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Identification of the Fur-Binding Site in Regulatory Region of the Vulnibactin-Receptor Gene in *Vibrio vulnificus*

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The *Vibrio vulnificus vuuA* gene, of which expression is repressed by a complex of iron and ferric uptake regulator (Fur), was characterized to localize the Fur-binding site in its upstream regulatory region. *In silico* analysis suggested the presence of two possible Fur-binding sites; one is a classical Fur-box and the other is a previously reported distinct Fur-binding site. Site-directed mutagenesis and DNase I protection assays revealed the binding site for the iron–Fur complex, which includes an extended inverted repeat containing a homologous sequence to the classical Fur-box.

Keywords: Fur, vulnibactin-receptor gene, *Vibrio vulnificus*

It has been reported that iron uptake systems, such as low-molecular-weight iron chelators and siderophores, are essential for pathogenic interactions in many *Vibrio* spp. [5, 11, 15]. Among various siderophores excreted by vibrios, a catechol-based siderophore is called vibriobactin in *V. cholerae* [7] and vulnibactin in *V. vulnificus* [13]. Iron ions complexed with catechol-siderophores are transported by a series of uptake components; for example, an outermembrane-bound receptor protein for siderophore (VuuA or ViuA), a periplasmic ABC-type transporter of siderophore (FatB), and a cytoplasmic protein interacting with siderophore (VuuB or ViuB) [11].

V. vulnificus is a human pathogen causing a fatal primary septicemia, gastroenteritis, or serous wound infections [6, 16]. Three genomes of *V. vulnificus* strains have been reported (GenBank Accession No of CP002470.1 for MO6-24/O [14]; AE016796.2 for CMCP6 [8]; and BA000038.2 for YJ016 [2]), and all of them show the presence of the genes coding for the vulnibactin-mediated iron uptake components. *vuuA* (VVM_02499: gene number from the strain of MO6-24/O) and *vuuB* (VVM_02490) are interrupted by several genes, although *vuuA–B* comprise an operon in *V. cholerae* [1]. The *fatB* gene (VVM_02497) is located upstream of *vuuA*, but the orientation is opposite.

The *vuuA* gene has been cloned and the role of its gene product has been studied *in vivo*. Expression of the *vuuA* gene has been reported to be regulated by an iron-responsive transcription factor, ferric uptake regulator (Fur), *via* repressing its transcription, which has been shown by Northern analysis of transcripts [17] and 2-D gel analysis of proteomes of wild-type and *fur* mutant *V. vulnificus* [12]. Fur-mediated repression of the *vuuA* gene expression was speculated by an *in silico* analysis of the *vuuA* upstream DNA sequence, suggesting the presence of a nucleotide sequence homologous to the Fur-box (5'-GATAATGATAATCATTATC-3') [17].

Recently, it was reported that *V. vulnificus* Fur is able to bind to a distinct DNA sequence including an AT-rich direct repeat (5'-AAATTGTN$_4$AAATTGT-3') that is not homologous to the Fur box at all [9]. Sequence analysis of the *vuuA* upstream region showed the presence of the classical Fur-box sequence (overlapped with -35 region) as well as the distinct Fur-binding site (overlapped with -10 region), both of which can be speculated to be involved in transcription repression *via* competition with RNA polymerase (Fig. 1). Since the location of the Fur-binding site for *vuuA* gene expression has not yet been defined, we have performed site-directed mutagenesis, subsequent transcription fusion assay, and DNase I footprint assay to exactly localize the binding site.

Prior to localization of a Fur-binding site, the dependence of *vuuA* expression on iron and Fur was confirmed by using a transcription reporter constructed by fusing the *vuuA* upstream region with the *luxAB* genes. The PCR primers used for fusion construction are designated in Fig. 1. The resultant transcription fusion, p*vuuA*::*luxAB*, was introduced into the Δ*fur* mutant [9] and the wild-type

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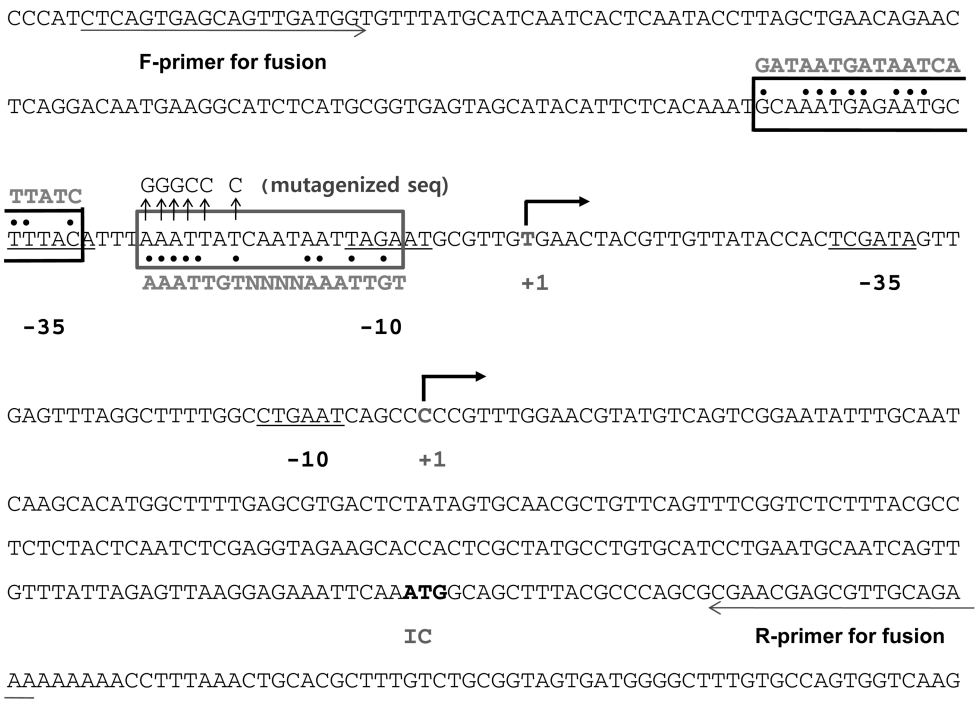


Fig. 1. Upstream region of the *vuuA* gene.
Two transcription initiation sites and the corresponding promoters, which have been identified by Webster and Litwin [17], are denoted by +1 and -35/-10, respectively. The tentative Fur-binding sites, which show the homologies with the classical Fur-box [4] or the distinct Fur-binding site [10], are presented with black and gray boxes, respectively. The PCR primers used for construction of the transcription reporter fusions, p*vuuA*::*luxAB* and p$vuuA_{MT}$::*luxAB*, are designated by F- and R-primer for fusion. The nucleotides changed in the mutated fusion, p$vuuA_{MT}$::*luxAB*, are shown above the distinct Fur-binding site with vertical arrows.

MO6-24/O [18]. As shown previously by Webster and Litwin [17], the expression of the transcription fusion was also significantly increased in the Δ*fur* mutant compared with the wild-type (Fig. 2A). In addition, its expression in wild-type is also similarly increased when the iron-chelator, 2,2'-dipyridyl, was supplied in the growth medium. Transcription in the Δ*fur* mutant, however, was not affected by exogenous addition of iron-chelator, suggesting that

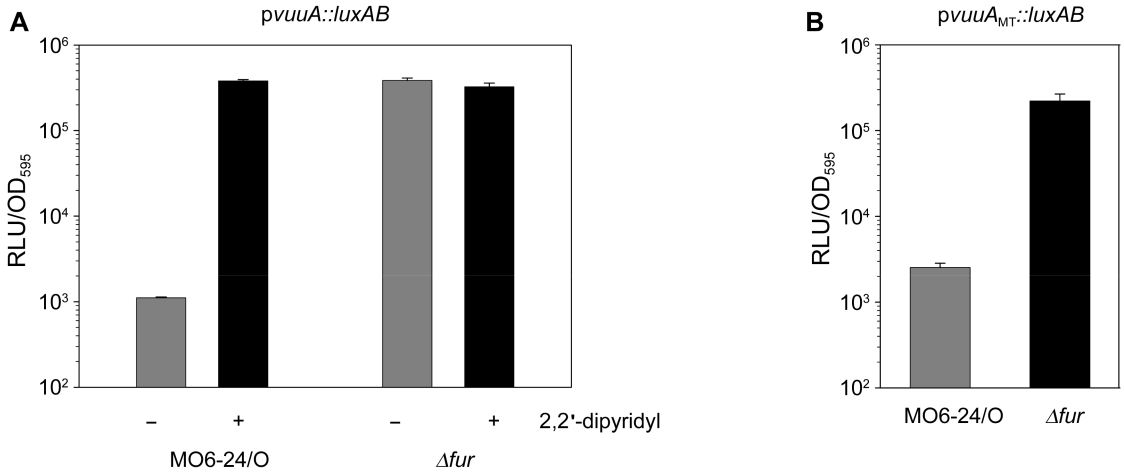
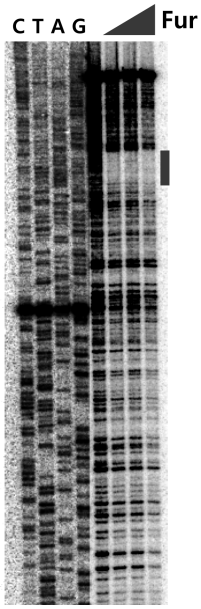


Fig. 2. Effects of Fur and iron availability on the expression of p*vuuA*::*luxAB* and p$vuuA_{MT}$::*luxAB*.
Wild-type and Δ*fur* mutant *V. vulnificus* carrying either p*vuuA*::*luxAB* (**A**) or p$vuuA_{MT}$::*luxAB* (**B**) were grown in LBS medium supplemented with 3 μg/ml tetracycline. Iron in the medium was depleted by adding 0.2 mM 2,2'-dipyridyl. Aliquots of each culture were sampled at the mid-exponential phase and determined for their cell masses (OD_{595}) and bioluminescence (relative light units [RLU]). Luciferase activities are expressed as normalized values, by dividing RLU by the OD_{595} of each sample. The activities of two independent experiments were averaged and are presented with their SD values.

A



B

GATAATGATAATCATTATC
CA**AATGCAAATGAGAATGCTTTACATTT**AAATTATCAATAATTAGAATGCGTTGT
-35 -10 +1

Fig. 3. Localization of the Fur-binding site.
DNase I footprinting assay was performed to localize the Fur-binding site in the regulatory region of the *vuuA* gene. The ^{32}P-labeled 452 bp DNA fragment of the *vuuA* promoter region (8 nM) was incubated with increasing amounts of the recombinant Fur protein (lanes 1 ~ 4: 0, 1.5, 2.9, and 5.8 μM), which had been reconstituted with 1 mM $FeSO_4$ as described previously [3], and the reactions were then treated with DNase I. The reaction mixtures were resolved on a 6% polyacrylamide sequencing gel alongside the sequencing ladder. The protected region designated by a vertical line (**A**) is illustrated with the corresponding sequences with bold-faced nucleotides (**B**). The sequence homologous to the classical Fur-box is shown by aligning with the consensus sequence (gray-colored nucleotides). The presence of an inverted repeat sequence (5'-AAATGCAAAN$_9$TTTAC ATTT-3') is denoted by opposing lines.

both iron and Fur play a repressional role together *via* iron–Fur complex formation.

In silico analysis revealed two possible Fur-binding sites around one (the more upstream promoter) of two promoters for the *vuuA* gene (Fig. 1); one is homologous to the classical Fur-Box and the other is homologous to a distinct Fur-binding site that includes a direct repeat. To investigate the involvement of each tentative site in being bound by Fur, the distinct Fur site was subjected to a site-directed mutagenesis to alter the nucleotides, as designated in Fig. 1, and then used for construction of a transcription reporter fusion (p*vuuA*$_{MT}$::*luxAB*).

Expression of the mutant fusion, however, showed basically the same as the original fusion in both the wild-type and Δ*fur* mutant (Fig. 2B). Thus, it suggests that this tentative site is not required for Fur regulation of *vuuA* expression. Thus, the second candidate, a homologous sequence to the classical Fur-box, was subjected to a site-directed mutagenesis and the transcription fusion including this mutated DNA was constructed. However, the resultant fusion failed to produce light (data not shown), possibly owing to alteration in nucleotides in the upstream region that might be important in expression. Therefore, we performed a DNase I footprint assay to directly localize the Fur-binding site.

DNase I footprint assay using iron-treated Fur protein and a DNA probe encompassing from -412 to +40 (relative to its initiation codon) clearly revealed the protected region localizing at -49 ~ -31 (relative to its transcription start site), which is partially overlapped with the -35 region (Fig. 3A). In the middle of the protected region, the sequence 5'-GCAAATGAGAATGCTTTAC-3' (identical nucleotides underlined), homologous to the consensus Fur-box sequence, is present. However, the number of nucleotides in the protected region is 26 nucleotides, suggesting that the binding site is extended compared with the 19 nucleotide-long Fur-box. Interestingly, the inverted-repeat sequence (5'-AAATGcAAAN$_9$TTTaCATTT-3') was also discernable in the protected region, but it is not known, at present, the possible role of the inverted repeat in the interaction with Fur (Fig. 3B). Interestingly, the upstream region of the *fatB* gene (VVM_02497), which is speculated to be regulated by an iron–Fur complex, also showd the presence of both the Fur-box and the same inverted repeat sequence in its putative promoter region (data not shown). Therefore, further study is needed to characterize the role of the inverted sequence, which is overlapped with the Fur-box, in the tendency of Fur to expand its binding region.

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