentration, which is mediated mainly by a global regulator, the ferric uptake regulator (Fur) (15).

Fur complexed with iron ions binds to a 19 bp-nucleotide sequence called the Fur box, which is usually located in the promoter regions of iron-regulated genes, preventing their transcription by competing with RNA polymerase for promoter regions (9). For example, *Escherichia coli* Fur represses the transcription of dozens of genes required for iron acquisition (2) and oxidative stress responses (34).

Some genes have been found to be positively regulated by Fur and iron (11, 12). This positive regulation by Fur is achieved through a function of a small RNA (sRNA), RyhB (24). RyhB RNA decreases the stability of the transcripts of several genes, including *sodB*, *sdhC*, *fumA*, and *bfr*, whose gene products utilize iron as a cofactor. This inhibitory effect of sRNA on target mRNAs occurs through an Hfq-mediated pairing between sRNA and the target mRNA, resulting in promoted degradation of mRNAs by RNase E (23). Since the expression of the *ryhB* gene is repressed by Fur in *E. coli*, the observed positive effect of Fur on the expression of some genes is indirect.

An RNA chaperone, Hfq was first discovered as a factor required for phage Qβ replication in *E. coli* (4) and is a small (11.2 kDa), heat-stable basic protein present as a hexameric structure (1). In *E. coli*, Hfq binds several other sRNAs (39). The DsrA RNA along with Hfq stimulates translation of *rpoS* mRNA but represses translation of *hns* mRNA (20, 22). Proteomic analysis to identify mRNA targets of Hfq revealed that it inhibits translation of the *sodB* and *fur* mRNAs in *E. coli* (35). However, limited information is available regarding regulation by sRNAs for *Vibrio* species. *Vibrio cholerae* mutants lacking the *hfq* gene are avirulent due to the attenuated ability to colonize the small intestines in a mouse model (10). *V. cholerae* RyhB is also regulated by the iron-dependent repressor, Fur, and it was found to interact with Hfq (3).

In contrast to the indirect control of genes by Fur via sRNA and Hfq, Fur was found to regulate its own expression by directly binding to *fur* upstream regions in *Helicobacter pylori* (7). DNase I-footprinting assays indicate that Fur binds to multiple sites with differential affinities, and the in vitro interactions between Fur and these sites vary in response to iron concentrations in the reaction mixture. One site bound by an iron-free form of Fur was involved in derepression of transcription. Binding of Fur to the other sites, which have higher affinity, is required for repression of the *fur* gene in an iron-dependent manner (8).

The present study found that intracellular levels of Fur were increased under iron-limited conditions. Thus, the role of Hfq, which is known to act at a posttranscriptional level, was examined in relation to the *fur* expression. As previously reported, it was found that *fur* gene expression in *V. vulnificus* is positively regulated by Fur when the iron concentration is limited (21). This study also revealed evidence for a unique regulatory mechanism for *fur* expression in *V. vulnificus*, in which Fur

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Strains		
E. coli		
DH5α	λ$^-$ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Laboratory collection
SM10λ*pir*	*thi-1 thr leu tonA lacY supE recA*::Rp4-2-Tc::Muλ*pir*; OriT of RP4; Km^r	31
JM109	*endA1 recA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^-) *relA1 supE44* Δ(*lac-proAB*)[F'*traD3 6proAB lacI*^{q}ZΔ*M15*]	QIAGEN
V. vulnificus		
MO6-24/O	Clinical isolate	37
HLM101	MO6-24/O; Δ*fur* Km^r	21
HLM102	MO6-24/O; Δ*hfq* Km^r	This study
Plasmids		
pRK415	IncP *ori*; broad-host-range vector; *oriT* of RP4; Tc^r	18
pHK0011	pRK415, a promoterless *luxAB*; Tc^r	16
pHL01	pHK0011, *fur*::*luxAB*	21
pBluescript II SK(+)	Cloning vector; Ap^r, *lac* promoter (*lacZ*); f1; ColE1	Stratagene
pSKhfq_up	pBluescript II SK(+) with 1,062-bp upstream region of *hfq*	This study
pSKhfq_up/down	pSKhfq_up with 1,029-bp downstream region of *hfq*	This study
pUC4K	*nptI*; Ap^r; Km^r	Pharmacia
pSKhfq_up/km/down	pSKhfqup/down with 1.2-kb *nptl* gene; Ap^r; Km^r	This study
pDM4	Suicide vector; *ori*R6K; Cm^r	26
pDMΔhfq	pDM4 with ApaI/SacI fragment of pSKhfqup/km/down; Cm^r; Km^r	This study
pQE30	Expression plasmid for expression of recombinant proteins with the N-terminal His tag	QIAGEN
pQE30-fur	pQE30 containing 471-bp *V. vulnificus fur*-containing region; Ap^r	This study
pGEM-T Easy	Cloning vector; Ap^r	Promega
pGEMT-fur	pGEM-T Easy containing 1,075-bp *V. vulnificus fur* upstream and coding region; Ap^r	This study
pGEMT-fur$_{mt}$	pGEMT-fur, but with mutation in the putative Fur-binding site; Ap^r	This study
pHL03	pHK0011; *fur*$_{mt}$::*luxAB*	This study

controls its own expression in a positive way via binding to a distinct nucleotide sequence.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 1. *E. coli* strains used for plasmid DNA preparation and the conjugational transfer of plasmid were grown in Luria-Bertani medium supplemented with appropriate antibiotics at 37°C. *V. vulnificus* strains were grown in Luria-Bertani medium supplemented with 2% (wt/vol) NaCl (LBS) at 30°C, unless stated otherwise. All medium components were purchased from Difco, and the chemicals and antibiotics were purchased from Sigma.

Western blot analysis. Two oligonucleotides, fur_overF (5′-CGGCGGATCC ATGTCAGACAATAACCAAGC-3′; underlined sequence denotes a PstI restriction site), were used to amplify a 479-bp DNA fragment containing the complete open reading frame (ORF) of the *fur* gene from the genomic DNA of *V. vulnificus*. BamHI and PstI sites located at both ends of the resultant *fur* DNA were used to clone into pQE30, an expression plasmid (QIAGEN), to generate a plasmid pQE-fur. Recombinant Fur was overexpressed in *E. coli* JM109 by adding isopropyl-β-D-thiogalactopyranoside at a concentration of 1.0 mM and was purified using a Ni^+-nitrilotriacetic acid affinity column as directed by the manufacturer (QIAGEN). The purified recombinant Fur was used to raise polyclonal antibodies by three immunizations of Sprague-Dawley rats (200 μg of Fur protein per each immunization) at 3-week intervals. Cell lysates of various *V. vulnificus* strains (wild type and Δ*hfq* mutant) were prepared by sonication in TNT buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) (30). Forty micrograms of each bacterial lysate was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond P membrane (Amersham). The membrane was incubated with polyclonal antibodies against Fur (1:5,000 dilution) and then with alkaline phosphatase-conjugated rabbit anti-rat immunoglobulin G (IgG; 1:1,000 dilution) (Sigma). Immunoreactive protein bands were visualized using the nitroblue tetrazolium—5-bromo-4-chloro-3-indolyphosphate (NBT-BCIP) system (Promega).

Construction of an *hfq* knockout mutant strain. An *hfq* upstream region of 1,062 bp was amplified from the genomic DNA of *V. vulnificus* MO6-24/O using two primers, hfq_upF (5′-CCCAAGCTTACGAATCCGATTGCGTCAACG-3′; underlined sequence denotes a HindIII restriction site) and hfq_upR (5′-AAACTGCAGTGTAGAGATTGCCCCTTAGCC-3′; underlined sequence denotes a PstI restriction site). The PCR product was then cloned into a plasmid, pBluescript SKII (+), to produce pSKhfq_up (Table 1). A 1,029-bp DNA fragment containing a downstream region of the *hfq* gene was made using primers hfq_downF (5′-AAACTGCAGATCGTCCAAGCGAGAAATCGG-3′; underlined sequence denotes the restriction site for PstI) and hfq_downR (5′-GGACTAGTTCTATCTTGTTCATCACCACG-3′; underlined sequence denotes a SpeI restriction site) and cloned into the corresponding sites of pSKhfq_up to result in pSKhfq_up/down. A 1.2-kb kanamycin-resistance gene was isolated from pUC4K (Pharmacia) and inserted into the PstI site of pSKhfq_up/down to produce pSKhfq_up/km/down. A 3,291-bp DNA fragment of pSKhfq_up/km/down digested with HindIII and SpeI was ligated to a suicide vector, pDM4 (26), to generate pDMΔhfq. *E. coli* SM10 λ *pir* strain carrying pDMΔhfq was conjugated with *V. vulnificus* MO6-24/O, and the exconjugants were then selected on TCBS (thiosulfate-citrate-bile salts-sucrose) medium supplemented with 2 μg ml^{-1} chloramphenicol. Colonies with characteristics indicating a double homologous recombination event (resistance to 5% sucrose and sensitivity to chloramphenicol) were further confirmed by PCR using the primers hfq_upF and hfq_downR, and the strain was named HLM102. The resultant Δ*hfq* mutant, HLM102, lost a main portion of the coding region of Hfq from amino acid residues 8 to 79 and instead had the *nptI* gene responsible for resistance to kanamycin.

DNase I footprinting. A 482-bp DNA fragment of the *fur* upstream region was amplified by PCR using a labeled fur_RT primer (5′-CACAACCGTTAGGCC AACGACATCATGAAG-3′) and an unlabeled fur_F3 primer (5′-ATACTCCC GCCATACTGAGTACGATTGGCG-3′). The binding of recombinant Fur protein to the labeled *fur* promoter was performed for 30 min at 37°C in a reaction buffer containing 10 mM BisTris-borate (pH 7.5), 5 μg ml^{-1} sonicated salmon sperm DNA, 5% glycerol, 100 μM $MnCl_2$, 100 μg ml^{-1} bovine serum albumin, 1 mM $MgCl_2$, and 40 mM KCl. The reaction mixture was treated with DNase I (9.4×10^{-3} U) for 1 min at room temperature, and the reaction was ended by adding a stop buffer (20 mM NaCI, 20 mM EDTA, 1% SDS, and 250 μg ml^{-1} tRNA). After precipitation with ethanol, the digested DNA products were resolved on a 6% polyacrylamide sequencing gel alongside sequencing ladders. Sequencing ladders were generated from pGEMT-fur, a plasmid containing the *fur* upstream region using labeled fur_RT primer and the AccuPower DNA sequencing kit (Bioneer).

Site-directed mutagenesis of the *fur* promoter. The putative Fur-binding site includes a pair of direct repeats, AAATTGT, located at −112 to −118 and −123 to −129 relative to the *fur* transcriptional start site. Direct repeats were mutagenized into GGGCCGC, using primers carrying the 12-bp substitution. To amplify the *fur* promoter region between −335 and −106 relative to the *fur* transcriptional start site, two primers, fur_R4 (5′-CCAGTTGCGGCCCAATAGCGGCCCGCAAAGAGAGCTAGGAAGGC-3′; underlined sequences denote the altered bases) and fur_upF (5′-CCCCGGTACCACTCCCGCCATACTGAG-3′), were utilized. Another set of primers, fur_F4 (5′-CGGGCCGCTATTGGGCCGCAACTGGTCAGATGAGCAATATAAGCG-3′; underlined sequences denote the altered bases) and fur_RT (5′-CACAACCGTTAGGCCAACGACATCATGAAG-3′), was used to produce the *fur* promoter region between −149 and +145 relative to the *fur* transcriptional start site. Two PCR products (a 241-bp PCR product using fur_upF and fur_R4 and a 294-bp PCR product using fur_F4 and fur_RT) were used as template DNAs to produce the mutagenized *fur* DNA fragment, encompassing the segment from −335 to +145, using the primers fur_upF and fur_RT. The resultant mutagenized *fur* promoter DNA was cloned into pGEM-T Easy vector (Promega) to produce pGEMT-fur_{mt}. Then, a DNA fragment containing the mutagenized Fur-binding site, which covers the same region of the *fur* upstream region present in pHL01 (21), was amplified using pGEMT-fur_{mt} and the primers fur_upF and fur_upR (5′-ACCAGGATCCTTTAGCGCTTGGTTATTGTCT-3′). The PCR product was digested with KpnI and XbaI and then ligated to KpnI/XbaI-digested pHK0011, which contained the promoterless *luxAB* genes (16). The resultant plasmid, pHL03, was mobilized into wild-type and Δ*fur* mutant *V. vulnificus* by conjugation, and the exconjugants were selected in TCBS medium supplemented with 3 μg ml^{-1} tetracycline. The light produced by these cells was measured in the presence of 0.006% (vol/vol) *n*-decyl aldehyde using a luminometer (TD-20/20 Luminometer; Turners Designs). Specific bioluminescence was calculated by normalizing the relative light units (RLU) with respect to cell mass (optical density at 600 nm [OD_{600}]) as described previously (21).

Statistical analyses. Results were expressed as the means ± standard deviations from four independent experiments. Statistical analysis was performed using a Student's *t* test (SYSTAT program, SigmaPlot version 9; Systat Software Inc.). Differences were considered significant if *P* values were <0.01. Data with *P* values of <0.001 are indicated by two asterisks, whereas data with *P* values between 0.001 and 0.01 are indicated by a single asterisk (see Fig. 3).

RESULTS

Increased formation of Fur protein under iron-limited conditions. *V. vulnificus* cells at exponential and stationary phases were treated with an iron chelator, 2,2′-dipyridyl, to challenge the cells under iron-limited conditions, and the levels of Fur proteins from the challenged cells were compared to the levels from control cells grown in the same medium but without addition of the iron chelator. Western blot analysis showed that the extracts of *V. vulnificus* grown in the presence of iron chelator demonstrated approximately twofold increased amounts of Fur in comparison with *V. vulnificus* cultivated in the absence of iron chelator (Fig. 1). This result raises a possibility that the expression of the *fur* gene might be modulated at the posttranscriptional level by a negative regulator, of which expression is repressed by the iron-Fur complex and derepressed by iron chelators. An example for such a regulator is the sRNA from *E. coli* (24).

Role of Hfq in the synthesis of Fur protein. The Fur protein is a well-known repressor of diverse iron-regulated genes in an Fe^{2+}-dependent manner (13). However, some genes were identified as positively regulated by Fur in *E. coli* such as those encoding tricarboxylic acid cycle enzymes, fumarase and succinate dehydrogenase (11, 14). Positive regulation of these genes by Fur has been explained by the concerted actions of an sRNA, RyhB, and an RNA-binding protein, Hfq, which decrease the mRNA levels and/or the translation efficiency of these genes (24). Fur functions as a positive regulator for these target genes in an indirect manner, by which Fur represses the expression of the *ryhB* gene. Thus, we first examined if the autoregulation of the *fur* gene in *V. vulnificus* might occur at a posttranscriptional level via actions of an sRNA and Hfq. BLASTP searches of the *V. vulnificus* databases (*V. vulnificus* strain CMCP6, GenBank accession no. NC_004459.1; *V. vulnificus* strain YJ016, GenBank accession no. NC_005139.1) using the amino acid sequence of the Hfq protein from *V. cholerae* (NP_230001) revealed an ORF composed of 86 amino acid residues, which shows 94% and 87% amino acid identities with *V. cholerae* Hfq (NP_230001) and *E. coli* Hfq (NP_418593), respectively.

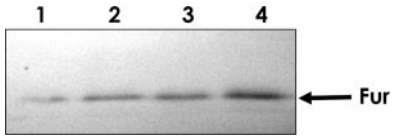

FIG. 1. Effect of iron availability on intracellular levels of Fur protein in wild-type *V. vulnificus*. *V. vulnificus* grown in LBS medium at the exponential phase and at the stationary phase were used for estimating cellular contents of Fur protein. Iron in the medium was depleted by adding 0.2 mM 2,2′-dipyridyl to the early exponential cultures (OD_{600} of 0.1). Forty micrograms of each bacterial lysate was fractionated by SDS-PAGE. The blotted membrane was treated with polyclonal antibodies raised against recombinant Fur and then with alkaline phosphatase-conjugated rabbit anti-rat IgG. Upon incubation with the NBT-BCIP system, the Fur protein (17 kDa) appeared as an immunoreactive band, as indicated by the arrow. Lane 1, lysate prepared from exponential phase *V. vulnificus* in medium without iron chelator; lane 2, lysate prepared from exponential phase *V. vulnificus* in medium with iron chelator; lane 3, lysate prepared from stationary phase *V. vulnificus* in medium without iron chelator; and lane 4, lysate prepared from stationary phase *V. vulnificus* in medium with iron chelator.

Deletion of the *hfq* gene from the chromosome of the mutant *V. vulnificus* was confirmed by PCR using the primers hfq_upF and hfq_downR. The resultant PCR product from the Δ*hfq* mutant *V. vulnificus*, HLM102, was 3.6 kb, whereas the intact *hfq* gene in the wild type produced a smaller PCR product of 2.4 kb (data not shown).

To verify that the *hfq*-homologous ORF, which was deleted in HLM102, has an analogous function to *E. coli* Hfq, the level of RpoS protein in HLM102 was compared to that of RpoS in wild-type *V. vulnificus* by Western blot analysis using polyclonal antibodies raised against recombinant RpoS from *V. vulnificus* (27). It was reported that Hfq controls the stability of the *rpoS* mRNA and that *hfq* mutant *E. coli* synthesizes a decreased amount of RpoS (22). Decreased amounts of RpoS protein were also observed in the mutant *V. vulnificus* HLM102 in comparison with levels in the wild-type (Fig. 2A), suggesting that *V. vulnificus* Hfq carries an analogous function to *E. coli* Hfq, at least in respect to the regulation of sigma factor σ^S.

Intracellular levels of Fur protein were examined by Western blot analysis in wild type and the Δ*hfq* mutant *V. vulnificus* using polyclonal antibodies specific to recombinant Fur protein from *V. vulnificus* (Fig. 2B). No obvious alteration in the amount of Fur protein was detected in the Δ*hfq* mutant in comparison with that of wild-type *V. vulnificus* under both exponential and stationary phases. These results suggest that positive autoregulation of *fur* expression is not mediated by Hfq.

Autoregulation of the *fur* gene at the transcriptional level in *V. vulnificus*. A previous report of *fur* gene expression in *V.*

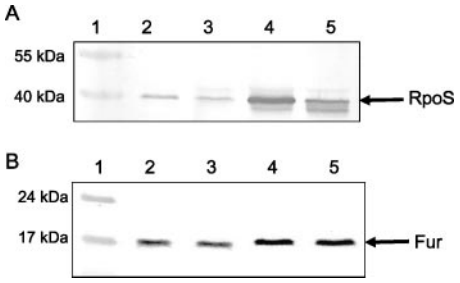

FIG. 2. Effect of the Δ*hfq* mutation on the intracellular levels of RpoS and Fur protein. Lysates of wild type and Δ*hfq* mutant grown at the exponential phase (OD_{600} of 0.5) and at the stationary phase (OD_{600} of 2.0) were used for estimating cellular contents of RpoS (A) and Fur protein (B). Forty micrograms of each bacterial lysate was fractionated by SDS-PAGE. The blotted membrane was treated with polyclonal antibodies raised against recombinant RpoS or Fur and then with alkaline phosphatase-conjugated rabbit anti-rat IgG. Upon incubation with the NBT-BCIP system, the Fur protein or RpoS protein appeared as an immunoreactive band, indicated by an arrow. Lanes 1, protein size marker; lanes 2, wild type at exponential phase; lanes 3, Δ*hfq* mutant at exponential phase; lanes 4, wild type at stationary phase; and lanes 5, Δ*hfq* mutant at stationary phase.

vulnificus indicated that Fur positively modulates its own expression in an unidentified way (21). The expression of a *fur*::*luxAB* transcriptional fusion (pHL01) in Δ*fur* mutant *V. vulnificus* was reduced to 36% of that of wild type (Fig. 3). The effect of iron ion on expression of the *fur* gene was also examined by adding an iron chelator, 2,2′-dipyridyl, to cultures at the early exponential stage (OD_{600} of ~0.1). While the expression of the *fur*::*luxAB* fusion was increased more than twofold under the iron-depleted conditions in wild type, its expression in the Δ*fur* mutant was not altered by the addition of the iron

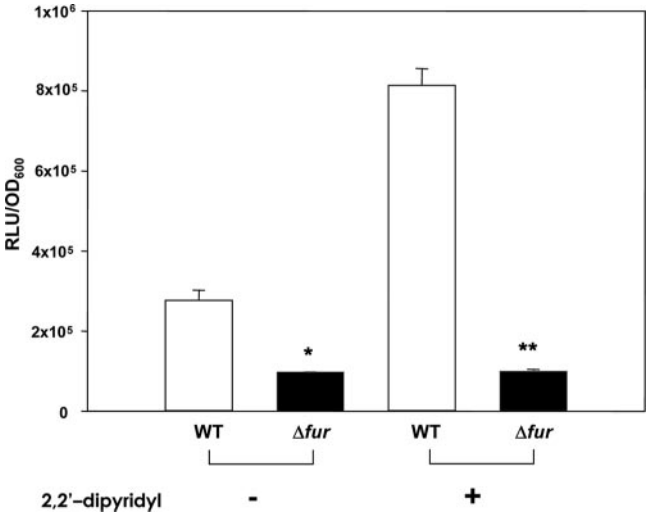

FIG. 3. Effect of iron availability on *fur*::*luxAB* (pHL01) expression in the wild-type and in the Δ*fur* mutant *V. vulnificus*. Wild type (WT) and Δ*fur* mutant *V. vulnificus* carrying pHL01 were grown in LBS medium supplemented with 5 μg ml^{-1} tetracycline. Iron in the medium was depleted by adding 0.2 mM 2,2′-dipyridyl to the early exponential cultures (OD_{600} of 0.1). The *fur*::*luxAB* activities were normalized by dividing the number of RLUs by the OD_{600} value. The *fur*::*luxAB* activities of four independent cultures at exponential phase (OD_{600} of 0.5) were averaged and are indicated with their standard deviations.

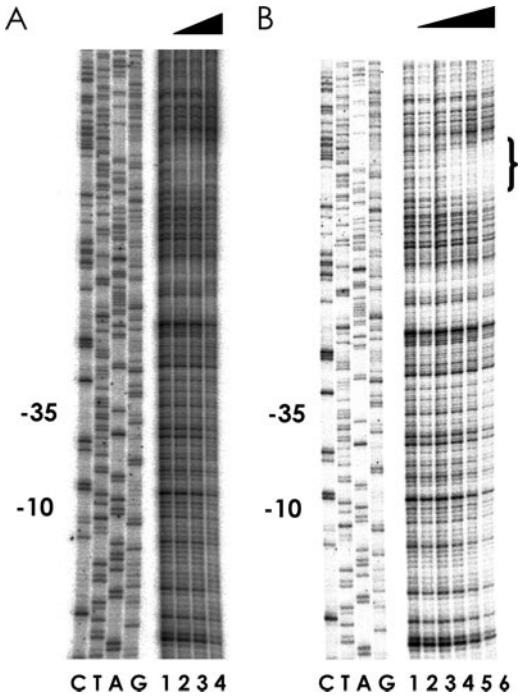

FIG. 4. Binding of Fur protein to the upstream region of the *fur* gene. DNase I-footprinting assays were performed to localize the Fur-binding site in the regulatory region of the *fur* gene in the absence of iron chelator (A) or in the presence of iron chelator (B). The ^{32}P-labeled 482 bp-DNA fragment of the *fur* promoter region was incubated with increasing amounts of Fur protein, ranging from 1.5 to 17 μM, and the binding reactions were then treated with DNase I. The reaction mixtures were resolved on a 6% polyacrylamide sequencing gel alongside the sequencing ladder derived from the plasmid pGEMT-fur. The protected region of the *fur* promoter is shown by a bracket. (A) Lane 1, DNA without Fur; lanes 2 to 4, DNA with recombinant Fur protein at 5.8, 12.0, and 17.0 μM, respectively. (B) Lane 1, DNA without Fur; lanes 2 to 6, DNA with recombinant Fur protein at 1.5, 2.9, 5.8, 12.0, and 17.0 μM, respectively.

chelator. These results indicate that the Fur protein activates its own expression, and the extent of its activation is elevated in the presence of iron chelator added to the growth medium. Subsequent experiments were performed to define the mechanism by which Fur protein acts as a positive regulator for its own expression.

Specific binding of Fur to the *fur* promoter. Since *fur* expression in *V. vulnificus* was not found to be regulated indirectly via Hfq, the possibility for direct interaction of Fur protein was investigated by DNase I-footprinting assays using recombinant Fur protein and a DNA encompassing the *fur* upstream region. A ^{32}P-labeled 482-bp *fur* promoter (covering from −337 to +145 nucleotide positions relative to the *fur* transcriptional start site) was incubated with increasing amounts of recombinant Fur protein ranging from 1.5 to 17.0 μM and then treated with DNase I. As a control, the labeled *fur* DNA without preincubation with Fur was also treated with DNase I in the same manner. After the reaction mixtures were subjected to denaturing polyacrylamide gel electrophoresis, the DNase I-digested patterns were observed in an autoradiograph. When Fur protein at a concentration of more than 2.9 μM was added to the binding reaction containing an iron

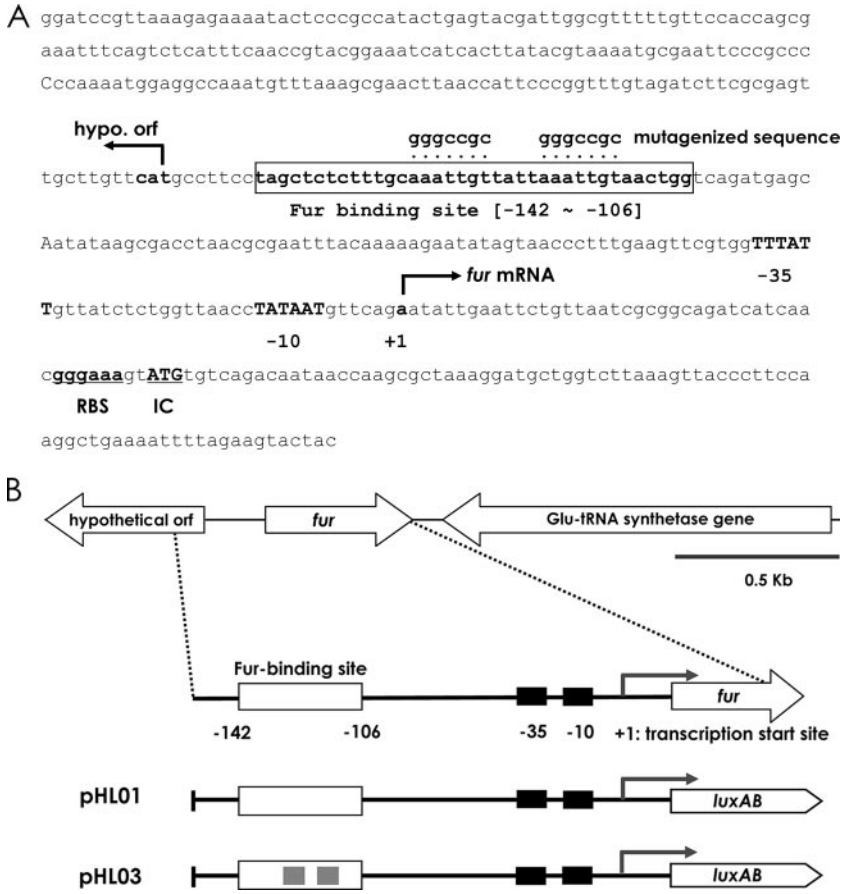

FIG. 5. Upstream region of the *fur* gene of *V. vulnificus* MO6-24/O. (A) The putative −10 and −35 sequence of the *fur* promoter are indicated in bold capitals. The transcriptional initiation site for the *fur* gene is represented with an arrowhead. Both the ribosomal binding sequence (RBS) and initiation codon (IC) for Fur protein are underlined. The Fur-protected region in the *fur* promoter is marked in a box and is located from nucleotides −142 to −106 with respect to the transcriptional start site of the *fur* gene. Mutagenized bases in the mutant *fur* promoter are indicated above the Fur binding site. (B) Two transcriptional fusions with the wild type (*fur*::*luxAB*; pHL01) or mutated Fur binding site (fur_{mt}::*luxAB*; pHL03) are represented in a schematic picture. The putative −10 and −35 sequences of the *fur* promoter are indicated in closed boxes, whereas the transcriptional initiation site for the *fur* gene is shown as an arrowhead. The Fur-binding region is displayed as an open box, and the altered region in the mutant *fur* promoter is indicated by gray boxes.

chelator, a portion of the *fur* promoter appeared as a region protected from DNase I, located between nucleotides −142 and −106 (5′-TAGCTCTCTTTGCAAATTGTTATTAAATT GTAACTGG-3′) with respect to the transcriptional start site of the *fur* mRNA (Fig. 4B). However, a protected region was barely observed in the *fur* upstream region, regardless of the concentration of Fur protein, if an iron chelator was not added to reaction mixture (Fig. 4A).

Role of Fur-P_{fur} interaction in *fur* expression. A subsequent experiment evaluated whether the protected region of the *fur* upstream region (P_{fur}) was indeed a Fur-binding site and if the interaction between Fur and the protected DNA sequence is essential for autoregulation of the *fur* gene. In the putative Fur-binding site, a pair of direct repeats, AAATTGT, was found at two positions from −112 to −118 and from −123 to −129 with respect to the transcriptional start site of the *fur* mRNA (Fig. 5A). To elucidate the role of these repeats in Fur-binding, the repeats were mutagenized into GGGCCGC. The mutagenized P_{fur} (P_{furM}) was used for constructing a fur_{mt}::*luxAB* transcriptional fusion (Fig. 5B) as well as for DNase I-footprinting with recombinant Fur protein.

P_{furM} did not show any region protected by Fur protein from DNase I under any tested conditions, regardless of the addition of iron chelator (Fig. 6A and B), suggesting that the mutagenized P_{fur} was unable to interact with Fur and that both or one of the repeats in the original P_{fur} may be a critical component in the Fur-P_{fur} recognition.

The role of Fur as an activator for expression of its coding gene was also confirmed in an additional experiment utilizing the P_{furM}. A fur_{mt}::*luxAB* transcriptional fusion (pHL03) was constructed, in which the inserted DNA covers the same region of the *fur* upstream region in pHL01 (21) but contains the mutagenized Fur-binding site. In the Δ*fur* mutant *V. vulnificus*, expression of the *fur*::*luxAB* fusion containing wild-type P_{fur} was decreased to approximately less than 40% of that in wild-type *V. vulnificus* (Fig. 3 and 6C and D). The degree of expression of the fur_{mt}::*luxAB* fusion in the wild type was highly reduced (Fig. 6C), and its expression was comparable to fur_{mt}::*luxAB* expression in the Δ*fur*

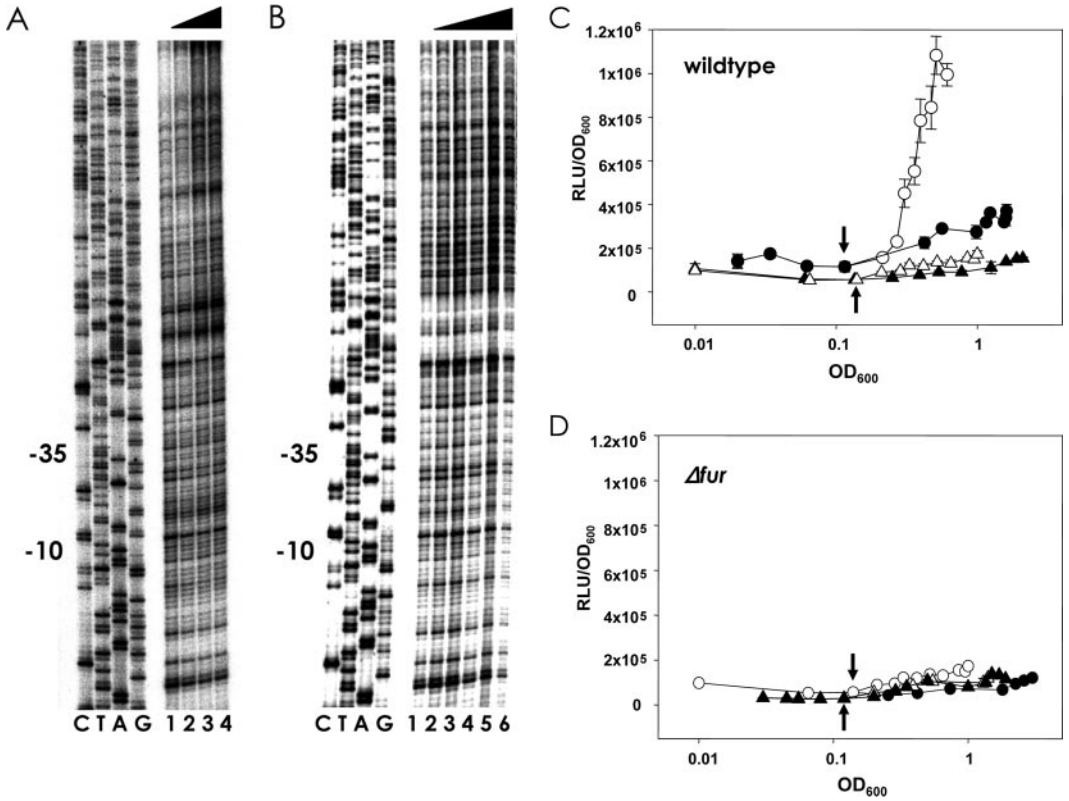

FIG. 6. Effect of the mutated Fur binding site on Fur-P_{fur} interaction (A and B) and expression of fur_{mt}::*luxAB* (pHL01) and fur_{mt}::*luxAB* (pHL03) (C and D). Binding of Fur to the mutated Fur binding site was examined by a DNase I-footprinting assay in the absence of iron chelator in panel A or in the presence of iron chelator in panel B. The ^{32}P-labeled 482 bp-DNA fragment of the *fur* promoter region with the mutated Fur binding site was incubated with increasing amounts of Fur protein, ranging from 1.5 to 17 μM, and then treated with DNase I. The reaction mixtures were resolved on a 6% polyacrylamide sequencing gel alongside the sequencing ladder derived from the plasmid pGEMT-fur. (A) Lane 1, DNA without Fur; lanes 2 to 4, DNA with recombinant Fur protein at 5.8, 12.0, and 17.0 μM, respectively. (B) Lane 1, DNA without Fur; lanes 2 to 6, DNA with recombinant Fur protein at 1.5, 2.9, 5.8, 12.0, and 17.0 μM, respectively. Wild-type (C) and Δ*fur* mutant (D) strains carrying pHL01 or pHL03 were grown in LBS medium supplemented with 5 μg ml^{-1} tetracycline and measured for luciferase activity. Iron in the medium was depleted by adding 0.2 mM 2,2′-dipyridyl, an iron chelator, into the cultures at the time points indicated with arrows. Luciferase activities are expressed as normalized values: number of RLUs divided by the OD_{600} value of each sample. Values are shown for the activity of *fur*::*luxAB* (pHL01) in the absence (closed circles) or presence (open circles) of an iron chelator and for fur_{mt}::*luxAB* (pHL03) containing the mutated Fur binding site in *V. vulnificus* strains cultivated without (closed triangles) or with (open triangles) the addition of an iron chelator.

mutant and *fur*::*luxAB* expression in the Δ*fur* mutant (Fig. 6D). This result indicates that the mutated *fur* upstream region is no longer influenced by Fur and therefore suggests that Fur activates expression of the *fur* gene by directly binding to P_{fur}. These data were strengthened by a fusion analysis in which the expression of both the *fur*::*luxAB* and the fur_{mt}::*luxAB* fusions was monitored in the presence or absence of an iron chelator, 2,2′-dipyridyl. In wild-type *V. vulnificus*, activity of the *fur*::*luxAB* fusion was induced with an iron chelator, whereas there was no detectable increase in expression of the fur_{mt}::*luxAB* in the same strain of *V. vulnificus* (Fig. 6C). In a Δ*fur* mutant *V. vulnificus*, no apparent increase of luciferase was observed with the addition of the iron chelator regardless of the kinds of fusion (Fig. 6D). These results indicated that expression of the *fur* gene is not influenced by iron when it contains the mutagenized Fur-binding site.

DISCUSSION

Fur protein is well known as a repressor of iron-responsive genes (13). The list of Fur-controlled genes has been extended by finding a subset of genes which seem to be activated by Fur in the presence of iron (15). This activating function of Fur is achieved through the action of Hfq protein and RyhB RNA, whose expression is repressed by the iron-Fur complex under iron-rich conditions (24). Thus, the positive regulation by Fur occurs indirectly at the posttranscriptional level in this case.

In this study, higher levels of Fur protein were observed upon incubation of *V. vulnificus* cells in the presence of an iron chelator (Fig. 1). This led us to examine the possibility that Fur controls its own expression at the posttranscriptional level via the sRNA-Hfq system. The Hfq proteins from different organisms showed a high conservation of their N termini containing the Sm1 sequence motif, whereas there is considerable variation at their C-terminal regions (32). An Hfq-homologous protein in *V. vulnificus* was identified (GenBank accession no. NP_760222). The N terminus of the *V. vulnificus* Hfq protein showed strong conservation with the N-terminal regions of other Hfq proteins, whereas the C-terminal region of *V. vulnificus* Hfq showed little homology with other homologous proteins. Overall, the putative Hfq of *V. vulnificus* displays

87%, 92%, and 94% identities with the proteins of *E. coli*, *Salmonella enterica* serovar Typhimurium, and *V. cholerae*, respectively. One of the reported functions of Hfq in *E. coli* is that it controls the amount of RpoS by increasing the translational efficiency of *rpoS* mRNA along with another sRNA, DsrA (22). Deletion of this *hfq*-homologous ORF also resulted in a decrease in the amount of RpoS in *V. vulnificus* (Fig. 2A), suggesting that the gene product of the *hfq*-homologous ORF in *V. vulnificus* has functions analogous to the Hfq found in other bacterial species. In addition, the *V. vulnificus* chromosome contains a putative gene which is highly homologous to the known *rhyB* sequences (25). Thus, it is probable that *V. vulnificus* Hfq could be involved in the control of several genes in collaboration with various sRNAs including RyhB.

If the Hfq-RyhB system is involved in *fur* autoregulation, the amount of Fur should be increased in an *hfq*-deficient mutant *V. vulnificus*. The intracellular levels of Fur in the Δ*hfq V. vulnificus* strain were measured and found to be similar to those in the wild-type strain, indicating that Hfq-RyhB is not responsible for *fur* autoregulation in *V. vulnificus* (Fig. 2B). This result was strengthened by quantitative reverse transcription-PCR analysis of the *fur* transcript in wild-type and Δ*hfq V. vulnificus*, which was not affected by the *hfq* mutation at all (H.-J. Lee and S.-J. Park, unpublished data). Thus, it is not likely that activation of the *V. vulnificus fur* by Fur is indirectly regulated by the sRNA-Hfq system.

An observation that Fur activates its own expression in *V. vulnificus* (21) (Fig. 3) raised a question as to how the positive control of the *fur* gene by its own gene product could occur. This mode of *fur* autoregulation is distinct in that the level of activation of *fur* expression was highly elevated under iron-depleted conditions (Fig. 3) in contrast to iron-dependent indirect activation of certain genes by Fur (24).

A plausible explanation for the positive autoregulation of the *fur* gene in *V. vulnificus* is that Fur directly binds the upstream region of the *fur* gene under iron-depleted conditions and then activates its expression. In *H. pylori*, Fur regulates the transcription of the *fur* gene by binding to multiple sites upstream of the *fur* gene in response to the level of iron concentrations (7). In this case, an iron-complexed Fur or an iron-free Fur binds to each operator region with different binding affinities, which results in differential regulatory effects on *fur* expression (8).

V. vulnificus Fur also controls its own expression in a positive way by directly binding to the upstream region of P_{fur}, which is located at positions −142 to −106 relative to the transcriptional start site of the *fur* gene (Fig. 4B). Fur binding to this site in *V. vulnificus* occurs only when the iron concentration is low, similar to when the iron-free Fur binds to the distal operator in *H. pylori*. However, an interaction between iron-free Fur and the corresponding site resulted in derepression of *fur* transcription in *H. pylori*. In contrast to multiple Fur-binding sites in upstream regions of P_{fur} in *H. pylori*, no additional Fur-binding sites were found in the upstream regions of P_{fur} in *V. vulnificus*.

Fur binding to this Fur-binding site occurred when a high concentration of Fur protein (higher than 2.9 μM) was present in the binding reaction containing an iron chelator (Fig. 4B). This finding demonstrates that this Fur-binding site has a lower affinity for Fur than the Fur-binding sites in *E. coli* and the other transcriptional factor-binding sites in *V. vulnificus*, in which DNA-binding proteins showed apparent binding to corresponding DNA sites at nanomolar concentrations (17, 19, 28). Requirement of a high concentration of Fur for protection of the *fur* promoter against DNase I digestion raises the possibility that most of the recombinant Fur protein prepared might be inactive for binding. Otherwise, a form of Fur protein capable of binding the *fur* promoter, i.e., an iron-free Fur protein, can be converted into other form(s) of Fur unable to bind to the same region, and the former may be present in a minor amount in our Fur binding reactions. Low-affinity binding sites of Fur have been also documented in other bacterial species (5, 6, 8, 29). In *Neisseria meningitides*, one of the two Fur binding sites in the *fur* upstream region was shown to have a low affinity (5). The *furA* of *Mycobacterium tuberculosis* was also regulated by its own gene product via direct binding, and its binding required a relatively high concentration of FurA protein at the level of micromolar concentrations (29). Measurements of intracellular levels of Fur indicate that a large amount of Fur protein is constitutively produced in *V. cholera*, and the cellular level of Fur is further increased under some conditions in *E. coli* (36, 40). These reports suggest that the direct interaction between the *fur* promoter and Fur indeed occurs in *V. vulnificus*, perhaps when Fur protein is accumulated at high concentrations under some physiological conditions. The physiological function of Fur autoregulation in *V. vulnificus* should be revealed in further investigations.

The Fur binding sequence in P_{fur} of *V. vulnificus* is a 37-bp sequence containing two direct repeats of 5′-AAATTGT-3′ (Fig. 5A). These Fur-boxes, binding sites for an iron-complexed Fur, contain a 19-bp consensus sequence, 5′-GATAA TGATAATCATTATC-3′ (19). No such consensus sequences were found in the *fur* promoter region protected by Fur in *V. vulnificus*. The upstream region of P_{fur} from *H. pylori*, to which iron-free Fur specifically binds, also did not show any homologous sequences to classical Fur-boxes (8). Sequence analysis of the *V. vulnificus* genome reveals the presence of the direct repeats of AAATTGT in the upstream regions of several putative iron-related ORFs (data not shown). It remains to be further studied if these genes are regulated by iron-free Fur and if this repeated sequence is, in fact, involved in positive regulation by Fur.

Site-directed mutagenesis of the repeat sequence within a Fur-protected region resulted in abolishment of the interaction between Fur protein and the P_{fur} of *V. vulnificus* (Fig. 5A and B and 6B). This result indicates that the regions from −112 to −118 and/or from −123 to −129 are, indeed, critical sequences for Fur binding to the P_{fur}. Disruption of the P_{fur}-Fur interaction also decreased transcription of *fur*::*luxAB* expression regardless of the presence of functional Fur (Fig. 6C and D). In addition, induction of *fur* expression by an iron chelator was abolished when the Fur-binding site of P_{fur} was mutated. These data strongly support an important role of Fur-P_{fur} interaction in positive autoregulation of *fur* expression under iron-depleted conditions.

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