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

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Received 28 November 2006/Accepted 16 January 2007

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 the 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 (furmt::luxAB). Expression of furmt::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, famA, 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 lns 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 hfg 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
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 conjugal 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’-CGGGGATCCATGTCAGGACTGACAAATACACGGC-3’) and *fur*downR (5’-AACTGCAGATCGTCCAAGCGAGAAATCGG-3’); underlined sequence denotes a HindIII restriction site) and *hfq*downF (5’-AAACTGCAGTGTAGAGATTGCCCCTTAGCC-3’) and an unlabeled *fur*F3 primer (5’-ATATCCCGCCTACTGAGTATAGGCC-3’); underlined sequence denotes an ApRI restriction site). The PCR product was then cloned into a plasmid, pBluescript II SK(+), 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 and *hfq*downR, and the strain was named HLM102. The resultant DNA fragment was digested with HindIII and SpeI was ligated to a suicide vector, pDM4 (26), and cloned into the corresponding sites of pSKhfqup to result in pSKhfq_up/down. A 1.2-kb kanamycin-resistance gene was isolated from pUC4K (Pharmacia) and inserted into the *E. coli* strain carrying pSKhfq_up/down to produce pSKhfq_upkm/down. A 2.391-bp DNA fragment of pSKhfq_upkm/down digested with HindIII and SpeI was ligated to a suicide vector, pDM4 (26), to generate pDMhfq. *E. coli* SM10 *Δ fur* strain carrying pDMhfq 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*downK, 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 analogous recombination event (resistance to 5% sucrose and sensitivity to chloramphenicol) were further confirmed by PCR using the primers *hfq*upF and *hfq*downK, 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 analogous recombination event (resistance to 5% sucrose and sensitivity to chloramphenicol) were further confirmed by PCR using the primers *hfq*upF and *hfq*downK, and the strain was named HLM102.

**DNaSe I footprinting.** A 482-bp DNA fragment of the *fur* upstream region was amplified by PCR using a labeled fur_RT primer (5’-CGAACTGAGTATAGGCCACCAGCATCATGAG-3’) and an unlabeled fur_F3 primer (5’-ATATCGATGCCACTGGAAGAGAGGCTTTAGCC-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 Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20 (30). Forty micromolars 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).

**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’-CCCCACGTACATCGGAATCCGGAGGTGCGTCAAAG-3’); underlined sequence denotes a HindIII restriction site) and *hfq*upR (5’-AACCTGCAATGTAAGATGGCCCTTTAGCC-3’); underlined sequence denotes a PstI restriction site). The PCR product was then cloned into a plasmid, pBluescript SKI (+, 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 and *hfq*downR, and the strain was named HLM102. The resultant DNA fragment was digested with HindIII and SpeI was ligated to a suicide vector, pDM4 (26), 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 *E. coli* strain carrying pSKhfq_up/down to produce pSKhfq_upkm/down. A 2.391-bp DNA fragment of pSKhfq_upkm/down digested with HindIII and SpeI was ligated to a suicide vector, pDM4 (26), to generate pDMhfq. *E. coli* SM10 Δ fur strain carrying pDMhfq 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*downK, and the strain was named HLM102. The resultant Δhfq mutant, HLM102, lost a main portion of the coding region of *Hfq* from amino acid residues 8 to 79 and instead had the analogous recombination event (resistance to 5% sucrose and sensitivity to chloramphenicol) were further confirmed by PCR using the primers *hfq*upF and *hfq*downK, and the strain was named HLM102. The resultant Δhfq mutant, HLM102, lost a main portion of the coding region of *Hfq* from amino acid residues 8 to 79 and instead had the analogous recombination event (resistance to 5% sucrose and sensitivity to chloramphenicol) were further confirmed by PCR using the primers *hfq*upF and *hfq*downK, and the strain was named HLM102.
Site-directed mutagenesis of the fur promoter. The putative Fur-binding site includes a pair of direct repeats, AAATTTG, located at −112 to −118 and −123 to −129 relative to the fur transcriptional start site. Direct repeats were mutated into GGCGCGCG, 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′-CAACGACGCAAGGGGCATACCCGCAGAAGCACG-3′; underlined sequences denote the altered bases) and fur_upF (5′-CCCCGGATCCACCTGCGAGAGCTTC-3′), were utilized. An other set of primers, fur_F4 (5′-CGGCGCGCGGTTAGGGGCGGTCATGCGAT GACAAATAAAGCG-3′; underlined sequences denote the altered bases) and fur_RT (5′-CAACCGTCATTGCGGCGGTCATGCGAT GACAAATAAAGCG-3′), were 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_RT) were used as template DNAs to produce the mutagenized Fur-binding site, which covers the same region of the DNA fragment containing the mutagenized Fur-binding site, which covers the same region of the fur upstream region present in PHL03 (21), was amplified using pGEM-T Easy vector (Promega) to produce pGEMT-furup_furrt. A DNA fragment containing the mutagenised Fur-binding site, which covers the same region of the fur upstream region present in PHL01 (21), was amplified using pGEM-T-furup_furt and the primers fur_upF and fur_upR (5′-ACACAGGATCTTT AGCGGCGCTGATGATGTCT-3′). The PCR product was digested with KpnI and XbaI and then ligated to KpnI/XbaI-digested pHK0011, which contained the promoterless luxCdB genes (16). The resultant plasmid, pHLO3, was mobilized into wild-type and Δfur 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-acetyl α-diphenylamine (TD-20/20 Luminometer, Turner Designs). Specific bioluminescence was calculated by normalizing the relative light units (RLU) with respect to cell mass (optical density at 600 nm [OD₆₀₀]) as described previously (21).

Statistical analyses. Results were expressed as the means ± standard deviations from four independent experiments. Statistical analysis was performed using 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 absence 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 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 Fe²⁺-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 Y1016, 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 57% amino acid identities with V. cholerae Hfq (NP_230001) and E. coli Hfq (NP_418593), respectively.

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 Δhfq 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. cholerae (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 σ⁷.

Intracellular levels of Fur protein were examined by Western blot analysis in wild type and the Δhfq 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 Δhfq 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. vulnificus.
V. vulnificus indicated that Fur positively modulates its own expression in an unidentified way (21). The expression of a fur:luxAB transcriptional fusion (pHL01) in Δfur 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\textsubscript{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 Δfur mutant was not altered by the addition of the iron 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 \(^{32}\text{P}\)-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 \(\mu\text{M}\), 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 \(\mu\text{M}\), 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 \(\mu\text{M}\), respectively.
chelator, a portion of the fur promoter appeared as a region protected from DNase I, located between nucleotides −142 and −106 (5'-TAGCTCTTTTGCAAATTGTTATTAAATTGTAACCTGG-3') 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<sup>mt</sup>::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.

**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<sup>mt</sup>::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.

The role of Fur-P<sub>fur</sub> interaction in fur expression. A subsequent experiment evaluated whether the protected region of the fur upstream region (P<sub>fur</sub>) 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<sub>fur</sub> (P<sub>furM</sub>) was used for constructing a fur<sup>mut</sup>::luxAB transcriptional fusion (Fig. 5B) as well as for DNase I-footprinting with recombinant Fur protein.

P<sub>furM</sub> 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<sub>fur</sub> was unable to interact with Fur and that both or one of the repeats in the original P<sub>fur</sub> may be a critical component in the Fur-P<sub>fur</sub> recognition.

The role of Fur as an activator for expression of its coding gene was also confirmed in an additional experiment utilizing the P<sub>furM</sub> A fur<sup>mut</sup>::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 Δfur mutant *V. vulnificus*, expression of the fur::luxAB fusion containing wild-type P<sub>fur</sub> 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<sup>mut</sup>::luxAB fusion in the wild type was highly reduced (Fig. 6C), and its expression was comparable to fur<sup>mut</sup>::luxAB expression in the Δfur
mutant and fur::luxAB expression in the 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 Pfur. These data were strengthened by a fusion analysis in which the expression of both the fur::luxAB and furmt::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 furmt::luxAB in the same strain of V. vulnificus (Fig. 6C). In a Δfur 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 shows 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 *rhfB* 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 Δhfq *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 Δhfq *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 *Pfur*, 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 *Pfur* in *H. pylori*, no additional Fur-binding sites were found in the upstream regions of *Pfur* 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 meningitidis*, 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. cholerae*, 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 *Pfur* 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 *Pfur* 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 *Pfur* 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 *Pfur*. Disruption of the *Pfur*-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 *Pfur* was mutated. These data strongly support an important role of Fur-*Pfur* interaction in positive autoregulation of *fur* expression under iron-depleted conditions.

**ACKNOWLEDGMENTS**

This study was supported by the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science and Technology (grant MG05-0201-5-0 to S.-J.P. and K.-H.L.), Republic of Korea.
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