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Cys-92, Cys-95, and the C-Terminal 12 Residues of the *Vibrio harveyi* Ferric Uptake Regulator (Fur) are Functionally Inessential

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Ferric uptake regulator (Fur) is a global regulator involved in multiple aspects of bacterial life. The gene encoding the *Vibrio harveyi* Fur (Fur_{Vh}) was cloned from a pathogenic *V. harveyi* strain isolated from diseased fish. Fur_{Vh} shares 77% overall sequence identity with the *Escherichia coli* Fur (Fur_{Ec}) and could complement a mutant of Fur_{Ec}. Like Fur_{Ec}, Fur_{Vh} possesses two cysteine residues at positions 92 and 95, yet unlike Fur_{Ec}, in which these cysteine residues constitute part of the metal ion coordination site and hence are vital to the repressor activity, C92 and C95 of Fur_{Vh} proved to be functionally inessential. Further study identified a *Vibrio* Fur signature sequence, which is preserved in all the ten *Vibrio* Fur proteins that have been discovered to date but in none of the non-vibrio Fur proteins. Site-directed and random mutation analyses of the signature residues, the cysteine residues, and seven highly charged amino acid residues indicated that D9, H32, C137, and K138 of Fur_{Vh} are functionally important but D9, C137, and K138 can be replaced by more than one functional substitutes. Systematic deletion analysis demonstrated that the C-terminal 12 residues of Fur_{Vh} are functionally inessential. These results (i) indicated that the activation mechanism, or certain aspects of which, of Fur_{Vh} is possibly different from that of Fur_{Ec}; and (ii) suggested that it is not very likely that the C-terminal 12 residues play any significant role in the activation or stability of Fur_{Vh}; and (iii) provided insights into the potential function of the local structure involving C137 and K138.

***Keywords*:** *Vibrio harveyi*, ferric uptake regulator, transcriptional regulator, mutagenesis

To most living organisms iron is qualified as an essential nutrient as it is required in a number of fundamental biological activities that are vital for most forms of life (Crosa, 1997; Ratledge and Dover, 2000). In Gram-negative bacteria the processes of iron acquisition are controlled in a general fashion by the ferric uptake regulator (Fur), a metalloregulatory protein that is activated by Fe^{2+} and some other divalent transition metal ions (Bagg and Neilands, 1987; Ochsner *et al*., 1995). In the absence of its cognate metal ions, Fur exists largely as an inactive monomer or oligomer; binding of Fe^{2+} causes dimerization and activation of the protein. The activated Fur acts primarily as a transcriptional regulator that controls the expression of genes with diverse functions (Hantke, 2001). In most cases, regulation by Fur is achieved through interaction between Fur and the target promoter at an operator site termed Fur box, a 19 bp palindrome that is relatively conserved among the Gram-negative bacteria. Recently a genus-specific consensus Fur box has been proposed for the vibrios (Ahmad *et al*., 2008), which is characterized by the sequence of 5'-AATGANAAT NATTNTCATT-3'.

Studies of the *Escherichia coli* Fur (Fur_{Ec}) and the *Pseudomonas aeruginosa* Fur (Fur_{Pa}) have indicated that these Fur proteins possess three functional domains – the helix-turn-helix DNA binding domain, the protein-protein dimerization domain, and the metal ion responsive domains (Coy and Neilands, 1991; Saito *et al*., 1991; Pohl *et al*., 2003). The DNA binding domain is located at the N-terminal 1∼82 region, which forms four α-helices and two β-strands. In Fur_{Ec}, mutation of H32 in this domain and deletion of the first 9 residues involved in α_1-helix formation reduced the activity of the repressor (Coy and Neilands, 1991; Coy *et al*., 1994). Likewise, in Fur_{Pa}, interruption of α_1-helix formation by A10G mutation inactivated the protein (Barton *et al*., 1996). Two metal ion coordination sites have been proposed for Fur_{Ec}, one, the regulatory site, is involved in the binding of Fe^{2+}; occupation of this site by Fe^{2+} induces a conformational change that leads to the interaction of the DNA-binding domain of Fur with the target DNA. The other ion coordination site is called the Zn^{2+} structural site, which binds Zn^{2+} with high-affinity and is involved presumably in the structural stability of the protein. The Zn^{2+} coordination site of Fur_{Ec} is constituted by C92, C95, and two other residues (Jacquamet *et al*., 1998; Gonzalez De Peredo *et al*., 1999); in consequence of their structural roles, C92 and C95 are essential to the operation of Fur_{Ec} (Coy *et al.*, 1994). The functional importance of C92 and C95 probably accounts for the observation that these two residues are preserved in a large number of Fur proteins, including those of the vibrios. Fur_{Pa}, however, contains only one cysteine residue,

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which proves to be functionally dispensable (Lewin *et al*., 2002). Structural analysis of Fur_{Pa} indicated that it also possesses a Zn^{2+} coordination site which, unlike that of $Fur_{Ec,}$ is formed by a Zn^{2+} ion bound to two histidine residues (H32 and H89) and two glutamic acid residues (E80 and E100).

Vibrio is a large genus covering more than 30 species, including some noted human and aquaculture pathogens, such as *V. parahaemolyticus*, *V. anguillarum*, and *V. harveyi*. To date nine *Vibrio* Fur have been identified, some of which are known to be functional homologues of Fur_{Ec}. All the *Vibrio* Fur possess the cysteine residues that are counterparts of the C92 and C95 of Fur_{Ec}, but their structural and functional roles are largely unknown. Studies of *V. anguillarum* Fur revealed a structural Zn^{2+} site involving the cysteine residues (Zheleznova *et al*., 2000); however it is not clear whether C92 and C95 play any specific role in Zn^{2+} coordination. Recently Liu *et al*. (2007) have analyzed the *V. alginolyticus* Fur and found that there exist extensive similarities between *V. alginolyticus* Fur and Fur_{Pa} in the overall structure and domain features, including the DNA binding site, the regulatory and the structural ion binding sites.

In this study we reported the identification and analysis of the *fur* gene from a pathogenic strain of *V. harveyi*, which

Table 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primers	Relevant characteristics or sequences (5'→3')	Source or reference
Strains		
Escherichia coli		
DH5α	Host strain for general cloning	TaKaRa (China)
H1681	*fur-31, fhuF::λplacMu*	Heidrich *et al*. (1996)
NCK	*λ⁻△lacX74 rpsL galOP308 fur*	Wang *et al*. (2008)
Vibrio harveyi		
T4	Fish pathogen	Zhang and Sun (2007)
Plasmids		
pBR322	Ap^R; general cloning vector	New England BioLabs
pL1	Ap^R; pBR322 derivative containing P_{lac}	This study
pLEF	Ap^R; pL1 expressing fur_{Ec}	This study
pLVF	Ap^R; pL1 expressing fur_{Vh}	This study
pET258	Kn^R; expression plasmid	Zhang and Sun (2007)
pETVF	Kn^R; pET258 expressing fur_{Vh}	This study
pSC13	Tc^R; promoter probe plasmid	Wang *et al*. (2008)
pS104	Tc^R; pSC13 carrying P_{psuA}-*lacZ* fusion	This study
Primers[a]		
10F1	AATAATCAGGCGCTGAAGNNNGCGGGCTTAAAAGTAACC	
95F1	CACCTTGTTTGTCTGGATNNNGGTGAAGTTATTGAATTTTC	
137F1	AATGCAGCGACGGTTCTNNNAAAGATAATCCAGACGCAC	
138F1	CAGCGACGGTTCTTGCNNNGATAATCCAGACGCACA	
LacPF1	GAATTCATTTAAATGCAGCTGGCACGA (EcoRI-SwaI)	
LacPR4	GGATCCACACAACATACGAGC (*Bam*HI)	
MF11	GACTGCCAGGAAATCAGTGCTGAAGATTTG	
MR11	ACTGATTTCCTGGCAGTCTGGCTG	
PSUF1	GATATCTTGTGTTTTTAGGGTAAATA (*Eco*RV)	
PSUR1	GATATCGTTTAGTTGTTATAAAGC (*Eco*RV)	
VF1	CCCTTTGAAGTTCGTGGT	
VF48	GATATCTGTTAAATCGCTGCAGA (*Eco*RV)	
VR3	CACTAGGTGGTCGTGGTG	
VR52	GCGCGATATCTTATTTTACTGGTTTGTGTG (*Eco*RV)	
VR60	AGAACCGTCGCTGCATTTG	
VR61	GCAAGAACCGTCGCTGC	
VR62	CCCGGGACGAGAATGACTATCGCAATG	
VR64	ATCCAGACAAACAAGGTGGTCG	
VR65	CTTCAGCGCCTGATTATT	
VR67	CGCGATATCTTATTTTACTGGTTTGTGTGC	

[a] Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends.

is an important aquaculture pathogen and can infect a number of cultured marine species including shrimp, fish, and oyster. Our results indicated that there exists a high level of sequence identity between *V. harveyi* Fur (Fur_{Vh}) and Fur_{Ec}, yet the cysteines that are functionally essential in Fur_{Ec} were found dispensable in Fur_{Vh}, which suggested the possibility that Fur_{Vh} may employ an activation process that is different from that employed by Fur_{Ec}.

Materials and Methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1. All *Escherichia coli* strains were grown in Luria-Bertani lysis broth (LB) or M9 minimal medium (Miller, 1992) at 37°C with appropriate antibiotics, which were supplemented at the following concentrations: ampicillin (Ap), 100 μg/ml; kanamycin (Kn), 50 μg/ml; tetracycline (Tc), 15 μg/ml. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was supplemented at 40 μg/ml. *Vibrio harveyi* strains were grown in LB medium at 28°C.

Plasmid and strain constructions

The plasmids used in this study are listed in Table 1. To construct pL1, the *rrnB* transcription terminator of pTrcHis (Invitrogen) was ligated into pBR322 at between the EcoRV-BsaBI sites, resulting in pBRB; the *lacO*-less P_{lac} of pEGFP (Clontech) was amplified with primers LacPF1/LacPR4 and the PCR products were inserted into pBRB at between the EcoRI-BamHI sites, yielding pLS; a *Bam*HI linker was then inserted into pLS at between the BamHI-SmaI sites, resulting in pL1. pLVF and pLEF were generated by inserting fur_{Vh} and fur_{Ec} into pL1 at the *Sma*I site. pL1 derivatives carrying the mutant fur_{Vh} were constructed by inserting the mutant fur_{Vh}, which were generated by using the method of overlap extension PCR (Ho *et al*., 1989), at the *Sma*I site of pL1. To construct fur_{Vh} libraries with random mutations at D9, H32, C95, C137, and K138, the 5' and 3' portions of fur_{Vh} with overlapping ends were generated by PCR with primer pairs VF48/VR65 and 10F1/VR62, VF48/MR11 and MF11/VR67, VF48/VR64 and 95F1/VR62, VF48/VR60 and 137F1/VR62, VF48/VR61 and 138F1/VR62, respectively, followed by fusion PCR with primers VF48/VR52. The PCR products were ligated into pL1 at the *Sma*I site and the ligation mix was introduced into H1681 by transformation. The transformants were plated on MacConkey agar plates for the identification of lactose-fermenting strains. pS104 was constructed by inserting the 104 bp DNA (generated by PCR with primers PSUF1/PSUR1) upstream of the *psuA* gene of *V. alginolyticus* into the promoter probe plasmid pSC13 at the *Swa*I site.

DNA and molecular techniques

Plasmid preparation, PCR amplifications, purification of PCR products, and genomic DNA preparation were carried out as described previously (Zhang and Sun, 2007). Restriction endonucleases and modifying enzymes were purchased from New England BioLabs (China) and used in accordance with the manufacturer's specifications.

Cloning of fur_{Vh}

Degenerate PCR was performed to amplify an internal 376 bp DNA fragment of fur_{Vh} by using primers VF1/VR3 and T4 genomic DNA as the template. The up- and down-stream regions of this 376 bp DNA were obtained by genome walking as described previously (Zhang and Sun, 2007).

Preparation of the recombinant Fur_{Vh}

The coding sequence of fur_{Vh} was inserted into pET258 at the NdeI-XhoI sites, resulting in plasmid pETVF, which was introduced into BL21(DE3) (Tiangen, China) by transformation. The His-tagged recombinant Fur_{Vh} was purified from BL21(DE3)/pETVF by using nickel-nitrilotriacetic acid beads as described previously (Zhang and Sun, 2007).

Antisera

Antisera to the recombinant Fur_{Vh} was prepared by subcutaneously injecting an adult New Zealand White rabbit with 250 μg of purified recombinant Fur_{Vh} mixed in complete Freund's adjuvant, followed by a boost with the same amount of Fur_{Vh} in incomplete Freund's adjuvant 25 days later. A second boost was performed 12 days post the first boost. The rabbit was bled 14 days after the second boost and the blood was collected, from which the sera were obtained by centrifugation.

Western and immunoblot

Cells were grown in LB medium to OD_{600}~0.9 and lysed with the lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, and 8 M urea, pH 8.0). The lysed cells were centrifuged at 4°C for 10 min; the supernatant was electrophoresed in 0.1% sodium dodecyl sulfate (SDS)/15% polyacrylamide gels. After electrophoresis, the proteins in the gels were transferred to nitrocellulose membranes. Immunoblotting was performed as described previously (Martin *et al.*, 2004) and the Fur_{Vh} proteins were detected by using rabbit anti-Fur_{Vh} antibodies.

β-Galactosidase assay

This was carried out as described by Sun *et al*. (1998).

H_2O_2 survival test

This was performed essentially as described by Quatrini *et al.* (2005). Briefly, cells were grown in LB medium to OD_{600} 0.5; half of the cell culture was removed and grown separately in LB medium supplemented with 15 mM H_2O_2 while the other half of the cell culture was grown continuously in the absence of H_2O_2. After 20 min growth at 37°C, the cells were chilled on ice for 10 min and then diluted in fresh LB medium. The diluted cells were plated on LB agar plates and, after overnight incubation at 37°C, the colonies that appeared on the plates were enumerated. Survival rates were presented as percentages of the colonies derived from the H_2O_2-treated cells compared to those derived from the untreated cells.

Database search and nucleotide sequence accession numbers

DNA and amino acid sequence search was conducted using the BLAST programs at the NCBI (National Center for

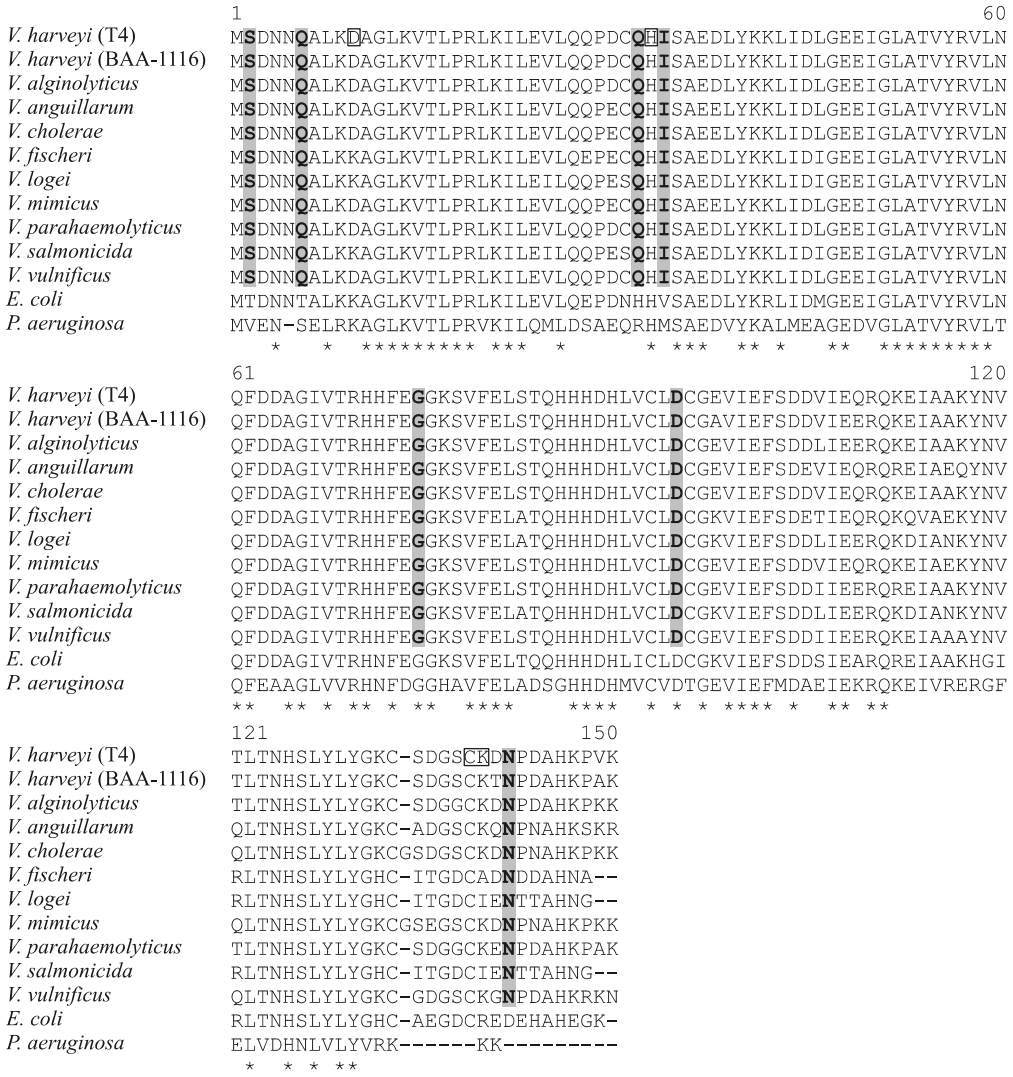

Fig. 1. Alignment of the sequences of the *Vibrio* Fur, Fur_{Ec}, and Fur_{Pa}. Asterisks indicate residues that are identical among all the listed Fur proteins. Shaded and bold residues constitute the *Vibrio* Fur signature. Boxed residues are essential to the activity of Fur_{Vh}. The GenBank accession no. of the *Vibrio* Fur (from top to bottom in the figure) are as follows: EF197913, ABU70316, AY957395, AAA16861, AAA27519, AAW85305, CAD26842, BAD24855, BAC59096, CAD26839, and AAO08713.

Biotechnology Information). The nucleotide sequence of the fur_{Vh} region has been deposited in GenBank databases under the accession number EF197913.

Results

Fur_{Vh} could complement the mutant phenotype of an *E. coli* strain defective in fur_{Ec}

fur_{Vh} was obtained from the *V. harveyi* strain T4, a pathogenic fish isolate, by degenerate PCR and genome walking. fur_{Vh} encodes a protein of 149 amino acids which differ at two positions from the Fur protein of BAA-1116, the *V. harveyi* strain that has been sequenced at the genome scale (GenBank accession no. CP000789). Outside the *V. harveyi* species, the closest homologues of Fur_{Vh} are found among the other vibrios (Fig. 1), of which the Fur proteins of *V. alginolyticus* and *V. parahaemolyticus* share the highest (97 and 95%, respectively) sequence identities with Fur_{Vh}. The overall sequence identity between Fur_{Vh} and Fur_{Ec} is 77%, most of which is located at the N-terminal DNA binding region (1~82), within which the similarity of these two proteins reaches 95%.

Given the high level of similarity between Fur_{Vh} and Fur_{Ec}, especially at the DNA binding domain, it was likely that Fur_{Vh} could function as a Fur_{Ec} substitute. To investigate whether or not this was the case, we utilized the *fur*-defective *E. coli* strain H1681 (Heidrich *et al*., 1996). H1681 carries a promoterless *lacZ* gene fused to the promoter (named P_{fhu}) of the gene *fhuF*; since P_{fhu} is a target promoter of Fur_{Ec}, it can be repressed by Fur_{Ec} or the functional homologues of Fur_{Ec}. When H1681 is transformed with a plasmid expressing a heterologous *fur* such as fur_{Vh}, the ability of the heterologous Fur to repress P_{fhu} can be monitored by the level of *lacZ* expression which can be de-

termined by β-galactosidase assay. A high level of β-galactosidase activity will be an indicator of a low binding affinity of the heterologous *fur* to P_{fhu}. In our case, H1681 was transformed separately with the plasmids pLVF and pLEF that constitutively express fur_{Vh} and fur_{Ec}, respectively. The transformants were subjected to β-galactosidase assay, which showed that the β-galactosidase activities of H1681/pLVF and H1681/pLEF (10.1 and 7.8 Miller units, respectively) were, respectively, 94.7 and 95.9% lower than that of H1681 transformed with the control plasmid pL1 (189 Miller units), suggesting that Fur_{Vh} repressed expression of the *lacZ* reporter gene to an extent similar to that effected by Fur_{Ec}. Consistently, the presence of the iron chelator dipyridyl, which inactivates Fur by depleting iron, increased the β-galactosidase activities of H1681/pLVF and H1681/pLEF (178 and 172 Miller units, respectively) to a level approaching that of H1681/pL1. These results indicated that Fur_{Vh} could interact with and repress transcription from the target promoter of Fur_{Ec}.

To further examine the ability of Fur_{Vh} to complement the defectiveness of Fur_{Ec}, H_2O_2 survival test was performed upon H1681/pL1, H1681/pLEF, H1681/pLVF, and H1681 harboring pK138E, which expresses a mutant fur_{Vh} (see below). The result showed that the survival rates of H1681/pL1, H1681/pLEF, H1681/pLVF, and H1681/pK138E after treatment with 15 mM H_2O_2 were, respectively, 4.9, 46.7, 43.9, and 5.2%, suggesting that Fur_{Vh} could, like Fur_{Ec}, confer upon H1681 the ability against H_2O_2 challenge.

Taken together, these results demonstrated that Fur_{Vh} could act as an effective Fur_{Ec} substitute.

Identification and significance analysis of the *Vibrio* Fur signature

To date, Fur of ten (including Fur_{Vh}) different *Vibrio* species have been known at the sequence level. Of these, the Fur proteins of *V. alginolyticus* and *V. parahaemolyticus* exhibit

Table 2. β-Galactosidase activities of H1681 harboring pL1 derivatives that express fur_{Vh} variants bearing single amino acid substitutions or C-terminal deletions

Strain H1681 harboring	Fur_{Vh}		β-Galactosidase activity (%)	
	Substitution	Deletion	− DP	+ DP
pL1	-	-	100	100
pLVF	Wild type	-	6.8	91.1
pQ5A	Q5A	-	10.9	96.5
pQ31A	Q31A	-	7.1	89.3
pI33A	I33A	-	9	94
pD94A	D94A	-	8.6	90.2
pN140A	N140A	-	10.7	104
pD9K	D9K	-	90.3	ND
pH32E	H32E	-	94.8	ND
pK116E	K116E	-	12	87.8
pK131E	K131E	-	10.6	92.6
pK138E	K138E	-	91.2	ND
pK145E	K145E	-	7.2	114.2
pK148E	K148E	-	6.1	95.9
pC30N	C30N	-	5.5	93.8
pC92S	C92S	-	11	102.9
pC95S	C95S	-	7.1	96.4
pC132S	C132S	-	7.5	110
pC137S	C137S	-	34.3	91.6
pDC1	-	C1	10.4	98.2
pDC2	-	C2	9.2	97.5
pDC8	-	C8	10.9	99
pDC12	-	C12	11.2	92.1
pDC16	-	C16	24.1	94.7
pDC31	-	C31	89.5	ND
pDC34	-	C34	92.7	ND
pDC44	-	C44	85.2	ND
pDC53	-	C53	90.8	ND

For β-galactosidase assays, cells were cultured to OD_{600}~1 in M9 minimal medium supplemented with 50 μM $FeCl_3$ or 50 μM 2, 2'-dipyridyl (DP). The values are shown in percentages of the β-galactosidase activity of H1681/pL1. Data are the means calculated from the results of at least three independent experiments. ND, not determined.

high sequence identities (95~97%) to Fur_{Vh} whereas those of *V. logei* and *V. salmonicida* exhibit relatively low sequence identities (84~85%) to Fur_{Vh}. Sequence comparison revealed that all *Vibrio* Fur share a common feature, which is having Ser, Gln, Gln, Ile, Gly, Asp, and Asn at positions 1, 5, 31, 33, 74, 94, and 140 (note: the residues of Fur_{Vh} were numbered, for the convenience of comparison, according to the numbering tradition of $Fur_{Ec,}$ with the first amino acid Met being ignored), respectively (Fig. 1). The position-associated conservedness of these residues appears in all the Fur proteins discovered in the vibrios but is absent, as a collective feature, in the Fur proteins of other bacterial species. Based on its universal and exclusive presence in the vibrios, S1, Q5, Q31, I33, G74, D94, and N140, as a collective characteristic, was designated the *Vibrio* Fur signature. To investigate their potential significance in the functioning of Fur_{Vh}, Q5, Q31, I33, D94, and N140 were each mutated to alanine; the mutant fur_{Vh} were cloned into pL1, resulting in plasmids pQ5A, pQ31A, pI33A, pD94A, and pN140A, respectively, which constitutively express the respective mutant fur_{Vh}. H1681 was transformed separately with each of these plasmids and the transformants were assayed for Fur activity in the form of β-galactosidase production, which showed that the β-galactosidase activities of H1681 harboring pQ5A, pQ31A, pI33A, pD94A, and pN140A were comparable to that of H1681/pLVF in both the absence and the presence of dipyridyl (Table 2). Hence, although preserved throughout the vibrios, Q5, Q31, I33, D94, and N140 could be functionally substituted by alanine.

D9, H32, and K138 were functionally important

Studies of DtxR, the Fur homologue of *Corynebacterium diphtheriae*, have demonstrated that mutation of a highly charged residue at the C-terminal domain alters the activation process of DtxR (Sun *et al*., 1998; Love *et al*., 2004). To investigate whether a similar phenomenon could be induced in Fur_{Vh}, glutamic acid substitution was performed upon the C-terminal highly charged residues K116, K131, K138, K145, K148, and H32, the last one was included for the reason that it is known to be an essential residue in

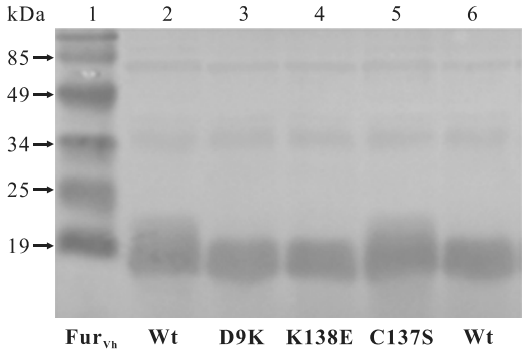

Fig. 2. Western immunoblotting analysis of Fur_{Vh} production in H1681 expressing fur_{Vh} variants. H1681/pLVF, H1681/pD9K, H1681/pK138E, and H1681/pC137S that express the wild type fur_{Vh}, the mutant fur_{Vh} bearing D9K, K138E, and C137S substitutions, respectively, were grown in LB medium to OD_{600} 0.9. The cells were lysed and the whole cell proteins were run in 0.1% SDS/15% polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes. Immunoblotting was performed by using rabbit anti-Fur_{Vh} antibodies. Lane 1, molecular weight markers. kDa, kilo Dalton. Wt, wild type Fur_{Vh}.

Fur_{Ec} and *V. alginolyticus* Fur (Coy *et al*., 1994; Liu *et al*., 2007). In addition, lysine substitution was performed upon D9, which was chosen because it is located in the DNA binding α_1-helix and, though it is conserved in seven of the ten known *Vibrio* Fur, it varies to a lysine residue in the other three species. The mutant fur_{Vh} were cloned into pL1 and the resulting plasmids, pK116E, pK131E, pK138E, pK145E, pK148E, pH32E, and pD9K that express the respective mutants as indicated by their names, were introduced into H1681 by transformation. The transformants were assayed for β-galactosidase production, which showed that except for H1681/pD9K, H1681/pH32E, and H1681/pK138E, which exhibited 13-fold more β-galactosidase activity than H1681/pLVF, all other transformants displayed β-galactosidase activities that were 0.9~1.76 fold of that displayed by H1681/pLVF (Table 2). To examine whether the increased

Table 3. β-Galactosidase activities of H1681 expressing functional fur_{Vh} variants bearing mutations at D9, K138, C95, and C137, respectively

D9 mutation	β-Gal activity (%)	K138 mutation	β-Gal activity (%)	C95 mutation	β-Gal activity (%)	C137 mutation	β-Gal activity (%)
D9I	7.4	Wt	6.8	Wt	6.8	C137A	10.1
D9L	7.1	Wt	6.8	C95N	6.9	C137I	7.0
D9L	7.1	Wt	6.8	C95N	6.9	C137I	7.0
D9M	6.9	K138I	5.3	C95N	6.3	C137I	7.0
D9Q	5.8	K138I	5.3	C95N	6.3	C137stop	11.6
D9S	7.5	K138I	5.3	C95N	6.3	C137stop	11.6
D9V	7.5	K138P	6.7	C95N	6.3		
D9V	7.5	K138P	6.7	C95T	7.2		
D9W	5.2	K138R	5.4				
D9W	5.2	K138V	6.2				

For β-galactosidase assays, cells were cultured to OD_{600}~1 in M9 minimal medium supplemented with 50 μM $FeCl_3$. The β-galactosidase (β-Gal) activities are shown in percentages of that of H1681/pL1, which is set as 100. Data are the means of at least three independent experiments. Wt, wild type; C137stop, C137 was substituted by a stop codon.

β-galactosidase activities in H1681/pK138E and H1681/pD9K were due to reduced production or instability of the mutant Fur_{Vh}, western immunoblotting was performed upon the whole cell proteins of these strains and the result showed that the amounts of Fur_{Vh} in these two strains approximated that in H1681/pLVF (Fig. 2). Similar result was obtained with western immunoblotting analysis of Fur_{Vh} production in H1681/pH32E (data not shown). These results indicated that the increased β-galactosidase activities in H1681/pH32E, H1681/pK138E, and H1681/pD9K were due to reduced activities of the mutant Fur_{Vh}, which implied that H32, K138, and D9 were essential to the repressor activity of Fur_{Vh}.

With the above results, we wondered whether D9, H32, and K138 were truly indispensable. We speculated that, although these three residues could not be functionally substituted by glutamic acid and lysine, there was the possibility that they might be functionally substituted by some other amino acids. To examine this possibility, libraries of *fur*$_{Vh}$ bearing random mutations at D9, H32, and K138, respectively, were ligated into pL1; H1681 was transformed with the ligation mix and the transformants were selected on MacConkey agar (Heidrich *et al*., 1996) plates for white (i.e. expressing functional *fur*$_{Vh}$) colonies. MacConkey agar is a culture medium designed for the identification of lactose-fermenting bacteria which appear red on the plates. In our case, red H1681 colonies on MacConkey agar plates would be an indicator that in these colonies the *lacZ* gene was expressed and β-galactosidase was produced; on the other hand, white colonies would be an indicator that *lacZ* expression was repressed. The result showed that ten white colonies were obtained from among $\sim 1\times10^5$ red ones bearing D9 random mutations. Similarly, ten white colonies were obtained from among $\sim 2\times10^5$ red ones bearing K138 random mutations. However, no white colonies were found among the $\sim 2\times10^5$ colonies bearing H32 random mutations. The plasmids contained in the white colonies were analyzed at the sequence level, which showed that, of the 10 functional D9 substitutions, three were D9L mutants, four were D9V and D9W mutants (two for each) and the remaining three were, respectively, D9M, D9Q, and D9S mutants (Table 3). Of the 10 functional K138 substitutions, three proved to be wild type, three were K138I mutants, two were K138P mutants, and the remaining two were K138R and K138V mutants (Table 3). Like the wild type Fur_{Vh}, all the functional Fur_{Vh} mutants were sensitive to dipyridyl (data not shown). These results demonstrated that, although D9 and K138 could not be functionally replaced by lysine and glutamic acid, respectively, they could be so replaced by, for D9, relatively a wide range of uncharged residues and, for K138, nonpolar hydrophobic as well as positively charged residues. In contrast, H32 appeared to be "unreplaceable" in the sense that no functional substitute could be found for it under the experimental conditions described above.

C137, but not C92 or C95, was functionally important

As mentioned earlier, C92 and C95 of Fur_{Ec} are functionally essential on account of their participation in the formation of the Zn^{2+} structural site. Fur_{Vh} possesses five cysteine residues, four (including C92 and C95) of which corresponding to those in Fur_{Ec}. To examine their potential significance in the operation of Fur_{Vh}, these cysteine residues were mutated individually to serine or, in the case of C30, asparagine, which is the counterpart in Fur_{Ec}. The plasmids pC30N, pC92S, pC95S, pC132S, and pC137S that express the mutant *fur*$_{Vh}$ as indicated by their names were introduced into H1681 by transformation. Subsequent β-galactosidase assay showed that the β-galactosidase activity of H1681/pC137S was 5-fold more than that of H1681/pLVF, whereas the β-galactosidase activities of all other transformants were 0.8∼1.6 fold of that of H1681/pLVF (Table 2). Western immunoblot showed that the amount of Fur_{Vh} in H1681/pC137S was similar to that in H1681/pLVF (Fig. 2). These results indicated that C137S substitution impaired the activity of Fur_{Vh} whereas all other substitutions had no apparent effect on Fur_{Vh} activity.

To further determine the potential essentialness of C95 and C137, their functional substitutes were screened from random mutation libraries as described above for D9 and K138. The results showed that of the 8 functional Fur_{Vh} selected from the C95 random mutation library, one was wild type, six had C95N substitutions, and one had C95T substitution (Table 3). Of the 6 functional Fur_{Vh} selected from the C137 random mutation library, one bore C137A mutation, three bore C137I mutations, and two bore deletions of the C-terminal 12 amino acid residues (C12) as a result of replacing C137 by a stop codon (Table 3). Like the wild type Fur_{Vh}, all the functional Fur_{Vh} mutants were sensitive to dipyridyl (data not shown). These results demonstrated that C95 could be functionally replaced not only by serine but also by two other uncharged polar amino acids whereas C137, though partially inactivated by serine substitution, could be functionally substituted by two hydrophobic amino acids.

The C-terminal 12 amino acid residues of Fur_{Vh} were functionally inessential

The above findings that deletion of C12 had no apparent effect on the activity of Fur_{Vh} promoted us to analyze the functional importance of the C-terminal residues of Fur_{Vh}. For this purpose, the C terminal 1, 2, 8, 12, 16, 31, 34, 44, and 53 residues (named C1 to C53, respectively) of Fur_{Vh} were each deleted and the respective mutant *fur*$_{Vh}$ were cloned into pL1. The resulting plasmids, pDC1 to pDC53, which constitutively express the truncated *fur*$_{Vh}$ bearing deletions of C1 to C53, respectively, were each introduced into H1681 by transformation. The transformants were assayed for β-galactosidase production, which showed that the β-galactosidase activities of H1681 transformed with pDC1, pDC2, pDC8, and pDC12 were 1.3∼1.6 fold of that of H1681/pLVF while the β-galactosidase activity of H1681/pDC16 was 3.5-fold of that of H1681/pLVF (Table 2); the presence of dipyridyl increased the β-galactosidase activities of these transformants to the level approaching that of H1681/pL1. In contrast, the β-galactosidase activities of H1681 transformed with pDC31, pDC34, pDC44, and pDC53 were 12∼13 folds higher than that of H1681/pLVF (Table 2). Western immunoblot assay showed that the amounts of Fur_{Vh} produced in H1681 transformed with pDC16, pDC31, pDC34, and pDC44 were comparable to that produced in

H1681/pLVF (data not shown). These results indicated that Fur_{Vh} bearing C-terminal deletions up to 12 residues still maintained most of the repressor activity.

Effect of Fur_{Vh} on the activity of a target promoter of the *V. alginolyticus* Fur

H1681 is a convenient and efficient system for the study of Fur proteins that are functional homologues of Fur_{Ec}. Fur_{Vh}, as shown above, is an effective Fur_{Ec} analogue, which was the basis for our having chosen H1681 as the genetic system for the analysis of Fur_{Vh}. Yet, since our study demonstrated that Fur_{Vh} differs from Fur_{Ec} in certain features, such as the functional qualities of the cysteine residues, it would be ideal to further examine Fur_{Vh} in a system that employs a native Fur_{Vh} target promoter. Literature searching indicated that no Fur_{Vh} targets have been reported to date. Although the genome sequencing data of the *V. harveyi* strain BAA-1116 reveal a couple of genes that are putatively iron-regulated, our study showed that the upstream regions (i.e. the putative promoter regions) of these genes, when cloned into a promoter probe plasmid, either exhibited no promoter activity or exhibited promoter activity that appeared not regulated directly by Fur_{Vh} (Sun, K. and L. Sun, unpublished data). It is known that in *V. alginolyticus* and *V. parahaemolyticus*, Fur directly regulates the expression of a gene named *psuA*, which is involved in siderophore utilization (Funahashi *et al*., 2002; Wang *et al*., 2007). Since *V. harveyi* is phylogenetically closely related to *V. alginolyticus* and *V. parahaemolyticus* and, as demonstrated above, Fur_{Vh} shares the highest sequence identity with the *V. alginolyticus* Fur (the two proteins differ in only three amino acids; Fig. 1), we decided to analyze the activity of Fur_{Vh} upon the *psuA* promoter (named P_{psuA}). For this purpose the low copy-number (with a pSC101 replication origin) promoter probe plasmid pS104 was constructed, in which the 104 bp DNA containing P_{psuA} and the Fur box was fused to a promoterless *lacZ* reporter

Table 4. β-Galactosidase activities of NCK/pS104 harboring pL1 derivatives that express fur_{Vh} variants bearing single amino acid substitutions or C-terminal deletions

NCK/pS104 harboring	Fur_{Vh}		β-Galactosidase activity (%)	
	Substitution	Deletion	− DP	+ DP
pL1	-	-	100	100
pLVF	Wild type	-	4.8	83.4
pQ5A	Q5A	-	5.9	93.6
pQ31A	Q31A	-	6.9	89.5
pI33A	I33A	-	5.5	91.7
pD94A	D94A	-	5.2	101.6
pN140A	N140A	-	7.0	87.5
pD9K	D9K	-	94.3	79.8
pH32E	H32E	-	88.3	89.0
pK116E	K116E	-	5.0	106.1
pK131E	K131E	-	6.8	88.5
pK138E	K138E	-	92.9	99.2
pK145E	K145E	-	6.1	103.2
pK148E	K148E	-	6	101
pC30N	C30N	-	7.2	97
pC92S	C92S	-	7.1	105.5
pC95S	C95S	-	6.1	99.4
pC132S	C132S	-	7.5	80.9
pC137S	C137S	-	81.7	101.9
pDC1	-	C1	5.1	92.8
pDC2	-	C2	5.5	89.3
pDC8	-	C8	5.4	86.5
pDC12	-	C12	5.8	94.5
pDC16	-	C16	11.6	112
pDC31	-	C31	98.9	ND
pDC34	-	C34	98	ND
pDC44	-	C44	72.4	ND
pDC53	-	C53	99.8	ND

β-Galactosidase assay was performed with cells that had been cultured to OD_{600}~1 in M9 minimal medium supplemented with 50 μM $FeCl_3$ or 50 μM 2, 2'-dipyridyl (DP). The values are shown in percentages of the β-galactosidase activity of NCK/pS104/pL1. Data are the means calculated from the results of three independent experiments. ND, not determined.

gene so that the activity of P_{psuA} can be monitored by β-galactosidase assay. pS104 was introduced into the *fur*-defective *E. coli* strain NCK by transformation. The transformant appeared as blue colonies on X-gal plate, suggesting that P_{psuA} is a functional promoter that can direct the expression of the *lacZ* reporter gene. NCK/pS104 was then transformed with pL1, pLVF, and the plasmids expressing all the fur_{Vh} mutants studied above. The transformants were assayed for β-galactosidase production, which showed that (i) the β-galactosidase activity of NCK/pS104/pLVF was 20.8-fold lower than that of NCK/pS104/pL1 (Table 4), suggesting that Fur_{Vh} could effectively repress the expression of P_{psuA}; (ii) the β-galactosidase activities of NCK/pS104 harboring pD9K, pH32E, pK138E, and pC137S were more than 17-fold higher than that of NCK/pS104/pLVF, suggesting that D9, H32, K138, and C137 were functionally essential; (iii) the β-galactosidase activities of NCK/pS104 harboring pDC1, pDC2, pDC8, and pDC12 were comparable to that of NCK/pS104/pLVF, suggesting that the C-terminal 12 residues of Fur_{Vh} were functionally inessential. These results demonstrated that the activities of Fur_{Vh} measured upon P_{psuA} were consistent with those measured upon the Fur_{Ec} target promoter.

Discussion

Previous studies have identified Fur homologues in nine members of Vibrios and the sequences of these *fur* alleles have been demonstrated to be useful as an alternative genetic marker for phylogenetic analysis of the vibrios (Colquhoun and Sorum, 2002). Similarly, we found that by the amino acid sequence of Fur *V. harveyi* is classified as closely related to *V. alginolyticus* and *V. parahaemolyticus* (95∼97% identity), moderately related to *V. mimicus* and *V. anguillarum* (91∼94% identity), and distantly related to *V. logei*, *V. salmonicida*, and *V. fischeri* (84∼85% identity), which is largely in accordance with the phylogenetic relationships generated by using the sequences of 16S rDNA, *recA*, and *rpoA* (Thompson *et al.*, 2004). Our results support the notion that phylogenetic analysis based on the sequence of Fur can serve as a complementary means to the traditional typing methods. The conservedness of Fur is in line with its role as a global regulatory protein that participates in multiple aspects of cellular life and, as such, probably has been preserved during evolution in a fashion similar to that in which the house keeping genes have been preserved.

Consistent with the above observation that individually each of the known *Vibrio* Fur proteins can serve as a species marker, collectively as a group these Fur proteins possess a common genetic feature, the *Vibrio* Fur signature, which can serve as a genus marker to distinguish the vibrios from the non-vibrios. The fact that five of the signature residues can be functionally replaced, on an individual level, by alanine suggests that the significance of the *Vibrio* Fur signature is probably more of evolutionary than of functional.

In Fur_{Ec}, K9, the counterpart of D9 in Fur_{Vh}, constitutes part of the N-terminal region that is unfolded in the unactivated protein but appears as a structured α-helix in the monomeric and activated form of Fur_{Ec} (Hamed and Al-Jabour, 2006; Pecqueur *et al.*, 2006). This N-terminal helix is conserved between Fur_{Ec} and Fur_{Pa} and is essential to DNA binding (Coy and Neilands, 1991; Barton *et al*., 1996; Pohl *et al*., 2003). The functional importance of D9 in Fur_{Vh} supports the idea that a similar N-terminal α-helix may exist in Fur_{Vh} and, like in Fur_{Ec}, its integrity is crucial to the activity of the repressor. It may be further speculated that functional maintenance of this helix presumably exerts certain structural constraints on a scale that is local, which may account for the observed essentialness of D9, or global, which may link the essentialness of the N-terminal D9 to that of the C-terminal C137 and K138. The fact that D9, C137, and K138 could be replaced by more than one other amino acid residues suggested the possibility that either these surrogating amino acid residues could act exactly like the native residues in the fulfillment of the roles played by the native residues or, more likely, considering the biochemical natures of the surrogates, which in some cases differ largely from those of the native residues, that the local structures involving D9, C137, and K138 are flexible and can tolerate certain alterations such as those caused by the substitution with the selected surrogating amino acids. The failure in the selection of a functional H32 substitute suggested that either this residue was truly unreplaceable or the size of the random library used in the selection was not large enough. In either case, it indicated that H32 was essential to the functional operation of Fur_{Vh}.

Of the two metal binding sites identified in Fur_{Pa} and Fur_{Ec}, the high-affinity Zn^{2+} binding site plays an important part in the shaping of the overall structure of the protein. Though the Zn^{2+} site has been found or proposed to exist in a number of Fur proteins, its location and coordinating ligands appear to be variable. While the Zn^{2+} site of Fur_{Pa} involves no cysteines (Lewin *et al*., 2002), that of Fur_{Ec} involves C92 and C95 and that of the *Bacillus subtilis* PerR, a member of the Fur family protein, involves the equivalent of C132 of Fur_{Ec} (Coy *et al*., 1994; Gonzalez De Peredo *et al*., 1999; Lee and Helmann, 2006). In *V. anguillarum* Fur, which possesses five cysteines corresponding to those in Fur_{Vh}, binding of Zn^{2+} is mediated by the cysteine residues (Zheleznova *et al*., 2000), though it is not clear which of the cysteines serve as the actual ligands. In *V. alginolyticus* Fur, C95, and C132 prove to be functionally insignificant (Liu *et al*., 2007). In Fur_{Vh}, we found that, except for C137, all other cysteine residues were functionally dispensable. Our results suggested the possibility that either Fur_{Vh} exhibits no structural Zn^{2+} site or no such structural Zn^{2+} site as it is found in Fur_{Ec}; it is more likely that Fur_{Vh} possesses a structural Zn^{2+} site that is supported by ligands other than C92 and C95. In either case, our findings imply that the activation mechanisms, or certain aspects of which, may be different between Fur_{Vh} and Fur_{Ec}. The finding that the activities of Fur_{Vh} and its mutants measured upon P_{psuA} correlated with those measured upon P_{fhu} indicated that despite the possible difference in the Zn^{2+} binding site, Fur_{Vh}, once activated, regulated P_{psuA} and P_{fhu} alike. These results suggested that the activation process, which may vary among different Fur proteins, is required for, but does not determine the efficiency of, DNA binding. Similar observations have been reported previously, for example, Fur_{Pa}, which possesses a Zn^{2+} coordination site that is completely differ-

ent from that of Fur_{Ec}, can recognize and repress transcription from the target promoter of Fur_{Ec} (Lewin *et al*., 2002); the DNA binding region of Fur_{Ec}, when fused to the C-terminal domain of the lambda phage repressor CI857, acts like the wild type Fur_{Ec} in the regulation of Fur_{Ec}-responsive promoters (Stojiljkovic and Hantke, 1995).

The essentialness of C137 and K138 and the inessentialness of the C-terminal 12 residues, which include C137 and K138, suggested that in the wild type Fur_{Vh} the C-terminal 12 residues are likely to play a minor role in the activation or operation of the repressor; C137S and K138E mutations may have induced certain structural changes that are inhibitory to the activation of Fur_{Vh}. This hypothesis can find support in the observation that Fur_{Vh} bearing C12 deletion was fully sensitive to dipyridyl, which rules out, to a large extent, the possibility that the C-terminal 12 residues are involved in the processes of Fe^{2+} coordination. It would be interesting to compare the *in vivo* biological effect of the wild type Fur_{Vh} with that of the mutant Fur_{Vh} bearing C12 deletion; it is possible that the C-terminal 12 residues, though appearing inessential to the activity of Fur_{Vh}, may have some yet unknown functions such as being the recognition/targeting site of proteins that interact with Fur_{Vh}, which may account for the preservation of the last 12 residues in Fur_{Vh}.

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