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Identification of Genes Involved in the Utilization of Hydroxamate Xenosiderophores in *Vibrio alginolyticus*

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Iron is an essential nutrient for bacterial survival. *Vibrio alginolyticus* is a pathogenic *Vibrio* species that produces vibrioferrin, a cognate siderophore for efficient iron acquisition in iron-limited environments. Many bacteria have developed mechanisms to utilize xenosiderophores produced by other microorganisms, in addition to using self-produced siderophores for iron acquisition. In this study, we found through a homology search using the whole genome sequence of *V. alginolyticus* NBRC 15630 that this bacterium has genes similar to those involved in the utilization of hydroxamate-based xenosiderophores, desferri-ferrichrome (DFC), desferrioxamine B (DFOB), and aerobactin (AERO), possessed by *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus*. In growth assays using an iron-limiting medium supplemented with each xenosiderophore, it was found that the *N646_3157* (*fhuA1*), *N646_0489* (*fhuA2*), *N646_0777* (*desA*), and *N646_4356* (*iutA*) are outer membrane receptor genes involved in the utilization of DFC, DFOB, and AERO and *N646_3158-3160* (*fhuC1D1B1*), and *N646_0486-0488* (*fhuC2D2B2*) are ATP-binding cassette transporter genes for both DFC and DFOB. Additionally, we demonstrated by reverse transcriptase-quantitative PCR and electrophoretic mobility shift assay that the *fhuC2D2B2A2* genes, which were newly identified in pathogenic *Vibrio* species, are an operon whose expression is probably regulated by Fur in response to iron availability.

Key words *Vibrio alginolyticus*, iron-limiting stress, siderophore, Fur regulation

INTRODUCTION

In bacteria and most living organisms, iron is an essential element for numerous fundamental biological processes, such as DNA synthesis, cellular respiration, and the activity of enzymes engaged in many key redox reactions.[1-3] Although iron is the fourth most abundant element on Earth, soluble ferrous iron (Fe^{2+}) is oxidized to water-insoluble ferric iron (Fe^{3+}) in aerobic natural environments, and iron exists as iron-binding proteins, such as transferrin and hemoglobin, in animal host environments. Therefore, in these environments, bacteria cannot freely acquire iron and are subjected to iron-limiting stress.[1] To overcome this iron-limiting stress, bacteria produce siderophores (ferric chelate molecules produced by microorganisms) and secrete them into the environment, solubilizing ferric iron as Fe^{3+}-siderophore complexes and taking them up via specific transporters.[1-3] In addition to using their self-produced siderophores, some bacteria have evolved systems to transport ferric iron complexes with exogenous siderophores (xenosiderophores) produced by other bacterial or fungal species.[2] This strategy may be highly advantageous for the survival and proliferation of these bacteria because it allows them to escape the bacteriostatic and competitive effects caused by heterologous siderophores that may coexist under various environmental conditions.[4]

In gram-negative bacteria, Fe^{3+}-siderophores are generally transported across the outer membrane by specific iron-repressible outer membrane receptors (IROMRs).[1,2] Fe^{3+}-siderophores that pass through the outer membrane are transported to the periplasmic space and through the inner membrane by ATP-binding cassette (ABC) transporters, consisting of periplasmic binding proteins, permeases, and ATP-binding proteins specific to each complex. In many bacteria, genes involved in iron acquisition are transcriptionally regulated by the iron-responsive transcriptional repressor Fur. Under iron-replete conditions, Fur forms a Fur-Fe^{2+} complex (holo-Fur) with ferrous iron, which acts as a corepressor and then binds to the Fur recognition site (Fur box), a 19-bp inverted repeat sequence (consensus sequence determined by *Escherichia coli*: 5′-GATAATGATAATCATTATC-3′) present in the promoter region of the iron acquisition genes, as a holo-Fur dimer, thereby repressing their transcription. However, under iron-limiting conditions, it becomes an unmetalled protein (apo-Fur), which loses its affinity for the Fur box and releases the transcriptional repression.[1,2]

Vibrio alginolyticus, which is a halophilic gram-nega-

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tive bacterium, is ubiquitous in temperate marine and brackish water.[5,6] This bacterium can cause gastroenteritis through consumption of raw seafood[7] and superficial wound and ear infections through contact with contaminated seawater.[5] Similar to *V. parahaemolyticus*, this bacterium possesses two operons, *pvsABCDE* and *pvuA1A2BCDE*, involved in biosynthesis/secretion and transport of the cognate siderophore vibrioferrin (VF),[8] and produces and uses VF under iron-limiting conditions for efficient iron acquisition.[9-12] Additionally, *V. parahaemolyticus* and other pathogenic *Vibrio* species such as *V. vulnificus* and *V. cholerae* can steal siderophores secreted by other microorganisms (e.g., desferri-ferrichrome (DFC) and desferrioxamine B (DFOB) secreted by fungi and aerobactin (AERO) produced by *Enterobacteriaceae*) to acquire iron.[13-17] However, little is known about the use of exogenous siderophores in *V. alginolyticus*. In this study, we identified *V. alginolyticus* genes involved in the transport of xenosiderophores containing hydroxamate moieties for iron chelation: DFC, DFOB, and AERO.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Primers *Vibrio alginolyticus* and *E. coli* strains used in this study are listed in Table 1. *Vibrio alginolyticus* strains were routinely cultured in Luria-Bertani (LB) medium containing 3% NaCl. *Escherichia coli* β2155,[18] a diaminopimelate auxotroph, was cultured in LB medium containing 0.5% NaCl with 0.5 mM diaminopimelate. LB media with and without 2,2'-bipyridyl, which is a synthetic chelator that binds both Fe^{2+} and Fe^{3+},[19] at 200 μM were used for growth as iron-limiting (−Fe) and replete (+Fe) media, respectively. When appropriate, ampicillin (100 μg/mL), carbenicillin (50 μg/mL), and chloramphenicol (10 μg/mL) were added to the medium. Primers used in this study are listed in Table 2.

Construction of *V. alginolyticus* Gene-Deletion Mutants Gene-deletion mutants were created through allelic exchange using the suicide vector pXAC623.[20] DNA fragments with deletions in the *V. alginolyticus pvsA* (*N646_3393*), *N646_3157*, *N646_0489*, *N646_0777*, *N646_4356*, *N646_3158* to *3160*, and *N646_0487* to *0488* genes were prepared through PCR-driven overlap extension[21] as previously described.[12] Then, they were ligated into *Xba*I-digested pXAC623. Each resulting plasmid was transformed into *E. coli* β2155 and mobilized into an appropriate *V. alginolyticus* strain by filter mating. The chloramphenicol-resistant merodiploids obtained were spread on VDS broth agar plates (1% polypeptone, 0.5% yeast extract, 30 mM NaCl, 55 mM KCl, 10% sucrose, and 2.5% agar) at 30°C for 48 h. Sucrose-resistant and chloramphenicol-sensitive colonies were selected, and the deleted DNA regions were confirmed using colony PCR (data not shown).

Growth Assay Stationary-phase *V. alginolyticus* cells were diluted to an optical density of 0.005 at 600 nm (OD_{600}) with the +Fe or −Fe medium. When needed, an appropriate siderophore, i.e. VF (prepared as described previously),[8] DFC (Sigma-Aldrich, St. Louis, MO, USA), DFOB (Sigma-Aldrich, St. Louis, MO, USA), or AERO (prepared as described previously),[22] was added to the −Fe medium at a concentration of 10 μM. The cultures were shaken at 70 rpm at 37ºC, and the OD_{600} was measured using a biophotorecorder TVS062CA (Advantec, Tokyo, Japan) every hour for 20 h.

Preparation of Total RNA Stationary-phase *V. alginolyticus* NBRC 15630 cells were inoculated into the +Fe or −Fe medium at a final OD_{600} of 0.005. The cultures were shaken at 37°C until the OD_{600} reached 0.4–0.6 and then treated with RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The treated cell pellets were maintained at −80ºC until RNA extraction. Total RNA samples were isolated using Isogen II reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Total RNA samples thus obtained were used for reverse transcriptase-quantitative PCR (RT-qPCR) and RT-PCR.

RT-qPCR Total RNA samples were treated with RNase-free DNase I, and a 0.5-μg aliquot of RNA was reverse transcribed using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) with a random hexamer primer (Takara, Shiga, Japan). qPCR analysis was performed using the primer pairs VagfhuC2-qF/VagfhuC2-qR, VagfhuB2A2-qF/VagfhuB2A2-qR, and VagfhuA2-qF/VagfhuA2-qR, specific for *fhuC2, fhuB2-fhuA2* intergenic region, and *fhuA2*, respectively (Table 2), and the Thunderbird SYBR qPCR Mix (Toyobo) in a Thermal

Table 1. *Vibrio alginolyticus* and *E. coli* Strains Used in This Study

Strain	Description[a]	Reference or source[b]
V. alginolyticus		
NBRC 15630 (= ATCC 17749)	Type strain	NBRC
VagD1	*pvsA* deletion mutant from NBRC 15630 (vibrioferrin non-producer mutant)	This study
VagD11	*N646_3157* deletion mutant from VagD1 (i.e., Δ*pvsA*Δ*fhuA1*)	This study
VagD12	*N646_0777* deletion mutant from VagD1 (i.e., Δ*pvsA*Δ*desA*)	This study
VagD13	*N646_4356* deletion mutant from VagD1 (i.e., Δ*pvsA*Δ*iutA*)	This study
VagD14	*N646_3158-3160* deletion mutant from VagD1 (i.e., Δ*pvsA*Δ*fhuC1D1B1*)	This study
VagD15	*N646_0489* deletion mutant from VagD11 (i.e., Δ*pvsA*Δ*fhuA1*Δ*fhuA2*)	This study
VagD16	*N646_0487-0488* deletion mutant from VagD1 (i.e., Δ*pvsA*Δ*fhuD2B2*)	This study
VagD17	*N646_0487-0488* deletion mutant from VagD14 (i.e., Δ*pvsA*Δ*fhuC1D1B1*Δ*fhuD2B2*)	This study
E. coli		
β2155	*thrB1004 pro thi strA hsdS* Δ(*lacZ*)ΔM15 (F' Δ(*lacZ*)M15 *lacI*^{q} *traD36 proA*$^+$ *proB*$^+$) Δ*dapA*::*erm*(Em^r), *pir*::RP4(::*kan*(Km^r) from SM10)	[18]
DH5α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR17*(r_K^- m_K^+), λ$^-$; host for cloning of pCold-VagFur	Promega
BL21(DE3)pLysS	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm lon* λ(DE3), pLysS (Cm^r)	Thermo Fisher Scientific

[a]Ap^r, ampicillin resistance; Cm^r, Chloramphenicol resistance; Em^r, erythromycin resistance; Km^r, kanamycin resistance. [b]NBRC, National Institute of Technology and Evaluation Biological Resource Center.

Table 2. Primers Used in This Study

Purpose for	Primer name	Sequence (5′–3′)[a]
construction of Δ*pvsA* fragment	VagpvsA-XbaI-#1 (including *Xba*I site)	CGTTACTCTAGAATACCGCTTGTGGCACATC
	VagpvsA-#2	ctgagtgatggagtgactgcATCGTTAACG
	VagpvsA-#3	gcagtcactccatcactcagCAACAGGTAC
	VagpvsA-XbaI-#4 (including *Xba*I site)	TCTGCGTCTAGATGAAACGCTTGGTCACAC
confirmation of *pvsA* deletion	VagpvsA-#5	TGTTGCTGCCTACACTACCG
	VagpvsA-#6	CAGTGCAAAATCAGCAGGAA
construction of Δ*fhuA1* fragment	VagfhuA1-XbaI-#1 (including *Xba*I site)	GGTCTTTCTAGAATGTTGCGCGTTCTTGATTAG
	VagfhuA1-#2	tacccatgcattatggtctcCATGGCAATC
	VagfhuA1-#3	gagaccataatgcatgggtaGACTACAGTG
	VagfhuA1-XbaI-#4 (including *Xba*I site)	TTTGAGTCTAGAAAGGGAAGTATCGTTTAGTG
confirmation of *fhuA1* deletion	VagfhuA1-#5	CGCCACTAAGAACAGCATCA
	VagfhuA1-#6	CGATTGTTGAGCCAGTAGCA
construction of Δ*fhuA2* fragment	VagfhuA2-XbaI-#1 (including *Xba*I site)	TTGTTGTCTAGATTAGCTTTGTTGGGTTGTTG
	VagfhuA2-#2	cgcaatgttcgaccaactgcACTGTCTTGC
	VagfhuA2-#3	gcagttggtcgaacattgcgTACGTTGATG
	VagfhuA2-XbaI-#4 (including *Xba*I site)	TCTACCTCTAGATTCCAAAGGGTGAGTTTGAC
confirmation of *fhuA2* deletion	VagfhuA2-#5	CCACTGGTGACCCAAGAAGT
	VagfhuA2-#6	AAAGGTCTCATACAAGCGAC
construction of Δ*desA* fragment	VagdesA-XbaI-#1 (including *Xba*I site)	CTCGGGTCTAGACCTCTTGGCTGTTCCTGAGC
	VagdesA-#2	tttagtctgttaacgcaatgCTTCGCTCAC
	VagdesA-#3	cattgcgttaacagactaaaAGAACTGACG
	VagdesA-XbaI-#4 (including *Xba*I site)	TCTGAATCTAGATACTGATAATCAAGCCCGAAC
confirmation of *desA* deletion	VagdesA-#5	GTGATTGATGTGACTCAAGC
	VagdesA-#6	GTGCATTACGATCAATGCTG
construction of Δ*iutA* fragment	VagiutA-XbaI-#1 (including *Xba*I site)	CCCATCTCTAGATGTCGGACGGAATCATGGCTTG
	VagiutA-#2	ctgctattaacctacaacaaCCAATTGCTC
	VagiutA-#3	ttgttgtaggttaatagcagCGATGACTTTG
	VagiutA-XbaI-#4 (including *Xba*I site)	TTACTTTCTAGACGGTCTAAGTACGCATCGAC
confirmation of *iutA* deletion	VagiutA-#5	GACGAAGCGCATATACATGG
	VagiutA-#6	TTAAGTTCCCAGAGCTGTTC
construction of Δ*fhuC1D1B1* fragment	VagfhuC1D1B1-XbaI-#1 (including *Xba*I site)	TAAACTTCTAGAGTCACCAATTAAGCTGATTG
	VagfhuC1D1B1-#2	acctaggaagtctgccagttTCTGTTCAGC
	VagfhuC1D1B1-#3	aactggcagacttcctaggtTAAGTATGAC
	VagfhuC1D1B1-XbaI-#4 (including *Xba*I site)	ATTAAGTCTAGAATACGTGGTGCGACGATCAAAAT
confirmation of *fhuC1D1B1* deletion	VagfhuC1D1B1-#5	GCACGATCTGAACTTAGCAC
	VagfhuC1D1B1-#6	CAAATACCCATCACCATTAACG
construction of Δ*fhuD2B2* fragment	VagfhuD2B2-XbaI-#1 (including *Xba*I site)	ATATGGTCTAGAGATGATGATCTCTGACCAC
	VagfhuD2B2-#2	ggtacaattgcacgtacgtgCTTTGTATCC
	VagfhuD2B2-#3	cacgtacgtgcaattgtaccAAGTAAGCTC
	VagfhuD2B2-XbaI-#4 (including *Xba*I site)	AGTTCCTCTAGACTTAACGGCTTTGCAATCAC
confirmation of *fhuD2B2* deletion	VagfhuD2B2-#5	CGACTACATCGTTGCATTAC
	VagfhuD2B2-#6	CAAAACGCAACTTAGCTAGC
RT-qPCR primers specific to *fhuC2*	VagfhuC2-qF	TACACAGCTGGTGACGTGGT
	VagfhuC2-qR	TGAGGCAAGTAGGCAATGTG
RT-qPCR primers specific to *fhuB2-A2* intergenic region	VagfhuB2A2-qF	GTCTCTCATTGGCGGTTTGT
	VagfhuB2A2-qR	CGACGTTGGTGTCAGCATAA
RT-qPCR primers specific to *fhuA2*	VagfhuA2-qF	TCGTTACAGCGAAATGGATG
	VagfhuA2-qR	CATTTGATGGGTCCAAAACC
RT-qPCR primers specific to 16S rRNA gene	Vag16S-qF	ATTACCGCGGCTGCTGG
	Vag16S-qR	CCTACGGGAGGCAGCAG
synthesis of cDNA using RT-PCR	VagfhuA2-Rev	TCTTGCTGCTGTCCAATCAC
RT-PCR primers specific to *fhuC2-D2* intergenic region	VagfhuC2D2-Fw	TGATTGCACAAGGCAATGTT
	VagfhuC2D2-Rev	CATCCTTCGTTTGAGGTGGT
RT-PCR primers specific to *fhuD2-B2* intergenic region	VagfhuD2B2-Fw	CCTCCGTTTCGCAGAGTTAG
	VagfhuD2B2-Rev	ACATCCAAGGTGCGAATAGC
RT-PCR primers specific to *fhuB2-A2* intergenic region	VagfhuB2A2-qF	See above
	VagfhuB2A2-qR	See above
construction of an insert fragment for T7 expression plasmid pCold-VagFur	Vagfur-cold-F (including *Nde*I site)	GGGAAAGCATATGTCAGATAATAATCAGGC
	Vagfur-cold-R (including *Eco*RI site)	TTTTTAAGAATTCAAACCCTTATTTTTTCGGTTTG
construction of probe 1 for EMSA	fhuC2pro-F1	TGTATTCACTGCGGCATTTTTCATTTC
	fhuC2pro-R (5′ fluorescein labeled)	CCCTACTTCACCTAATAGCC
construction of probe 2 for EMSA	fhuC2pro-F2	ATGATCTTGTTGATGTAATG
	fhuC2pro-R (5′ fluorescein labeled)	See above
construction of probe 3 for EMSA	fhuC2pro-F3	ATTTGTATCTTTGTTGTTATATAAAACC
	fhuC2pro-R (5′ fluorescein labeled)	See above
construction of probe 4 for EMSA	fhuC2pro-F1	See above
	fhuC2pro-del-#2	agatacaaattcaacaagatCATTGAGAGA
	fhuC2pro-del-#3	atcttgttgaatttgtatctTTGTTGTTATATAAAACC
	fhuC2pro-R (5′ fluorescein labeled)	See above

[a]Underlined sequences indicate restriction enzyme sites; lower-case letter sequences within primers #2 and #3 are complementary base pairs.

Cycler Dice Real-Time System III (Takara). Relative mRNA expression levels were determined using the comparative threshold cycle method with 16S rRNA expression as an internal control.

RT-PCR Total RNA samples were treated with RNase-free DNase I, and a 0.5-μg aliquot of RNA was reverse transcribed using ReverTra Ace reverse transcriptase with the primer VagfhuA2-Rev primer specific for *fhuA2* in a 10-μL reaction for 60 min at 42°C. One μL of the cDNA reaction mixture was then used as a template for PCR with the specific PCR primer pairs VagfhuC2D2-Fw/VagfhuC2D2-Rev, VagfhuD2B2-Fw/VagfhuD2B2-Rev, and VagfhuB2A2-qF/VagfhuB2A2-qR, specific for *fhuC2-D2, fhuD2-B2, and fhuB2-A2* intergenic regions, respectively (Table 2). As a negative control, PCR omitting prior reverse transcription was performed directly for the same RNA template to confirm the absence of contaminating chromosomal DNA. PCR products were electrophoresed through 2.0% agarose gels, stained with Midori Green Xtra (Nippon Genetics, Tokyo, Japan), and visualized with a Blue/Green LED transilluminator.

Overexpression and Purification of *V. alginolyticus* Fur The *V. alginolyticus fur* (*N646_2985*) was amplified using PCR using the primers Vagfur-cold-F and Vagfur-cold-R (Table 2). The PCR fragment was ligated into the *Nde*I and *Eco*RI sites of the pCold I expression vector (Takara), yielding the pCold-VagFur plasmid. *E. coli* BL21(DE3)pLysS (Thermo Fisher Scientific, Waltham, MA, USA) transformed with pCold-VagFur was cultivated in LB medium with carbenicillin and chloramphenicol at 37ºC to an OD_{600} of 0.5 and then incubated for 30 min at 4ºC. Expression of N-terminal histidine-tagged Fur (His-Fur) was induced by isopropyl thiogalactoside (final concentration, 0.5 mM) at 15ºC for 16 h. After harvesting, cells were disrupted through sonication in phosphate-buffered saline. His-Fur in the soluble lysate was purified using a HisTrap FF column (GE Healthcare, Chicago, IL, USA) according to the manufacturer's instructions. Purified His-Fur was desalted using a PD10 column (GE Healthcare, Chicago, IL, USA) equilibrated with buffer A (100 mM Tris-HCl, 100 mM KCl, and 2 mM DTT; pH 7.5) and concentrated using an ultrafiltration membrane filter. After buffer exchange and concentration, the His-Fur sample was placed in a store buffer (50 mM Tris-HCl, 50 mM KCl, 1 mM DTT, and 40% glycerol; pH 7.5), mixed with an equal volume of 80% glycerol, and stored at −20ºC until use. Protein concentration was assayed using the bicinchoninic acid method.

Electrophoretic Mobility Shift Assay (EMSA) of the Interaction between Fur and the Promoter Region of *fhuC2* The fluorescein-labeled probes 1, 2, and 3 for EMSA were amplified via PCR using the primer pairs fhuC2pro-F1/fhuC2pro-R, fhuC2pro-F2/fhuC2pro-R, and fhuC2pro-F3/fhuC2pro-R, respectively. Additionally, the fluorescein-labeled probe 4 containing a 25-base deletion in the *fhuC2* promoter region was prepared via PCR-driven overlap extension[21] using the primers fhuC2pro-F1, fhuC2pro-del-#2, fhuC2pro-del-#3, and fhuC2pro-R (Table 2), as previously described.[12] Binding of His-Fur to each probe was allowed to proceed for 30 min at room temperature in 10 μL of the reaction mixture containing 0.01% bovine serum albumin, 0.1 μg/μL poly(dI-dC), 20 nM labeled probe, 100 mM $MnCl_2$, and GST-IutR in the binding buffer (20 mM Bis-Tris borate, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, and 5% glycerol). For competition experiments, 1000 nM of unlabeled probe 1 was included in the reaction mixture. The reaction mixtures were then loaded onto a 5% native polyacrylamide gel and run with 0.5 × TBE buffer for 75 min at 100 V at room temperature. Gel images were analyzed using a ChemiDoc imager (Bio-Rad, Hercules, CA, USA) at an excitation wavelength of 460 nm and an emission bandpass filter of 515 nm.

RESULTS AND DISCUSSION

Homology Search for Genes Involved in Utilizing Hydroxamate Xenosiderophores in *V. alginolyticus* Many bacteria compete for iron acquisition in various environments by using their own siderophores and stealing xenosiderophores. The overall mechanism of iron acquisition via siderophores has been elucidated for pathogenic *Vibrio* species such as *V. parahaemolyticus*,[23] *V. cholerae*,[24] and *V. vulnificus*.[25] In this study, to investigate whether *V. alginolyticus* has a utilization system for hydroxamate xenosiderophores DFC, DFOB, and AERO like other pathogenic *Vibrio* species, we first searched for proteins that show homology to IROMRs for ferrichrome (FC; Fe^{3+}-bound DFC), ferrioxamein B (FOB; Fe^{3+}-bound DFOB), and Fe^{3+}-AERO (Fe^{3+}-bound AERO) in *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* from the whole-genome sequence of *V. alginolyticus* NBRC 15630.[26] The protein encoded by *V. alginolyticus N646_3157-3160* genes showed extremely high identities (95%, 94%, 76%, and 87%) with the proteins encoded by *V. parahaemolyticus* DFC utilization operon *fhuACDB*, which are IROMR, ATP-binding subunit, periplasmic binding protein, permease for FC transport, respectively (Fig. 1A). Moreover, the *V. alginolyticus* N646_3157-3160 proteins exhibited a fairly high degree of identities (65%, 75%, 55%, and 62%) with the *V. cholerae* FhuACDB (Fig. 1A). Furthermore, *V. alginolyticus* N646_0777 showed 50% identity with DesA, the FOB receptor of *V. vulnificus* (Fig. 1B), and N646_4356 showed respective identities of 91% and 67% with the Fe^{3+}-AERO receptors IutA of *V. parahaemolyticus* and *V. vulnificus* (Fig. 1C). Additionally, the *N646_3157*, *N646_0776*, *N646_0777*, and *N646_4356* genes contained putative Fur boxes involved in iron-responsive expression in their promoter regions, like the corresponding homologous genes in *V. parahaemolyticus*,[14,17] *V. cholerae*,[27] and *V. vulnificus*[15,16] (Fig. 1A-C and Table 3).

Identification of Genes Encoding IROMRs for Hydroxamate Xenosiderophores FC, FOB, and Fe^{3+}-AERO in *V. alginolyticus* To identify the genes encoding IROMRs for hydroxamate xenosiderophores, we constructed various deletion strains for their respective genes. All these deletion mutants exhibited similar growth rates in the +Fe medium (Fig. 2A). To clarify whether an IROMR gene, *N646_3157*, of *V. alginolyticus* found by homology search is involved in the utilization of FC, we performed growth assays using strains lacking the relevant genes. In the −Fe medium in which the wild-type strain NBRC 15630 was allowed to grow, the VagD1 strain, in which the *pvsA* gene for the biosynthesis of the cognate siderophore VF was deleted, showed reduced growth compared with the NBRC 15630 strain (Fig. 2B-D, open circles and squares). However, the addition of DFC to the −Fe medium restored the growth of the VagD1 strain (Fig. 2B, filled squares). This indicates that this bacterium can utilize DFC for iron acquisition. The VagD11 strain, in which the gene encoding N646_3157, which shows homology to FhuA, was deleted, exhibited suppressed growth com-

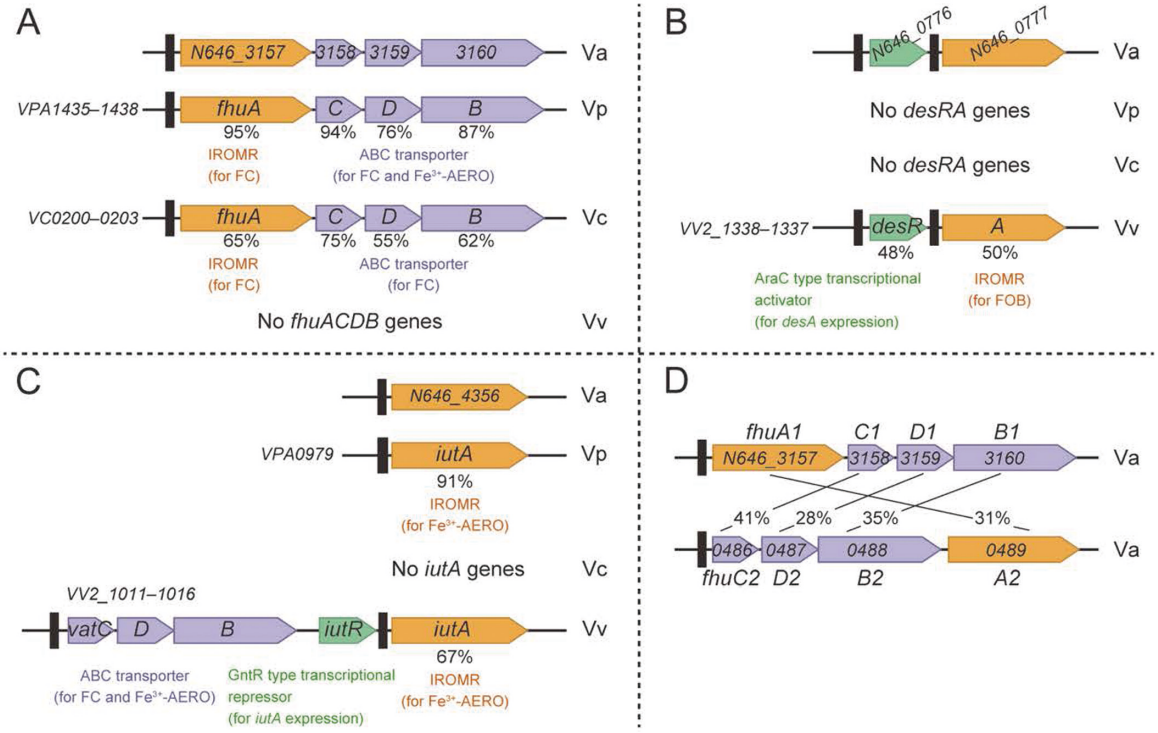

Fig. 1. Comparative Gene Arrangement of Genes Involved in the Transport of Hydroxamate Xenosiderophores in *V. alginolyticus* (Va), *V. parahaemolyticus* (Vp), *V. cholerae* (Vc), and *V. vulnificus* (Vv)

Arrows represent the genes and their orientations. Each gene arrow is labeled with a gene name and/or locus tag corresponding to the genome. Different colors represent the function of different genes: orange, IROMR gene; purple, genes encoding components of ABC transporter; green, transcriptional regulator gene. Panels A, B, and C show genes involved in utilizing DFC, DFOB, and AERO, respectively, in *V. alginolyticus* (Va), *V. parahaemolyticus* (Vp), *V. cholerae* (Vc) and *V. vulnificus* (Vv). In panels A, B, and C, the percentages shown under the gene arrows for Vp, Vc, and Vv indicate the amino acid identities between the protein encoded by each gene and the corresponding protein in Va. Panel D shows two *fhu* operons in Va. The percentages between the two *fhu* operons in Va indicate identities between the proteins encoded by each gene. The filled boxes indicate potential Fur boxes.

Table 3. Potential Fur Boxes Present in the Promoter Regions of Genes Involved in Utilizing Hydroxamate Xenosiderophores in *V. alginolyticus*

Locus tag	Gene	Potential Fur box sequence (5′–3′) [a]	The number of nucleotide-matches to the 19-bp consensus Fur box sequence of *E. coli*
N646_3157	*fhuA1*	tcaAATGATAATaATTAca	13 matches of the 19 nucleotides
N646_0776	*desR*	GATAATGATAcTCAaTtaa	14 matches of the 19 nucleotides
N646_0777	*desA*	acaAATaAcAATCATTATC	14 matches of the 19 nucleotides
N646_4356	*iutA*	GAgAgTGAgAATaAgTATC	14 matches of the 19 nucleotides
N646_0486	*fhuC2*	GATAATGAcAAcggTTtgC	13 matches of the 19 nucleotides

[a]Upper-case letter sequences indicate matches to the consensus sequence of 5′-GATAATGATAATCATTATC-3′ for *E. coli* Fur boxes.

pared with the wild-type strain but was not complete (Fig. 2B, filled triangles). This suggests that *V. alginolyticus* harbors an FC receptor gene other than *N646_3157*. Therefore, we searched the NRBC whole genome sequence for a protein that shows homology to N646_3157 and found N646_0489, which showed 31% identity to N646_3157 (Fig. 1D). Furthermore, in the upstream region of the gene encoding N646_0489, we found the *N646_0486-0488* genes, which encode ABC transporters with 41%, 28%, and 35% identities to N646_3158-3160 (Fig. 1D). To determine whether *N646_0489* encodes an FC receptor, a growth assay was performed using strain Vag15, in which the *N646_0489* gene was deleted from Vag11. The results showed that the Vag11 strain grew in the −Fe medium containing DFC, but the Vag15 strain exhibited a completely suppressed growth in this medium (Fig. 2B, filled diamonds). This suggests that the *N646_0489* gene also encodes an FC receptor as *N646_3157*. Based on the results obtained thus far, we named the *N646_3157-3160* and *N646_0486-0489* genes *fhuA1C1D1B1* and *fhuC2D2B2A2*, respectively (Fig. 1D).

Next, we performed growth assays using the VagD12 and VagD13 strains in which the *N646_0777* and *N646_4356* genes were deleted from the VagD1 strain. The growth of the VagD1 strain was restored by adding DFOB or AERO to the −Fe medium, whereas growth recovery by adding DFOB or AERO was lost in the VagD12 and VagD13 strains (Fig. 2C and D). These results suggest that the *N646_0777* (*desA*) and *N646_4356* (*iutA*) genes in *V. alginolyticus* encode receptors for FOB and Fe^{3+}-AERO, respectively.

Identification of Genes Encoding ABC Transporters for Hydroxamate Xenosiderophores FC, FOB, and Fe^{3+}-AERO in *V. alginolyticus* FhuCDB is an ABC transporter involved in the transport of hydroxamate-based siderophores in many bacteria, such as *E. coli*[28,29] and *Yersinia pestis*.[30] Moreover, in pathogenic *Vibrio* species, FhuCDB of *V. parahaemolyticus*[17] and VatCDB (FhuCDB homolog) of *V. vulnificus*[15,16,25] can import multiple hydroxamate-based siderophores into cells. Therefore, to examine whether *fhuC1D1B1* and *fhuC2D2B2* in *V. alginolyticus* are ABC transporter genes involved in the

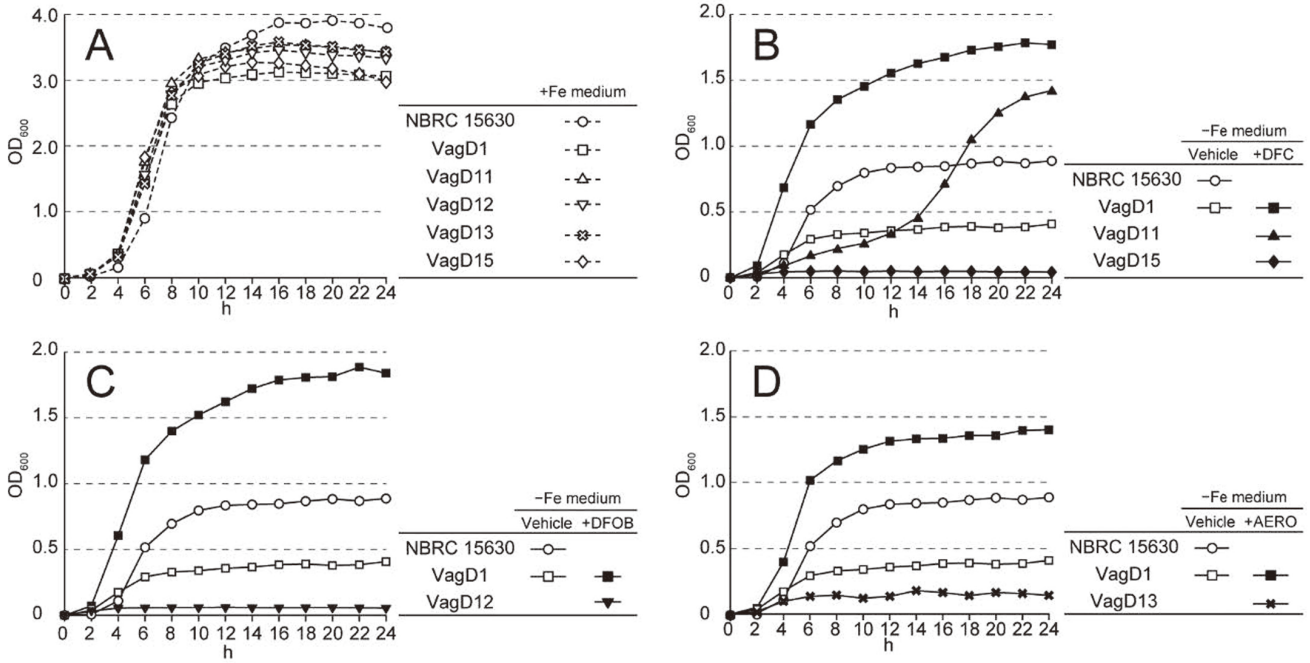

Fig. 2. Growth Curves of *V. alginolyticus* Deletion Mutants for Genes Encoding IROMRs for Hydroxamate Xenosiderophores

The growth rates of *V. alginolyticus* NBRC 15630, VagD1 and various IROMR gene mutants generated from VagD1 were assessed in the +Fe (A) and −Fe media with vehicle or a hydroxamate xenosiderophore: DFC (B); DFOB (C); or AERO (D). OD_{600} was measured every 2 h for 24 h. Representative results from three independent experiments are shown.

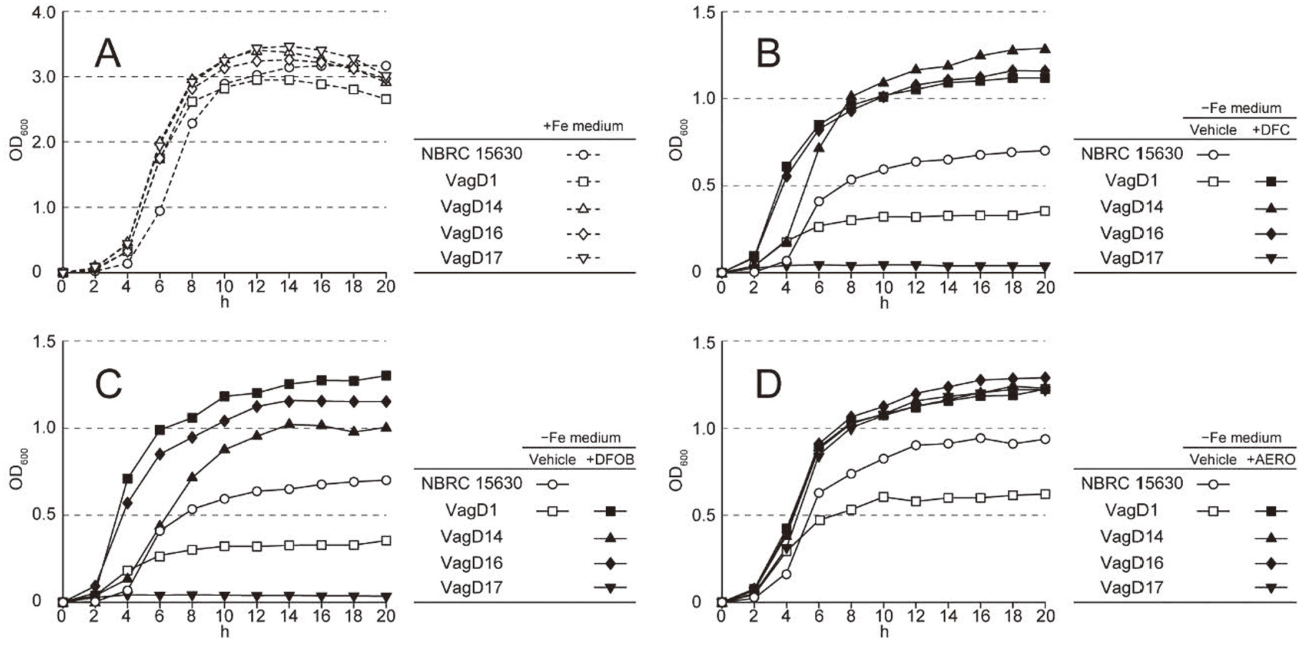

Fig. 3. Growth Curves of *V. alginolyticus* Deletion Mutants for ABC Transporter Genes for Hydroxamate Xenosiderophores

The growth rates of *V. alginolyticus* VagD1 and various ABC transporter gene mutants generated from VagD1 were assessed in the +Fe (A) and −Fe media with vehicle or a hydroxamate xenosiderophore: DFC (B); DFOB (C); or AERO (D). OD_{600} was measured every 2 h for 20 h. Representative results from three independent experiments are shown.

transport of hydroxamate siderophores, we generated deletion mutants of these genes and performed growth assays. All these deletion mutants exhibited similar growth rates in the +Fe medium (Fig. 3A). Although the VagD1 strain exhibited growth reduction compared to the NBRC 15630 strain in the −Fe medium (Fig. 3B-D, open circles and squares), VagD1, as well as *fhuC1D1B1* (VagD14) and *fhuD2B2* (VagD16) deletion mutants from their parental VagD1, showed growth promotion by adding DFC or DFOB to the −Fe medium (Fig. 3B and C, filled squares, triangles, and diamonds). How-

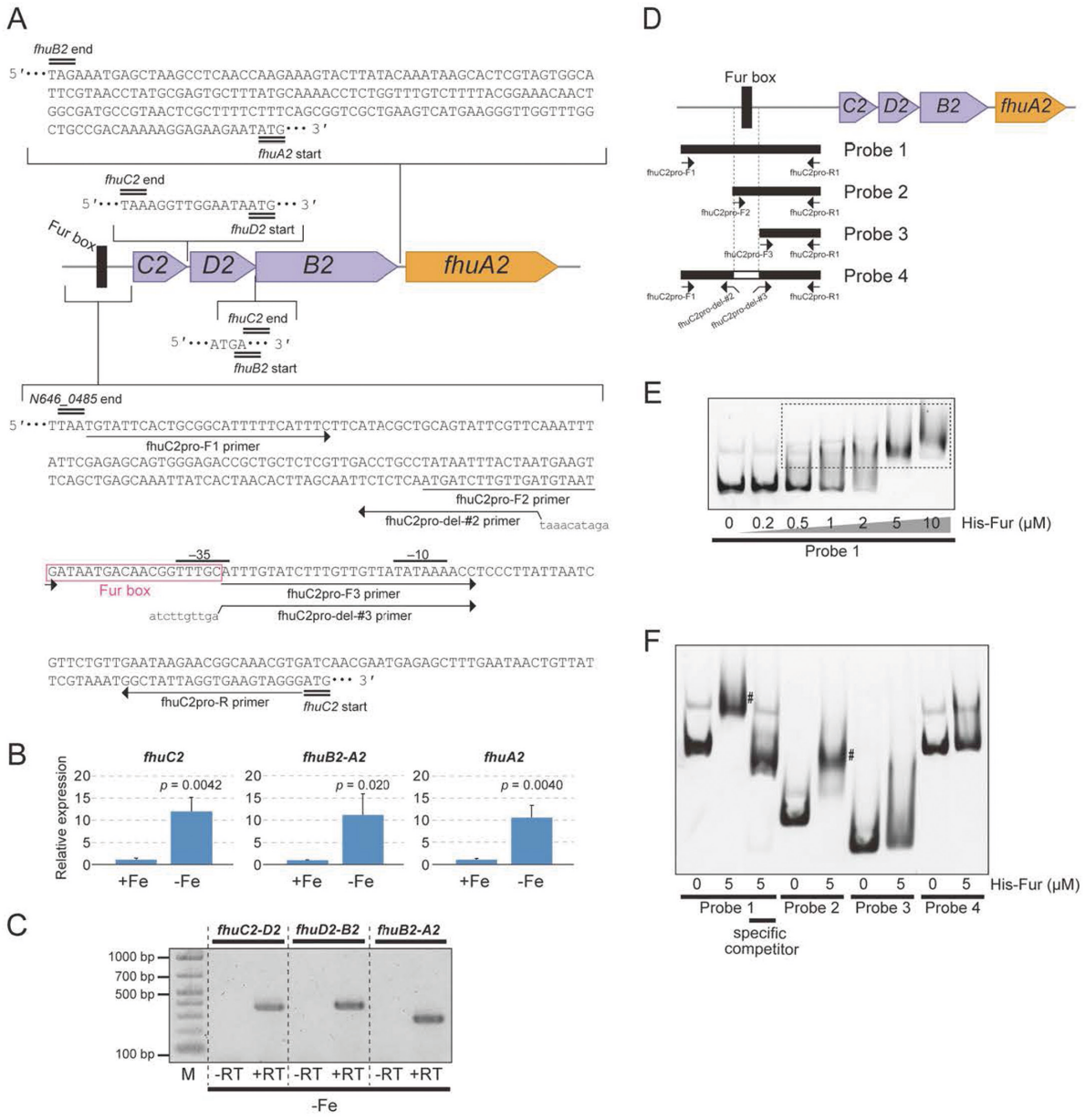

Fig. 4. Transcription Analysis of *fhuC2D2B2A2* Operon and Binding Analysis between the Upstream Region of *fhuC2* and Fur

(A) Nucleotide sequences of the upstream and intergenic regions of *the fhuC2D2B2A2* operon. Arrows represent genes and their orientations, and each gene arrow is labeled with a gene name. Different colors represent the function of different genes: orange, IROMR gene; purple, genes encoding components of ABC transporter. The putative –35 and –10 promoter elements, as well as the Fur box sequence in the upstream region of *fhuC2*, are indicated. The start and stop codons for each gene are shown. The locations of primers used to create the probes 1–4 used in EMSA are shown. (B) RT-qPCR analysis of the transcript of *fhuC2D2B2A2* operon. The amounts of mRNA for *fhuC2*, *fhuD2-B2* intergenic region, and *fhuA2* were evaluated using RT-qPCR using the total RNA samples extracted from *V. alginolyticus* NBRC 15630 cultivated in the +Fe or −Fe medium. The expression of each gene was normalized to that of the 16S rRNA gene, and each bar represents mean ± SD ($n = 3$). *P*-values were estimated using Student's *t*-test. (C) RT-PCR amplification of the transcript of *fhuC2D2B2A2* operon. Total RNA was extracted from *V. alginolyticus* NBRC 15630 cultivated in the −Fe medium. The RT-PCR products in the *fhuC2-D2*, *fhuD2-B2*, and *fhuB2-A2* intergenic regions were separated on a 2% agarose gel. +RT and -RT, RT-PCR was performed with and without reverse transcriptase, respectively. M, DNA size marker. (D) Schematic representation of EMSA probes 1–4 and primers used to generate them. The closed thick lines indicate the location of the probes, and the open line in probe 4 represents the deletion of the Fur box. (E) EMSA showing binding between the probe 1 and His-Fur. EMSA was conducted using 20 nM fluorescein-labeled probe 1 in the presence of His-Fur at the indicated concentrations. The bands enclosed by the dotted line square are complexes of GST-IutR with probe 1. (F) EMSA results for binding between probes 1–4 and His-Fur. EMSA was performed using 20 nM fluorescein-labeled probes 1–4 in the presence or absence of His-Fur at 5 μM. The unlabeled specific competitor (1000 nM) with the same sequence as probe 1 was used as needed. Sharps indicate mobility shifts of fluorescein-labeled probes 1 and 2.

ever, the deletion of both *fhuC1D1B1* and *fhuD2B2* (VagD17) resulted in no growth in −Fe medium, even in the presence of DFC or DFOB (Fig. 3B and C, filled inverted triangles). This suggests that *fhuC1D1B1* and *fhuC2D2B2* are common ABC transporter genes in hydroxamate siderophores involved in FC and FOB transport. However, the growth of VagD17 strains was observed in the −Fe medium supplemented with AERO (Fig. 3D), suggesting that *V. alginolyticus* has ABC transporter genes involved in the transport of AERO other than *fhuC1D1B1* and *fhuC2D2B2*, which warrant further investigation.

Expression of *fhuC2D2B2A2* Operon Iron-Regulated by Fur in *V. alginolyticus* Unlike other pathogenic *Vibrio* species, *V. alginolyticus* possesses two sets of *fhu* gene clusters, *fhuA1C1D1B1* and *fhuC2D2B2A2*. The *fhu* genes arranged in the order of *fhuC*, *fhuD*, *fhuB*, and *fhuA* have been identi-

fied as operons in *Actinobacillus pleuropneumoniae*,[31] but no examples of pathogenic *Vibrio* species possessing this gene cluster have been reported. Additionally, the expression of iron acquisition system genes is generally regulated by Fur depending on the iron concentration, but the expression of the *fhuCDBA* genes in *A. pleuropneumoniae* is not affected by the iron concentration.[31] Therefore, we investigated whether *fhuC2D2B2A2* found in *V. alginolyticus* is an operon whose expression is a subject to regulation by Fur. The *fhuC2*, *fhuD2*, and *fhuB2* genes were closely spaced or overlapped by several base pairs, whereas *fhuB2* and *fhuA2* were separated by as much as 200 bp (Fig. 4A). However, RT-qPCR analysis showed that the *fhuB2-A2* intergenic region, similar to *fhuC2* and *fhuA2*, was transcriptionally induced approximately 10-fold in the −Fe medium compared with the +Fe medium (Fig. 4B). Moreover, RT-PCR using the primer pairs designed to cover the *fhuC2-D2*, *fhuD2-B2*, and *fhuB2-A2* intergenic regions produced extension bands (Fig. 4C). These results strongly suggest that *fhuC2D2B2A2* is an operon whose expression is upregulated by iron limitation.

Additionally, a putative Fur box with 13 matches of 19 nucleotides to the *E. coli* consensus sequence was found upstream of *fhuC2,* overlapping the putative –35 element (Fig. 4A and Table 3). To confirm that Fur binds to this Fur box, EMSA was performed with a purified His-Fur together with the fluorescein-labeled probes 1 to 4 (Fig. 4D). As shown in Fig. 4E, His-Fur shifted the probe 1 in a concentration-dependent manner (0–10 μM). Furthermore, in a competitive EMSA using 1000 nM of unlabeled probe 1 (a specific competitor), the shift in probe 1 by the addition of His-Fur disappeared (Fig. 4F). This indicates the presence of a Fur-binding site in the upstream region of *fhuC2*. Moreover, the EMSA results showed that Fur could bind to probes 1 and 2, which contain the putative Fur box in the upstream region of *fhuC2* (Fig. 4F). In contrast, no band shift was observed with probes 3 and 4, which did not contain the putative Fur box, due to the addition of His-Fur (Fig. 4F). Therefore, since the Fur box in the upstream region of *fhuC2* shown in Fig. 4A functions as a Fur-binding site, the expression of *fhuC2D2B2A2* operon is thought to be regulated by Fur in response to iron availability.

In conclusion, we demonstrated that *V. alginolyticus* can grow under iron-limiting conditions using hydroxamate xenosiderophores DFC, DFOB, and AERO, in addition to its cognate VF, and identified genes involved in their utilization. Additional iron acquisition systems mediated by these xenosiderophores in *V. alginolyticus* may be advantageous for iron competition among resident microorganisms in their natural habitats. Moreover, iron uptake by siderophores is also essential for infections caused by many bacteria because the loss of this system significantly reduces the ability of pathogens to colonize the host.[32,33] Therefore, iron assimilation systems using xenosiderophores in *V. alginolyticus* may improve the chances of survival and proliferation of this bacterium in hosts. In the future, it will be of interest to clarify the utilization systems for other siderophores, such as catechol-based siderophores,[2] in addition to the utilization system for hydroxamate-based siderophores revealed in this study to clarify the iron acquisition system of this bacterium.

Conflict of interest The authors declare no conflict of interest.

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