

## Full Paper

# Domain analysis of the *Edwardsiella tarda* ferric uptake regulator

Kun Sun,<sup>1,2</sup> Shuang Cheng,<sup>1,2</sup> Fang Wang,<sup>1,2,3</sup> and Li Sun<sup>1,\*</sup>

<sup>1</sup> Institute of Oceanology, Chinese Academy of Sciences, Qingdao, PR China

<sup>2</sup> Graduate University of the Chinese Academy of Sciences, Beijing, PR China

<sup>3</sup> Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, PR China

(Received March 24, 2009; Accepted May 19, 2009)

Recent studies have shown that the ferric uptake regulator (Fur) of *Edwardsiella tarda* (Fur<sub>Et</sub>) shares high sequence identity with the *Escherichia coli* Fur (Fur<sub>Ec</sub>) at the N-terminal DNA-binding region. In the present study, the functional importance of the C-terminal region of Fur<sub>Et</sub> was investigated. It was found that Fur<sub>Et</sub> bearing deletion of the C-terminal 12 residues still possesses most of the repressor activity, whereas Fur<sub>Et</sub> bearing deletions of the C-terminal 16 and more than 16 residues are severely affected in activity. Domain swapping analyses indicated that the chimeric Fur proteins (Et75Ec73 and Et75Vh74) consisting of the N-terminal 1–75 region of Fur<sub>Et</sub> fused to the C-terminal 76–148 region of Fur<sub>Ec</sub> and the C-terminal 76–149 region of the *Vibrio harveyi* Fur (Fur<sub>Vh</sub>), respectively, are fully active. C92 of Fur<sub>Ec</sub> and C137 of Fur<sub>Vh</sub>, which are functionally essential in Fur<sub>Ec</sub> and Fur<sub>Vh</sub>, respectively, are also essential in Et75Ec73 and Et75Vh74, respectively. Further study identified an artificial Fur protein, EtMF54, which is composed of the N-terminal 49 residues of Fur<sub>Et</sub> and five artificial residues. Compared to Fur<sub>Et</sub>, EtMF54 possesses partial Fur activity that is iron-dependent. These results (i) indicate that there exist certain functional/structural compatibilities among Fur<sub>Et</sub>, Fur<sub>Ec</sub>, and Fur<sub>Vh</sub> at the C-terminal region; (ii) provide insights to the potential location of the regulatory ion-binding site of Fur<sub>Et</sub>.

**Key Words**—domain swapping; *Edwardsiella tarda*; ferric uptake regulator; mutagenesis

## Introduction

The ferric uptake regulator (Fur) is a global regulatory protein that is involved in diverse aspects of bacterial life (Hantke, 2001; Ratledge and Dover, 2000). Fur is a metalloregulatory protein that requires Fe<sup>2+</sup>, or other divalent transition metal ions, as a cofactor (Bagg and Neilands, 1987; Ochsner et al., 1995; Zhelezнова et al., 2000). Fur is synthesized as a biologically inactive monomer, which, upon binding of Fe<sup>2+</sup>, becomes activated via dimerization. The activated

Fur acts primarily as a transcription repressor and regulates the expression of target genes by binding to a 19-bp palindrome sequence termed Fur box. Fur in general possesses 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; Pohl et al., 2003; Saito et al., 1991). Structural and gene fusion analyses of the *Escherichia coli* Fur (Fur<sub>Ec</sub>) have demonstrated that the DNA-binding domain is located at the N-terminal 1–82 region, which forms the classical winged helix-turn-helix motif composed of three  $\alpha$ -helices and two  $\beta$ -strands plus a unique N-terminal  $\alpha$ -helix (Hamed and Al-Jabour, 2006; Pecqueur et al., 2006; Stojiljkovic and Hantke, 1995). Highly similar structural motifs are also found in the corresponding N-terminal region of the *Pseudomonas aeruginosa* Fur (Fur<sub>Pa</sub>). Fur<sub>Pa</sub>, how-

\* Address reprint requests to: Dr. Li Sun, Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, PR China.

Tel and Fax: 86-532-82898834

E-mail: lsun@ms.qdio.ac.cn

ever, differs from Fur<sub>Ec</sub> in the metal ion-binding domain. Two metal ion coordination sites have been identified or proposed to exist in Fur<sub>Ec</sub> and Fur<sub>Pa</sub>; one is the regulatory Fe<sup>2+</sup>-binding site. Occupation of Fe<sup>2+</sup> at this site enables the repressor to interact specifically with the Fur box sequence; the other metal ion coordination site is the structural Zn<sup>2+</sup>-binding site, so called because it is involved in high-affinity binding of Zn<sup>2+</sup> and presumably functions in the shaping of the overall structure of the repressor protein (Althaus et al., 1999; Gonzalez de Peredo et al., 1999; Jacquamet et al., 1998). The Zn<sup>2+</sup>-binding site of Fur<sub>Ec</sub> is formed by C92, C95, and two other residues, whereas that of Fur<sub>Pa</sub> involves no cysteine residues (Lewin et al., 2002; Pohl et al., 2003).

*Edwardsiella tarda* is an important aquaculture pathogen that can infect a large number of reared marine species. Recently, we have cloned and analyzed the Fur protein from a pathogenic *E. tarda* strain (Wang et al., 2008). The *E. tarda* Fur (Fur<sub>Et</sub>) is 149 residues in length (one residue longer than Fur<sub>Ec</sub>) and shares 90.6, 75.8, and 51.7% overall sequence identities with Fur<sub>Ec</sub>, the *Vibrio harveyi* Fur (Fur<sub>Vh</sub>), and Fur<sub>Pa</sub>, respectively. In the present study, we investigate the functional importance of the C-terminal region of Fur<sub>Et</sub>, which, like in all other known Fur proteins, contains sequence features that are species-specific. Our results indicate that there exist certain functional and structural compatibilities among Fur<sub>Et</sub>, Fur<sub>Ec</sub>, and Fur<sub>Vh</sub> at the C-terminal region.

## Materials and Methods

**Bacterial strains.** The bacterial strains used in this study are listed in Table 1. *Escherichia coli* was grown in Luria-Bertani lysis broth (LB) or M9 minimal medium (Miller, 1992) at 37°C with ampicillin (Ap; 100 µg/ml) or kanamycin (Kn; 50 µg/ml). *Edwardsiella tarda* was grown in LB medium at 28°C.

**Plasmid construction.** The plasmids used in this study are listed in Table 1 and Table 2. pEtN139, pEtN137, pEtN133, pEtN132, pEtN118, and pEtN75 were constructed by inserting the truncated *fur*<sub>Et</sub> (amplified by PCR with the primer TF39 paired with the primers TR60, TR62, TR63, TR64, TR65, and TR77, respectively) into pBT at the *Sma*I site. pEt75Ec73, pEt75Vh74, and pEt75Pa60 were created by the method of overlap extension PCR (Ho et al., 1989) as follows: the overlapping PCR amplifications were carried out

by using the primer pairs TF14/TR86 and CF6/CR4, TF14/TR87 and VF45/VR18, TF14/TR95 and PF3/PR3, respectively; the fusion PCR amplifications were performed with the primer pairs TF14/CFR4, TF14/VR18, and TF14/PR3, respectively. pEt75Ec73M and pEt75Vh74M were similarly constructed, except that the overlapping PCR amplifications were performed with the primer pairs CF6/CR4 and VF45/VR18, respectively, and the templates used were pC137S and pTF92, respectively. The plasmids pEt75Ec73, pEt75Vh74, and pTF were digested with *Swa*I and the fragments carrying *Et75Ec73*, *Et75Vh74*, and *fur*<sub>Et</sub> were inserted into pJRA at the *Eco*RV site, resulting in pJEt75Ec73, pJEt75Vh74, and pJFUR, respectively. To construct the plasmids expressing *EtMF54* mutants, PCR amplifications were performed using pEtMF54 as the template and TF39/TR78, TF39/54R1, and TF39/54R2 as the primer pairs, respectively; the PCR products were purified and inserted into pBT at the *Sma*I site, resulting in pEtMF54MD, pEtMF54M2, and pEtMF54M5, respectively.

**Construction of *fur* libraries.** The libraries encoding the 1–49 and 1–75 regions of Fur<sub>Et</sub> fused to random peptides were constructed by PCR using pTF as the template and TF76/CMR1 and TF76/CMR2 as the primer pairs, respectively; the PCR products were purified and inserted into pBT at the *Sma*I site. The recombinant plasmids were introduced into H1681 by transformation.

**Bacterial conjugation.** Bacterial conjugation was performed as described previously (Zhang et al., 2008b).

**Preparation of the recombinant Fur<sub>Et</sub>.** The coding sequence of *fur*<sub>Et</sub> was inserted into pET258 at the *Nde*I-*Xho*I sites, resulting in plasmid pETF2, which was introduced into BL21(DE3) (Tiangen, China) by transformation. The His-tagged recombinant Fur<sub>Et</sub> was purified from BL21(DE3)/pETF2 by using nickel-nitrilotriacetic acid beads as described previously (Zhang and Sun, 2007).

**Western immunoblotting.** Western immunoblotting was performed as described previously (Zhang et al., 2008a). Briefly, cells were grown in LB medium to an optical density at 600 nm (OD<sub>600</sub>) of 0.9 and lysed with the lysis buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 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

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

Strain or plasmid or primer	Relevant characteristics	Source or reference
<b>Strains</b>		
<i>E. coli</i> H1681	<i>fur-31, thuF::λplacMu</i>	Heidrich et al., 1996
<i>E. tarda</i> TX1	Fish pathogen	Zhang et al., 2008b
<b>Plasmids</b>		
pBT	Ap <sup>R</sup> , cloning vector	Wang et al., 2008
pC137S	Ap <sup>R</sup> , expressing <i>fur<sub>Vh</sub></i> bearing C137S mutation	Sun et al., 2008
pET258	Kn <sup>R</sup> , expression plasmid	Zhang and Sun, 2007
pETF2	Kn <sup>R</sup> , expressing <i>fur<sub>Et</sub></i>	This study
pJRA	Ap <sup>R</sup> , broad host range vector	Wang et al., 2008
pL1	Ap <sup>R</sup> , cloning vector	Sun et al., 2008
pTF	Ap <sup>R</sup> , expressing <i>fur<sub>Et</sub></i>	Wang et al., 2008
pTF92	Ap <sup>R</sup> , expressing <i>fur<sub>Et</sub></i> bearing C92S mutation	Wang et al., 2008
<b>Primers</b>		
	Sequences (5'→3') <sup>a</sup>	
54R1	TTATCCGCCAAAAGAGCCCTCCTCGCCCAT	
54R2	TTAACAGCCAAAACAGCCCTCCTCGCCCAT	
CF6	TGAGGGCGGTAAATCCGTATTGAAC	
CR4	<u>GATATC</u> AGGCTGGCTTATTGTC ( <i>EcoRV</i> )	
CMR1	TTAN <sub>27</sub> CTCCTCGCCCATGT	
CMR2	TTAN <sub>27</sub> GCCCTCAAAGTTATGGC	
PF3	TGAGGGCGGCCATGCCGTGTTCG	
PR3	CGCCGATATCTACTTCTTTCGCGCACG ( <i>EcoRV</i> )	
TF14	<u>GATATCGGATCC</u> AGGACAGAATCCGAATGA ( <i>EcoRV-BamHI</i> )	
TF39	GAGTGATATCCGTTCCGTAACGGC ( <i>EcoRV</i> )	
TF76	CCGTCGCGCATCCCGGGCACTATTATTTCCGTTCCGTA ( <i>SmaI</i> )	
TR60	TATGATATCTTAGCGGCAGTCGCCCT ( <i>EcoRV</i> )	
TR62	TATGATATCTTAGTCGCCCTCGGCG ( <i>EcoRV</i> )	
TR63	AATGATATCTTAGCAGTGGCCGTAGAG ( <i>EcoRV</i> )	
TR64	GATGATATCTTAGTGCCGTAGAGATAC ( <i>EcoRV</i> )	
TR65	CGCGATATCTTAGTGTTTTTGGCGATTTC ( <i>EcoRV</i> )	
TR77	ATAGGATCCCTTAGCCCTCAAAGTTATGG ( <i>BamHI</i> )	
TR78	TATGGATCTTACTCCTCGCCCATGT	
TR86	GGATTACCGCCCTCAAAGTTATGG	
TR87	GATTGCGGCCCTCAAAGTTATGG	
TR95	CATGGCCGCCCTCAAAGTTATGG	
VF45	TTGAGGGCGGCAATCAGTTTTTGAATT	
VR18	GATGGATCCTTATTTTACTGTTTGTGTGC ( <i>BamHI</i> )	

<sup>a</sup>Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends.

gels were transferred to nitrocellulose membranes and subjected to immunoblotting using rabbit anti-Fur<sub>Et</sub> antibodies, which were prepared as described previously (Zhang et al., 2008a) using the purified recombinant Fur<sub>Et</sub>.

**β-Galactosidase assay.** Cells were cultured in M9 minimal medium (Miller, 1992) at 37°C with and without 50 μM 2,2'-dipyridyl to an OD<sub>600</sub> of ~1. The cells were then used for β-galactosidase assay as described previously (Sun et al., 1998).

**Quantitative real-time PCR (qRT-PCR).** Total RNA

was extracted from cells grown in LB medium to OD<sub>600</sub> of 0.8 by using the SV total RNA isolation system (Promega). Six hundred nanograms of total RNA was reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase and random primers (Promega). qRT-PCR was carried out in an ABI 7300 Real-time Detection System (Applied Biosystems) by using the SYBR ExScript RT-PCR Kit (TaKaRa) as described previously (Zhang et al., 2008a). Each assay was performed in triplicate with the 16S rRNA as a control. All data are given in terms of relative mRNA

Table 2.  $\beta$ -Galactosidase activities of H1681 expressing *fur<sub>Et</sub>* variants.

Strain H1681 harboring	<i>fur</i>	$\beta$ -Galactosidase activity (Miller units)	
		– DP	+ DP
pBT	—	2,002.2 $\pm$ 14.4	2,015.9 $\pm$ 23.4
pTF	Wild-type <i>fur<sub>Et</sub></i>	66.3 $\pm$ 2.1	1,786.9 $\pm$ 41.4
pEtN139	<i>fur<sub>Et</sub></i> bearing deletion of the C-terminal 10 residues	78.9 $\pm$ 3.7	1,820 $\pm$ 27.3
pEtN137	<i>fur<sub>Et</sub></i> bearing deletion of the C-terminal 12 residues	95.8 $\pm$ 4.9	1,590.8 $\pm$ 30.1
pEtN133	<i>fur<sub>Et</sub></i> bearing deletion of the C-terminal 16 residues	214.8 $\pm$ 13.4	2,003.1 $\pm$ 47.7
pEtN132	<i>fur<sub>Et</sub></i> bearing deletion of the C-terminal 17 residues	479.9 $\pm$ 18.6	1,486.8 $\pm$ 13.5
pEtN118	<i>fur<sub>Et</sub></i> bearing deletion of the C-terminal 31 residues	1,278.5 $\pm$ 26.6	1,706.1 $\pm$ 18.6
pEtN75	<i>fur<sub>Et</sub></i> bearing deletion of the C-terminal 74 residues	1,826 $\pm$ 26.4	1,500 $\pm$ 29.4
pEt75Ec73	<i>fur<sub>Et</sub></i> - <i>fur<sub>Ec</sub></i> chimera	75.2 $\pm$ 3.7	2,034.1 $\pm$ 19.7
pEt75Ec73M	Et75Ec73 bearing C92 <sub>Ec</sub> S	1,926.6 $\pm$ 33	1,666.3 $\pm$ 35.4
pEt75Vh74	<i>fur<sub>Et</sub></i> - <i>fur<sub>Vh</sub></i> chimera	70 $\pm$ 4.3	1,629.1 $\pm$ 12.4
pEt75Vh74M	Et75Vh74 bearing C137 <sub>Vh</sub> S	1,752.5 $\pm$ 19.8	2,017 $\pm$ 18.3
pEt75Pa60	<i>fur<sub>Et</sub></i> - <i>fur<sub>Pa</sub></i> chimera	951.9 $\pm$ 15.4	1,699.7 $\pm$ 27.1
pEtMF54	1–49 of <i>fur<sub>Et</sub></i> fused to P5	233.4 $\pm$ 14.7	1,813.6 $\pm$ 8.7

For  $\beta$ -galactosidase assays, cells were cultured to OD<sub>600</sub>  $\sim$  1 in M9 minimal medium supplemented with or without 50  $\mu$ M 2,2'-dipyridyl (DP).

Data are the means of at least three independent experiments and presented as the means  $\pm$  standard errors. ND, not determined.

expressed as means plus or minus standard errors of the means (SE). Statistical analyses were performed by using the two-tailed *t*-test.

## Results

### *The C-terminal 12 residues of Fur<sub>Et</sub> are functionally essential*

Previous study of Fur<sub>Et</sub> has placed important roles on the N-terminal region and the C-terminal region involving C92 and C95; it is not clear as to the potential function of the very C-terminal residues. To investigate the functional importance of the C-terminal region of Fur<sub>Et</sub>, systematic deletion was performed whereby the C-terminal 10, 12, 16, 17, 31, and 74 residues were each deleted. The resulting mutant *fur<sub>Et</sub>*, which consist of the N-terminal 139, 137, 133, 132, 118, and 75 residues of Fur<sub>Et</sub>, respectively, were expressed from the plasmids pEtN139, pEtN137, pEtN133, pEtN132, pEtN118, and pEtN75, respectively. These plasmids, as well as the control plasmid pBT and the plasmid pTF, which expresses the wild-type *fur<sub>Et</sub>*, were introduced into the *fur*-defective *E. coli* strain H1681 (Heidrich et al., 1996) by transformation. H1681 carries a promoterless *lacZ* gene fused to *fhu*, a gene whose expression is repressed by Fur<sub>Ec</sub>; hence, in

H1681 the expression of *lacZ* is repressed by Fur<sub>Ec</sub>. When H1681 is transformed with a plasmid expressing a *fur<sub>Ec</sub>* homologue, such as *fur<sub>Et</sub>*, the ability of the heterologous Fur to repress *lacZ* expression can be determined by  $\beta$ -galactosidase assay. In our case, the results showed that the  $\beta$ -galactosidase activities of H1681/pEtN139 and H1681/pEtN137 were, respectively, 1.2- and 1.4-fold of that of H1681/pTF, whereas the  $\beta$ -galactosidase activities of H1681 transformed with pEtN133, pEtN132, pEtN118, and pEtN75 were 3.2- to 27.5-fold of that of H1681/pTF (Table 2). To examine whether the differences in  $\beta$ -galactosidase activity among these transformants were due to differences in the production/stability of the mutant Fur<sub>Et</sub>, Western immunoblotting was performed to analyze the Fur proteins produced in these strains. The results showed that the mutant and the wild-type Fur<sub>Et</sub> were produced at comparable levels (Fig. 1a and data not shown). Taken together, these results demonstrated that deletion of the C-terminal 10 and 12 residues had no drastic effect on the activity of Fur<sub>Et</sub>. Since the presence of the iron chelator 2,2'-dipyridyl, which inactivates Fur by depleting iron from the medium, drastically increased the  $\beta$ -galactosidase activities of H1681 harboring pEtN139, pEtN137, pEtN133, and pEtN132 (Table 2), the activities or residual activities of the mu-

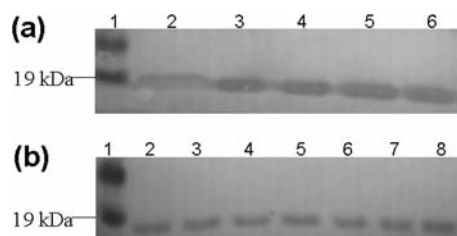


Fig. 1. Western immunoblotting analyses of Fur<sub>Et</sub> production in H1681 expressing fur<sub>Et</sub> variants.

(a) Whole cell proteins were prepared from H1681 harboring pTF, pEtN139, pEtN137, pEtN133, and pEtN132 (lanes 2–6, respectively) and run in a 0.1% SDS–15% polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and blotted with Fur<sub>Et</sub> antibodies. (b) Whole cell proteins were prepared from H1681 harboring pTF (lanes 2 and 3), pEt75Ec73, pEt75Vh74, pEt75Pa60, pEt75Ec73M, and pEt75Vh74M (lanes 4–8, respectively) and used for Western immunoblotting as described above. Lane 1 of both panels, molecular weight markers.

tant Fur<sub>Et</sub> expressed by these plasmids were iron-dependent.

*The C-terminal 76–149 region of Fur<sub>Et</sub> can be functionally replaced by the corresponding regions of Fur<sub>Ec</sub> and Fur<sub>Vh</sub>*

Since, as demonstrated above, the truncated Fur<sub>Et</sub> consisting of the N-terminal 75 residues (i.e., Fur<sub>Et</sub> bearing deletion of the C-terminal 74 residues) exhibits no repressor activity, we wondered whether its functional defectiveness could be remedied by fusion with the C-terminal region of Fur<sub>Ec</sub>. To investigate this question, region swapping was performed, by which the N-terminal 1–75 region of Fur<sub>Et</sub> was fused in-frame to the C-terminal 76–148 region of Fur<sub>Ec</sub>, which corresponds to the 76–149 region of Fur<sub>Et</sub> (Fig. 2). The resulting Fur chimera was designated Et75Ec73. The plasmid pEt75Ec73, which expresses Et75Ec73, was introduced into H1681 via transformation. The transformants were plated on MacConkey agar plates. MacConkey agar is a culture medium designed for the identification of lactose-fermenting bacteria, which form red colonies on the plate. In our case, if the chimeric Fur produced by the transformant was inactive, then the *lacZ* gene in H1681 would be expressed, resulting in the production of β-galactosidase, which would cause fermentation of the lactose in the MacConkey medium, and, as a result, the colonies would appear red on the plate. If, on the other hand, the chimeric Fur produced by the transformant was function-

	1	10	20	30	40	50
<i>E. tarda</i>	MTDNN	TALKK	AGLKV	TLPR	LKILEV	LQDPTCHHVSAEDLYKKLIDMGEEI
<i>E. coli</i>	MTDNN	TALKK	AGLKV	TLPR	LKILEV	LQEPDNNHHVSAEDLYKKLIDMGEEI
<i>V. harveyi</i>	MSDNN	QALKD	AGLKV	TLPR	LKILEV	LQPDCHHISAEDLYKKLIDMGEEI
<i>P. aeruginosa</i>	MVEN-	SELRK	AGLKV	TLPR	VKILQ	MLDSAEQRHMSAEDVYKALMEAGEDV
	*	*	*	*	*	*
	51	60	70	80	90	100
<i>E. tarda</i>	GLATVYRVLN	QFDDAGIV	TRHNFEGGKSVFELT	QQHHHDLICLD	CGKVI	
<i>E. coli</i>	GLATVYRVLN	QFDDAGIV	TRHNFEGGKSVFELT	QQHHHDLICLD	CGKVI	
<i>V. harveyi</i>	GLATVYRVLN	QFDDAGIV	TRHNFEGGKSVFELT	QQHHHDLICLD	CGEVI	
<i>P. aeruginosa</i>	GLATVYRVLN	QFEAAGLV	VRHNFEGGKSVFELT	QGHHDHLMVC	VDTEVI	
	*	*	*	*	*	*
	101	110	120	130	140	149
<i>E. tarda</i>	EFSDSIEKRQREIA	AKKHGIQLTNHSLY	LYGHCAEGDCRSDEALHDEKA			
<i>E. coli</i>	EFSDSIEKRQREIA	AKKHGIQLTNHSLY	LYGHCAEGDCREDEHAHEGK-			
<i>V. harveyi</i>	EFSDSIEKRQREIA	AKKHGIQLTNHSLY	LYGHCAEGDCRSCKNDPAHKPVK			
<i>P. aeruginosa</i>	EFMDAIEKRQREIA	VRERGFELVDHNLVLY	-----VRKKK-----			
	*	*	*	*	*	*

Fig. 2. Alignments of the Fur sequences of *E. tarda*, *E. coli*, *V. harveyi*, and *P. aeruginosa*.

Asterisks indicate residues that are identical among all four Fur proteins.

al, then it would repress *lacZ* expression, and the colonies would appear white on the plate. The result showed that the colonies of H1681/pEt75Ec73 were white on MacConkey medium, suggesting that Et75Ec73 possesses repressor activity. β-Galactosidase assays showed that the β-galactosidase activities of H1681/pEt75Ec73 in the absence and presence of 2,2'-dipyridyl were comparable to those of H1681/pTF cultured under the same conditions (Table 2), suggesting that, like the wild-type Fur<sub>Et</sub>, the chimeric Et75Ec73 requires iron as an activating factor. These results demonstrated that the C-terminal 76–149 region of Fur<sub>Et</sub> can be functionally replaced by the corresponding region of Fur<sub>Ec</sub>, which is 86% identical to the former.

To examine whether the compatibility at the C-terminal region was restricted to Fur<sub>Et</sub> and Fur<sub>Ec</sub>, a similar region swapping was performed upon Fur<sub>Et</sub> and the *Vibrio harveyi* Fur, Fur<sub>Vh</sub>, which, compared to Fur<sub>Ec</sub>, shares 15% less (75% vs 90%) overall identity with Fur<sub>Et</sub> (Fig. 2). The resulting chimeric Fur, Et75Vh74, is composed of the N-terminal 1–75 region of Fur<sub>Et</sub> fused in-frame to the C-terminal 76–149 region of Fur<sub>Vh</sub>, which is 63% identical to the corresponding region of Fur<sub>Et</sub>. β-Galactosidase assays showed that the β-galactosidase activities of H1681 harboring pEt75Vh74, which expresses Et75Vh74, were comparable to those of H1681/pTF (Table 2) in the absence and presence of 2,2'-dipyridyl (Table 2). Hence, Et75Vh74 functions like the wild-type Fur<sub>Et</sub>.

To further examine the flexibility of the C-terminal 76–149 region of Fur<sub>Et</sub>, a region swapping similar to that described above was performed between Fur<sub>Et</sub>



and Fur<sub>Pa</sub>. Fur<sub>Pa</sub>, which has been shown to be able to complement Fur<sub>Ec</sub> deficiencies (Lewin et al., 2002), contains 134 amino acid residues and shares 51.7% overall sequence identity with Fur<sub>Et</sub> (Fig. 2). In the resulting chimeric Fur (named Et75Pa60), the N-terminal 1–75 region of Fur<sub>Et</sub> is fused in-frame to the C-terminal 75–134 region of Fur<sub>Pa</sub> which is 41.9% identical to the corresponding region of Fur<sub>Et</sub>.  $\beta$ -Galactosidase assays showed that the  $\beta$ -galactosidase activity of H1681 harboring pEt75Pa60, which expresses *Et75Pa60*, was 14.4-fold higher than that of H1681/pTF (Table 2), suggesting that Et75Pa60 exhibited no repressor activity. Since Western immunoblotting analyses indicated that the amount of Et75Pa60 produced in H1681/pEt75Pa60 was comparable to that of the wild-type Fur<sub>Et</sub> produced in H1681/pTF (Fig. 1b), these results demonstrated that the C-terminal 76–149 region of Fur<sub>Et</sub> can not be functionally replaced by the corresponding region of Fur<sub>Pa</sub>.

*C92 of Fur<sub>Ec</sub> and C137 of Fur<sub>Vh</sub> are essential to the functioning of Et75Ec73 and Et75Vh74, respectively*

It is known that C92 of Fur<sub>Ec</sub> (named C92<sub>Ec</sub>) and C137 of Fur<sub>Vh</sub> (named C137<sub>Vh</sub>) are functionally essential (Coy et al., 1994; Sun et al., 2008). Since these two cysteine residues are contained within Et75Ec73 and Et75Vh74, respectively, we examined their potential significance in the functioning of the chimeric Fur. For this purpose, the plasmids pEt75Ec73M, which expresses *Et75Ec73* bearing a C92<sub>Ec</sub>S substitution, and pEt75Vh74M, which expresses *Et75Vh74* bearing a C137<sub>Vh</sub>S substitution, were introduced into H1681 by transformation. Western immunoblotting assays showed that the Fur proteins produced in the transformants were comparable to those produced in H1681/pEt75Ec73 and H1681/pEt75Vh74 (Fig. 1b).  $\beta$ -Galactosidase assays showed that the  $\beta$ -galactosidase activities of H1681/pEt75Ec73M and H1681/pEt75Vh74M were 25.6- and 25-fold more than those of H1681/pEt75Ec73 and H1681/pEt75Vh74, respectively (Table 2), suggesting that the mutant chimeric Furs were inactive. Hence, C92<sub>Ec</sub> and C137<sub>Vh</sub> are functionally essential in Et75Ec73 and Et75Vh74, respectively.

*Selection of a mini-Fur<sub>Et</sub> that possesses iron-regulated repressor activity*

With the above results, we wondered whether the C-terminal region of Fur<sub>Et</sub> could be functionally re-

placed by, in addition to derivatives of natural Fur proteins such as those described above, artificial peptides. To investigate this question, two libraries of random chimeric Fur<sub>Et</sub> were created, in which the N-terminal 1–49 and 1–75 regions of Fur<sub>Et</sub> were each fused to a library of random peptides. H1681 expressing the libraries was selected on MacConkey plates for white (i.e., active Fur-producing) colonies. One white colony with a red core was obtained from the library containing the 1–49 region of Fur<sub>Et</sub>. This colony harbors a recombinant plasmid (named pEtMF54) that expresses a chimeric *fur<sub>Et</sub>* (named *EtMF54*) encoding the 1–49 region of Fur<sub>Et</sub> fused to a five-residue peptide with the sequence of GCFGG (named P5).  $\beta$ -Galactosidase assays showed that the  $\beta$ -galactosidase activity of H1681/pEtMF54 was 3.4-fold higher than that of H1681/pTF. In the presence of 2,2'-dipyridyl, H1681/pEtMF54 was completely red on MacConkey medium and exhibited drastically increased  $\beta$ -galactosidase activity (Table 2). These results indicated that, compared to the wild-type Fur<sub>Et</sub>, EtMF54 possesses partial repressor activity which is iron-dependent.

To examine the significance of P5 in the functioning of EtMF54, H1681 was transformed with the plasmids pEtMF54MD, pEtMF54M2, and pEtMF54M5, which express *EtMF54* bearing P5 deletion, C2S substitution at P5, and G5S substitution at P5, respectively. The results showed that all the transformants formed red colonies on MacConkey plates, suggesting that EtMF54 lacking P5 is devoid of repressor activity and that C2 and G5 of P5 are functionally essential.

*Et75Ec73 and Et75Vh74 are functional in E. tarda*

Hirono et al. (1997) had observed that expression of the *E. tarda* hemolysin gene *ethB* was regulated by iron. Consistently, we found in a previous study that Fur<sub>Et</sub> repressed moderately the expression of *ethB* when the cells were cultured in LB medium (Wang et al., 2009). To examine whether Et75Ec73 and Et75Vh74 could function as effective regulators in the *E. tarda* genetic background, the effects of Et75Ec73 and Et75Vh74 on *ethB* expression were determined. For this purpose, the conjugative plasmids pJEt75Ec73, pJEt75Vh74, and pJFUR, which express *Et75Ec73*, *Et75Vh74*, and the wild-type *fur<sub>Et</sub>*, respectively, were conjugated into the pathogenic *E. tarda* strain TX1. Quantitative real time PCR analyses showed that *ethB* expressions in TX1/pJEt75Ec73, TX1/pJEt75Vh74, and TX1/pJFUR were comparable and were approximately 2-fold less

than that in TX1 harboring the control plasmid pJRA. These results suggested that in TX1, Et75Ec73 and Et75Vh74 function like Fur<sub>Et</sub>.

## Discussion

It has long been noted that the Fur family proteins are conserved at the N-terminal DNA-binding region, which accounts for the ability of most Fur proteins to recognize the same Fur box sequence. In contrast, the C-terminal region, represented by the sequence formed by residues corresponding to 83–148 of Fur<sub>Ec</sub>, differs widely among Fur proteins. Our previous study has shown the C-terminal 12 residues of Fur<sub>Vh</sub> are functionally inessential (Sun et al., 2008); similarly, in this study, we found that Fur<sub>Et</sub> bearing deletion of the C-terminal 10 and 12 residues still retain most of the repressor activity. These results suggest that the very C-terminal region of Fur<sub>Et</sub>, represented by the last 12 residues, is not very likely to play any key role in the activation process, which is in agreement with the observation that this region is entirely missing in some Fur proteins and, for those Furs that do possess it, there is generally no sequence conservation in this region.

Since the C-terminal region, as defined above, is presumably involved in the dimerization and metal ion-binding that are required for activation, the sequence, as well as length, variation in this region provides a genetic basis for variations in the mechanism of activation. In our study, we found that the C-terminal 73 residues of Fur<sub>Ec</sub> and the C-terminal 74 residues of Fur<sub>Vh</sub>, which differ in primary structure from the corresponding region of Fur<sub>Et</sub>, can successfully replace the corresponding region of Fur<sub>Et</sub>. These results suggest that either the C-terminal regions of Fur<sub>Ec</sub> and Fur<sub>Vh</sub> can form structural motifs that are functionally analogous to that formed by the counterpart region of Fur<sub>Et</sub>, or, alternatively, the substituting regions of Fur<sub>Ec</sub> and Fur<sub>Vh</sub>, when fused to the N-terminal region of Fur<sub>Et</sub>, can reconstitute into chimeric but functional domains with the residues of Fur<sub>Et</sub>. In either case, since C92<sub>Ec</sub> and C137<sub>Vh</sub> are as important in the chimeric Furs as they are in the native Fur<sub>Ec</sub> and Fur<sub>Vh</sub> respectively. The substituting regions of Fur<sub>Ec</sub> and Fur<sub>Vh</sub> in the chimeric Fur proteins are likely to adopt the same higher-order conformations as those adopted by these regions in the native Fur<sub>Ec</sub> and Fur<sub>Vh</sub>. The failure of the C-terminal 75–134 region of Fur<sub>Pa</sub> to replace the corresponding

region of Fur<sub>Et</sub> could be due to dissimilar domain structures formed by Fur<sub>Et</sub> and Fur<sub>Pa</sub> in this region, which is consistent with the low sequence identity between Fur<sub>Et</sub> and Fur<sub>Pa</sub> at this region.

Recently (Pecqueur et al., 2006), structural analyses of Fur<sub>Ec</sub> have indicated that the monomeric form of Fur<sub>Ec</sub> differs from the dimeric form in several aspects, notably in the N-terminal  $\alpha$ -helix and the C-terminal structure, and that the truncated Fur<sub>Ec</sub>, consisting of the N-terminal 1–82 residues, exhibits an overall structural topology resembling that of the activated Fur<sub>Ec</sub>, which suggests the possibility that it is the presence of the C-terminal region that, in the absence of the cognate metal ion, prevents the organization of the N-terminal region into an active higher-order structure. In our study, we found that the artificial Fur<sub>Et</sub>, EtMF54, which lacks the middle and C-terminal region of Fur<sub>Et</sub>, possesses partial repressor activity. Our results potentially favor the notion that, in the absence of activating metal ions, the C-terminal region of Fur<sub>Et</sub> may exert a negative modulating effect on the N-terminal region so that the latter is maintained in a structurally inactive state. The reduced activity of EtMF54 is likely due to the disruption of the winged helix-turn-helix motif. The fact that EtMF54 requires Fe<sup>2+</sup> as a cofactor for repressor activity suggests that the N-terminal 1–49 region of Fur<sub>Et</sub>, or at least the N-terminal 1–49 region of Fur<sub>Et</sub> in EtMF54, can interact with Fe<sup>2+</sup>. However, since certain residues of P5 in EtMF54 appear to be functionally essential, it is also possible that, in EtMF54, Fe<sup>2+</sup> is coordinated by residues of both Fur<sub>Et</sub> and the artificial peptide.

## Acknowledgments

This work was supported by the 973 Project of China Grant 2006CB101807 and the 863 High Technology Project Grant 2008AA092501.

## References

- Althaus, E. W., Outten, C. E., Olson, K. E., Cao, H., and O'Halloran, T. V. (1999) The ferric uptake regulation (Fur) repressor is a zinc metalloprotein. *Biochemistry*, **38**, 6559–6569.
- Bagg, A. and Neilands, J. B. (1987) Ferric uptake regulation protein acts as a repressor, employing iron (II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. *Biochemistry*, **26**, 5471–5477.
- Coy, M., Doyle, C., Besser, J., and Neilands, J. B. (1994) Site-

- directed mutagenesis of the ferric uptake regulation gene of *Escherichia coli*. *BioMetals*, **7**, 292–298.
- Coy, M. and Neilands, J. B. (1991) Structural dynamics and functional domains of the Fur protein. *Biochemistry*, **30**, 8201–8210.
- Gonzalez de Peredo, A., Saint-Pierre, C., Adrait, A., Jacquamet, L., Latour, J. M., Michaud-Soret, I., and Forest, E. (1999) Identification of the two zinc-bound cysteines in the ferric uptake regulation protein from *Escherichia coli*: Chemical modification and mass spectrometry analysis. *Biochemistry*, **38**, 582–589.
- Hamed, M. Y. and Al-Jabour, S. (2006) Iron (II) triggered conformational changes in *Escherichia coli* fur upon DNA binding: a study using molecular modeling. *J. Mol. Graph. Model.*, **25**, 234–246.
- Hantke, K. (2001) Iron and metal regulation in bacteria. *Curr. Opin. Microbiol.*, **4**, 172–177.
- Heidrich, C., Hantke, K., Bierbaum, G., and Sahl, H. G. (1996) Identification and analysis of a gene encoding a Fur-like protein of *Staphylococcus epidermis*. *FEMS Microbiol. Lett.*, **140**, 253–259.
- Hirono, I., Tange, N., and Aoki, T. (1997) Iron-regulated haemolysin gene from *Edwardsiella tarda*. *Mol. Microbiol.*, **24**, 851–856.
- Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene*, **77**, 51–59.
- Jacquamet, L., Aberdam, D., Adrait, A., Hazemann, J. L., Latour, J. M., and Michaud-Soret, I. (1998) X-ray absorption spectroscopy of a new zinc site in the fur protein from *Escherichia coli*. *Biochemistry*, **37**, 2564–2571.
- Lewin, A. C., Doughty, P. A., Flegg, L., Moore, G. R., and Spiro, S. (2002) The ferric uptake regulator of *Pseudomonas aeruginosa* has no essential cysteine residues and does not contain a structural zinc ion. *Microbiology*, **148**, 2449–2456.
- Miller, J. H. (1992) A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Ochsner, U. A., Vasil, A. I., and Vasil, M. L. (1995) Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: Purification and activity on iron-regulated promoters. *J. Bacteriol.*, **177**, 7194–7201.
- Pecqueur, L., D'Autreaux, B., Dupuy, J., Nicolet, Y., Jacquamet, L., Brutscher, B., Michaud-Soret, M. I., and Bersch, B. (2006) Structural changes of *Escherichia coli* ferric uptake regulator during metal-dependent dimerization and activation explored by NMR and X-ray crystallography. *J. Biol. Chem.*, **281**, 21286–21295.
- Pohl, E., Haller, J. C., Mijovilovich, A., Meyer-Klaucke, W., German, E., and Vesil, M. L. (2003) Architecture of a protein central to iron homeostasis: Crystal structure and spectroscopic analysis of the ferric uptake regulator. *Mol. Microbiol.*, **47**, 903–915.
- Ratledge, C. and Dover, L. G. (2000) Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.*, **54**, 881–941.
- Saito, T., Womlad, M. R., and Williams, R. J. P. (1991) Some structural features of the iron-uptake regulation protein. *Eur. J. Biochem.*, **197**, 29–39.
- Stojiljkovic, I. and Hantke, K. (1995) Functional domains of the *Escherichia coli* ferric uptake regulator protein (Fur). *Mol. Gen. Genet.*, **247**, 199–205.
- Sun, K., Cheng, S., Zhang, M., Wang, F., and Sun, L. (2008) Cys-92, Cys-95, and the C-terminal 12 residues of the *Vibrio harveyi* ferric uptake regulator (Fur) are functionally inessential. *J. Microbiol.*, **46**, 670–680.
- Sun, L., vanderSpek, J., and Murphy, J. (1998) Isolation and characterization of iron-independent positive dominant mutants of Diphtheria toxin repressor, DtxR. *Proc. Natl. Acad. Sci. USA*, **95**, 14985–14990.
- Wang, F., Cheng, S., Sun, K., and Sun, L. (2008) Molecular analysis of the fur (ferric uptake regulator) gene of a pathogenic *Edwardsiella tarda* strain. *J. Microbiol.*, **46**, 350–355.
- Wang, F., Zhang, M., Sun, K., Zhang, W., and Sun, L. (2009) Regulation of the *Edwardsiella tarda* haemolysin gene and luxS by EthR. *J. Microbiol. Biotechnol.* (in press). doi: 10.4014/jmb.0810.574.
- Zhang, W. and Sun, L. (2007) Cloning, characterization and molecular application of a beta-agarase gene from *Vibrio* sp. strain V134. *Appl. Environ. Microbiol.*, **73**, 2825–2831.
- Zhang, W., Sun, K., Cheng, S., and Sun, L. (2008a) Characterization of DegQ<sub>VH</sub>, a serine protease and a protective immunogen from a pathogenic *Vibrio harveyi* strain. *Appl. Environ. Microbiol.*, **74**, 6254–6262.
- Zhang, M., Sun, K., and Sun, L. (2008b) Regulation of autoinducer 2 production and luxS expression in a pathogenic *Edwardsiella tarda* strain. *Microbiology*, **154**, 2060–2069.
- Zheleznova, E. E., Crosa, J. H., and Brennan, R. G. (2000) Characterization of the DNA- and metal-binding properties of *Vibrio anguillarum* fur reveals conservation of a structural Zn<sup>2+</sup> ion. *J. Bacteriol.*, **182**, 6264–6267.