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NOTE

Molecular Analysis of the *fur* (ferric uptake regulator) Gene of a Pathogenic *Edwardsiella tarda* Strain

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The gene encoding the *Edwardsiella tarda* ferric uptake regulator (Fur_{Et}) was cloned from a pathogenic *E. tarda* strain isolated from diseased fish. Fur_{Et} shares 90% overall sequence identity with the *Escherichia coli* Fur (Fur_{Ec}) and was able to complement the mutant phenotype of a *fur*$_{Ec}$-defective *E. coli* strain. Mutational analysis indicated that C92S and C95S mutations inactivated Fur_{Et} whereas E112K mutation resulted in a superactive Fur_{Et} variant. Fur_{Et} negatively regulated its own expression; interruption of this regulation impaired bacterial growth, altered the production of certain outer membrane proteins, and attenuated bacterial virulence.

Keywords: Edwardsiella tarda, Fur, autoregulation, virulence, mutation

The ferric uptake regulator (Fur) is a metal ion-responsive transcription regulator that controls the expression of genes involved in diverse cellular functions. Fur is synthesized as an inactive apoprotein and becomes activated when bound to iron. In the presence of iron, Fur operates by interacting with its target promoter at a site termed Fur box that conforms, at various degrees, to the consensus sequence of GATAATGATAATCATTATC (Escolar *et al.*, 1999). In addition to Fe^{2+}, factors that regulate the expression of *fur* include the cAMP receptor protein, oxidative stress response regulators, and Fur itself (De Lorenzo *et al.*, 1998; Zheng *et al.*, 1999; Van Vliet *et al.*, 2000; Lowe *et al.*, 2001; Varghese *et al.*, 2007).

Edwardsiella tarda is a Gram-negative bacterium that can be pathogenic to a broad range of host, including humans. It is currently one of the most important fish pathogens that affect the aquaculture industries worldwide. To date a few virulence systems and factors have been discovered in *E. tarda* (Hirono *et al.*, 1997; Tan *et al.*, 2005; Zheng and Leung, 2007), yet the fundamental pathogenic mechanisms of this species remain largely unknown. As a first step to the understanding of the potential role of Fur in the pathogenicity of *E. tarda*, we cloned and analyzed the *E. tarda* fur (*fur*$_{Et}$) gene.

Fur_{Et} can function as an *E. coli* Fur (Fur_{Ec}) substitute

fur$_{Et}$ (GenBank accession no. EF197912) was cloned from TX1, an *E. tarda* strain isolated from diseased fish, by degenerate PCR and genome walking as described previously (Zhang and Sun, 2007). Fur_{Et} shares 90% overall sequence identity with Fur_{Ec}. To determine whether Fur_{Et} could act as a functional Fur_{Ec} surrogate, the plasmid pTF was constructed by inserting *fur*$_{Et}$ (generated by PCR with the primers EF21/ER29) at the *Sma*I site of pBT, which was created by inserting the P_{trc} promoter and the *rrnB* transcription terminator of pTrcHis (Invitrogen) into pBR322 at between the *Eco*RV-*Bsa*BI sites. Thus in pTF *fur*$_{Et}$ was constitutively expressed under P_{trc}. pTF and the control plasmid pBT were each introduced, by transformation, into the *E. coli* strain H1681, a *fur* mutant that carries a *fhuF-lacZ* fusion and hence can serve as a reporter of heterologous Fur activity (Heidrich *et al.*, 1996). The transformants, H1681/pTF and H1681/pBT, were plated on MacConkey agar (Heidrich *et al.*, 1996) plates supplemented with or without the iron chelator 2,2'-dipyridyl (200 μM). After 24 h growth, H1681/pTF appeared as white colonies in the absence and red colonies in the presence of 2,2'-dipyridyl, whereas H1681/pBT were red under all conditions. This result demonstrated that Fur_{Et} could recognize the target promoter of Fur_{Ec} and inhibit transcription therefrom.

Mapping of the *fur*$_{Et}$ promoter

To locate the *fur*$_{Et}$ promoter, a *fur*-defective *E. coli* strain, NCK, and a promoter probe plasmid, pSC13, were constructed as follows: the *cat* gene of pACYC184 was inserted into pGP704 (Table 1) at between the *Pst*I-*Sal*I sites, resulting in p704C; an internal fragment of *fur*$_{Ec}$ (generated by PCR with primers ECF1/ECR1) was inserted into p704C at the *Eco*RV site, yielding pCE, which was introduced into the strain S17-1λpir (Table 1) by transformation; the transformant was conjugated with N7K, a kanamycin-resistant variant of NK7047 (Table 1); the transconjugant was named NCK.

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

Strain, plasmid or primer	Relevant characteristics	Source or reference
E. coli strains		
BL21(DE3)	Host strain for expressing fur_{Et}	Novagen (USA)
DH5α	Host strain for general cloning	TaKaRa (China)
H1681	*fur-31, fhuF::λplacMu*	Heidreich *et al.* (1996)
NK7047	F⁻λ⁻△*lacX74 rpsL galOP308*	Sun *et al.* (1998)
NCK	NK7047 derivative; *fur*-defective	This study
S17-1λpir	RP4:2-Tc:Mu:Km Tn7λpir	Biomedal (Spain)
E. tarda strains		
TX1	Fish pathogen	This study
Plasmids		
pBT	Expression plasmid carrying P_{trc}	This study
pDN18	Broad host range vector	Marx and Lidstrom (2001)
pET258	Expression plasmid	Zhang and Sun (2007)
pJRA	pDN18 derivative	This study
pGP704	Suicide plasmid	Miller and Mekalanos (1988)
pJRTF	pJRA expressing fur_{Et}	This study
pSC6	Cloning vector	Sun *et al.* (1998)
pSC13	pSC6 derivative	This study
pSC270	pSC13 carrying P_{fur}	This study
pSC270M1	pSC13 carrying P_{fur} with mutated -10	This study
pSC270M2	pSC13 carrying P_{fur} with mutated -10	This study
pSC270M3	pSC13 carrying P_{fur} with mutated Fur box	This study
pTF	pBT expressing fur_{Et}	This study
Primers	Sequences (5'→3')[a]	
ECF1	CTGGCCTGAAAGTAACGCT	
ECR1	GCGAATGCCATGTTTTGCG	
EF1	TAAAGAAGGCTGGCCTGAAAG	
EF21	CTATTATTTTCCGTTCGGTAAC	
EF24	GGGATATCGGCCTTATAATGAATC	
ER2	ACTTTTCCGCAGTCAAGGCA	
ER23	GACTTTCAGGCCAGCTTTC	
ER29	<u>GATATC</u>TTAGGCCTTTTCGTCG (*Eco*RV)	
T48F47	TAAATGGCCCCGCGG	
T48R40	ATTATACCTCAAGAGTCCGAG	

[a] Underlined nucleotides are the restriction site of *Eco*RV.

pSC13 was created by replacing the *tox-tetR* of the low copy-number plasmid pSC6 (Table 1) with a promoterless *lacZ* gene, resulting in pSC11, which was then digested with *Sma*I and ligated to the Tc^R gene of pBR322. The 270 bp DNA fragment (named D270) upstream of the translation start of fur_{Et} was inserted into pSC13 at the *Swa*I site, resulting in pSC270, in which D270 was fused to the promoterless *lacZ* gene. pSC270 was introduced into NCK by transformation. The transformant, NCK/pSC270, manifested blue on the X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) plate, suggesting that D270 possessed an active promoter. Inspection of D270 revealed a putative σ^{70}-dependent promoter (named P_{fur}) with TTCCCA and TATAAT as the -35 and -10 box, respectively (Fig. 1). To examine the potential essentialness of P_{fur} to the promoter activity of D270, the -10 sequence of P_{fur} was "down"-mutated to ATTAAT and AAGAAT by site-directed mutagenesis using the method of overlap extension PCR (Ho *et al*., 1989). The mutated D270 were inserted into pSC13 at the *Swa*I site, resulting in pSC270M1 and pSC270M2, respectively, which were introduced into NCK by transformation and the transformants were subjected to β-galactosidase assay, which

270 atcgcctgccggcgcggatccccgtttaatagccggcaggtatttcagccatcaataaaaacgatagtgcgcagttc
205 gctgcgtcatttgtttctatcttcctcattattcgcgagttatatcagatctgcgccgcagacaccccgcgctgaccacg

EF24 → EF21 →
126 ccgcgccacggTTCCCAtttagggatatcggcct*TATAATgaatctcactatt*attttccgttcggtaacgg
-35 -10
56 ccccagggcgctgccgggcgctgtgacaggacagaatccga**ATG**

Fig. 1. Nucleotide sequence of D270. The numbers refer to the positions upstream from the translation start of *fur*$_{Et}$, which is shown in capital letter at the end of the sequence. The -35 and -10 box of P$_{fur}$ are capitalized and indicated. The Fur box is italicized. The arrows cover the sequences and indicate the directions of the primers EF21 and EF24.

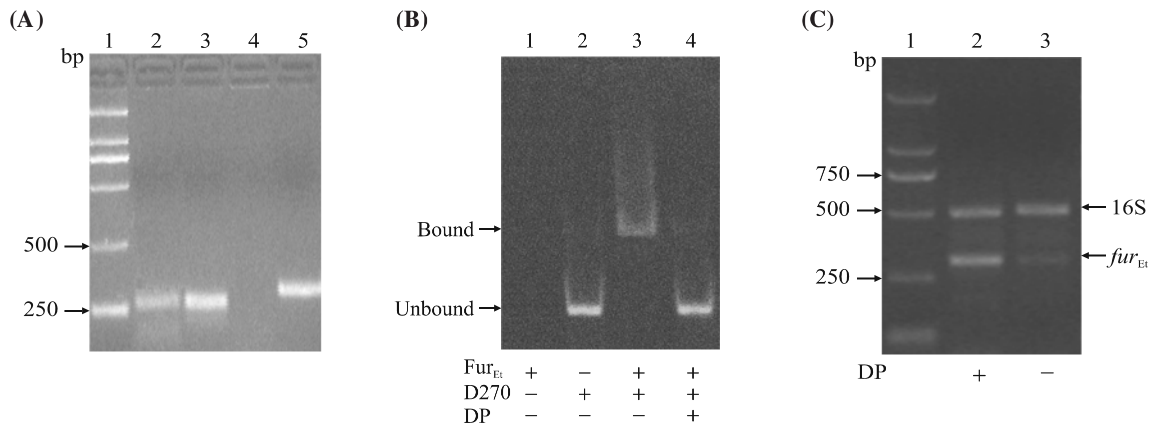

Fig. 2. (A) Determination of the transcriptional start of *fur*$_{Et}$. The cDNA corresponding to the 5' portion of *fur*$_{Et}$ mRNA was used in PCR with the primer pair EF21/ER23 (lane 2) and EF24/ER23 (lane 4). As positive controls, parallel PCR reactions were carried out using TX1 genomic DNA as the template and EF21/ER23 (lane 3) and EF24/ER23 (lane 5) as primers. Lane 1, molecular weight markers. (B) Specific binding of Fur$_{Et}$ to its own promoter region. EMSA was performed in the binding buffer containing the carboxyfluorescein-labeled D270 and the purified recombinant Fur$_{Et}$ in the presence or absence of 2,2'-dipyridyl (DP). (C) Expression of *fur*$_{Et}$ in relation to iron conditions. RT-PCR was performed with total RNA extracted from TX1 cultured in LB medium supplemented with or without 100 μM 2,2'-dipyridyl (DP). 16S rRNA was used as an internal control. Lane 1, molecular weight markers.

was performed as described previously (Sun *et al*., 1998). The result showed that the β-galactosidase activities of NCK/pSC270M1 and NCK/pSC270M2 (8.5 and 5.1 Miller units) were 80~88% lower than that of NCK/pSC270 (42 Miller units), suggesting that P$_{fur}$ was essential to the promoter activity of D270. To further examine whether P$_{fur}$ was the promoter of *fur*$_{Et}$ in TX1, the cDNA corresponding to the 5' portion of the *fur*$_{Et}$ mRNA was extended by using the reverse primer ER23 (corresponding to the 28~47 region of the *fur*$_{Et}$ coding sequence) and served as the template for PCR amplification using ER23 paired with two different forward primers: EF24 and EF21, locating at, respectively, 4 bp downstream of the -35 element of P$_{fur}$ and 8 bp downstream of the -10 element of P$_{fur}$ (Fig. 1). The result showed that only the PCR with the primer pair EF21/ER23 yielded positive products (Fig. 2A), suggesting that *fur*$_{Et}$ transcription started at between EF21 and EF24. Since P$_{fur}$ is the only apparent promoter that can give rise to such a transcription, it is very likely the actual *fur*$_{Et}$ promoter in TX1.

Fur$_{Et}$ negatively regulates transcription from P$_{fur}$

It is known that some Fur proteins regulate their own expression in a feedback manner (Delany *et al*., 2002, 2003). To investigate whether Fur$_{Et}$ had a similar functional aspect, NCK/pSC270 was transformed separately with pTF and the control plasmid pBT. Subsequent β-galactosidase assay indicated that the β-galactosidase activity of NCK/pSC270/pTF (14.6 Miller units) was 65% lower than that of NCK/pSC270/pBT (42 Miller units), suggesting that Fur$_{Et}$ repressed transcription from P$_{fur}$. To examine whether Fur$_{Et}$ could bind directly to D270 *in vitro*, electrophoresis mobility shift assay (EMSA) was performed by incubating Fur$_{Et}$ with carboxyfluorescein-labeled D270 at 25°C for 30 min in the binding buffer (Bes *et al*., 2001) with or without 8 mM 2,2'-dipyridyl. As a negative control, a 287 bp DNA fragment (D287) amplified by PCR from TX1 with the primer pair T48F47/T48R40 was included in the assay. After the incubation, the samples were run on a nondenaturing 8% polyacrylamide gel, which showed that Fur$_{Et}$ could bind specifically to D270 and this binding was sensitive to 2,2'-dipyridyl (Fig. 2B); in contrast, Fur$_{Et}$ did not bind to the negative control DNA D287 (data not shown). To further examine whether Fur$_{Et}$ regulated its own expression in TX1, *fur*$_{Et}$ expression was analyzed by reverse transcriptase (RT)-PCR, which was carried out as described by Martin *et al.* (2004) using total RNA extracted from TX1 grown at different conditions. The result showed that depletion of iron by 2,2'-dipyridyl caused ~5 fold increase in *fur*$_{Et}$ expression (Fig. 2C), which supported the idea that the expression of *fur*$_{Et}$ was subjected to negative autoregulation. In line with

Table 2. β-Galactosidase activities of H1681 transformed with pBT expressing fur_{Et} variants

Strain H1681 harboring	Fur_{Et}	Relative β-galactosidase activity (%)	
		in the absence of DP	in the presence of DP
pBT	-	100	100
pTF	Wild type	8.6	92
pTF92	C92S	94.6	89.5
pTF95	C95S	68.7	103
pTF132	C132S	10	90.8
pTF137	C137S	11.8	87.2
pTF97	K97E	11.7	114
pTF103	D103K	9.1	103.6
pTF104	E104K	8.3	111.7
pTF107	E107R	10.7	96
pTF109	R109E	9.6	90.1
pTF112	E112K	2.2	33.2
pTF136	D136K	7.3	86.4

β-Galactosidase assay was performed with cells grown to an OD_{600} of ~1.2 in LB medium in the presence or absence of 200 μM 2,2'-dipyridyl. The values are shown in percentages of the β-galactosidase activity of H1681/pBT, which is defined as 100. The data are the means of at least three independent experiments. DP, 2,2'-dipyridyl.

these observations, a putative Fur box consisting of 19 palindrome-forming nucleotides (5'-TATAATGAATCTCACTA TT-3') was identified in D270 at a region that overlaps P_{fur} (Fig. 1). To determine the potential importance of this putative Fur box in the functioning of Fur_{Et}, the palindrome of the Fur box was disrupted by mutating the CA to GT; the mutated D270 was cloned into pSC13 and the resulting plasmid, pSC270M3, was introduced into NCK and NCK/pTF by transformation. Subsequent β-galactosidase assay showed that the β-galactosidase activities of NCK/pSC270M3 and NCK/pSC270 were of similar levels, but the β-galactosidase activity of NCK/pTF/pSC270M3 (33.6 Miller units) was 2.3-fold higher than that of NCK/pTF/pSC270 (14.4 Miller units), suggesting that mutation of the Fur box had no effect on the activity of P_{fur} but severely reduced the repressive effect of Fur_{Et}, hence the putative Fur box was critical to the operation of Fur_{Et}. Taken together, these results demonstrated that Fur_{Et} can bind to its own promoter region and inhibit transcription from P_{fur}.

Mutations of Cys-92, Cys-95, and Glu-112 significantly alter the activity of Fur_{Et}

Fur_{Et} possesses four cysteine residues at the positions corresponding to those of the Fur_{Ec}. To examine their potential functional importance, serine substitution was performed upon each of these residues. In addition, the effect of substituting the highly charged amino acids at the C-terminal region of Fur_{Et}, i.e. Lys-97, Asp-103, Glu-104, Glu-107, Arg-109, Glu-112, and Asp-136 (numbered according to the numbering tradition of Fur_{Ec}), with amino acids of the opposite charge was also analyzed. The mutant fur_{Et}, which were generated by the method of overlap extension PCR (Ho *et al*., 1989), were cloned into pBT at the *Sma*I site, resulting in plasmids pTF92, pTF95, pTF132, pTF137, pTF97, pTF103, pTF104, pTF107, pTF109, pTF112, and pTF136 that constitutively express C92S, C95S, C132S, C137S, K97E, D103K, E104K, E107R, R109E, E112K, and D136K mutants, respectively. H1681 was transformed with each of these plasmids and the transformants were subjected to western immunoblotting assay which was performed as described by Martin *et al*. (2004) using rabbit anti-Fur_{Et} antibodies that were prepared by subcutaneously injecting an adult New Zealand White rabbit with 200 μg of purified recombinant Fur_{Et} mixed in complete Freund's adjuvant; the rabbit was boosted with the same amount of Fur_{Et} in incomplete Freund's adjuvant 3 weeks later and bled 12 days after the boosting. The recombinant Fur_{Et} used above was purified by using nickel-nitrilotriacetic acid beads (Zhang and Sun, 2007) from BL21(DE3) transformed with pEEF which was created by inserting the coding region of fur_{Et} into pET258 (Table 1) at between the *Nde*I-*Xho*I sites. The result of immunoblotting showed that the amount of each of the mutant Fur_{Et} produced in the respective transformants was comparable to that of the wild type Fur_{Et} produced in H1681/pTF (data not shown), suggesting that the mutations had not affected the expression or stability of the mutant Fur_{Et}. The H1681 transformants expressing the mutant Fur_{Et} were then subjected to β-galactosidase assay which showed that H1681/pC92S and H1681/pC95S displayed, respectively, ~11 and 8 fold increase in β-galactosidase activity comparing to H1681/pTF, whereas H1681/pE112K displayed ~4 fold decrease in β-galactosidase activity (Table 2). All other mutations had no significant effect on the activity of Fur_{Et}. The presence of 2,2'-dipyridyl inactivated all Fur_{Et} variants except for the E112K mutant, which still retained partial activity (Table 2). Taken together these results demonstrated that C92S and C95S mutations inactivated Fur_{Et} whereas E1123K mutation enhanced the activity of Fur_{Et}. Given the fact that in Fur_{Ec} Cys-92 and Cys-95 participate in the formation of the Zn coordination site and hence are functionally indis-

pensable (Coy *et al*., 1994; Jacquamet *et al*., 1998; Althaus *et al*., 1999; Gonzalez De Peredo *et al.*, 1999), our results suggest the possibility that in Fur_{Et} Cys-92 and Cys-95 may play a role that is analogous to that played by their counterparts in Fur_{Ec}. Our results also favor the notion that Glu-112, which is preserved among a number of Fur proteins and forms the 5th alpha helix of Fur_{Ec}, is involved in the activation of Fur_{Et}.

Overexpression of *fur_{Et}* affects bacterial growth and outer membrane protein synthesis

Since the expression of *fur_{Et}* was subjected to negative autoregulation, we wondered what would ensue from disruption of the regulated synthesis of Fur_{Et}. To investigate this question, the plasmid pJRTF was created as follows: the *bla* gene of pACYC177 (New England BioLabs) was inserted into pDN18 (Table 1) at the *Eco*RV site, resulting in pJRA, which was then digested with *Eco*RV and ligated to the SwaI fragment carrying the P_{trc}-*fur_{Et}* fusion of pTF, resulting in pJRTF. Thus in pJRTF, *fur_{Et}* was constitutively expressed under P_{trc}. pJRTF and the control plasmid pJRA were each transformed into S17-1λpir and the transformants were conjugated with TX1 by using the method of Herrero *et al.* (1990). The transconjugants, TX1/pJRTF and TX1/pJRTF, were examined for potential growth difference. The result showed that the growth of TX1/pJRTF was similar to that of TX1/pJRA under iron-deplete condition but different from the latter when iron was abundant, under which condition TX1/pJRTF exhibited slower doubling time and lower maximum cell density (Fig. 3A). To investigate whether the alteration in growth was accompanied by any alteration in protein production, the outer membrane proteins of TX1/pJRTF and TX1/pJRA were extracted as described by Hantke (1981) and electrophoresed on 0.1% SDS/12% polyacrylamide gels; the proteins were viewed after staining with Coomassie blue, which showed that, comparing to TX1/pJRA, TX1/pJRTF exhibited similar but distinctly different protein profiles (Fig. 3B), suggesting that overexpression of *fur_{Et}* changed the production of certain outer membrane proteins. It is noteworthy that all the proteins that distinguish TX1/pJRTF from TX1/pJRA appeared to be overproduced, which suggested that either the expression or the stability of these proteins was positively modulated by Fur_{Et}. Though Fur is known primarily as a transcription repressor, positive regulations by Fur have been found in several cases (Hall and Foster, 1996; Dubrac and Touati, 2000). Given these precedents, it is likely that the overproduced proteins observed in TX1/pJRTF represent products of those genes that are activated directly or indirectly by Fur_{Et}.

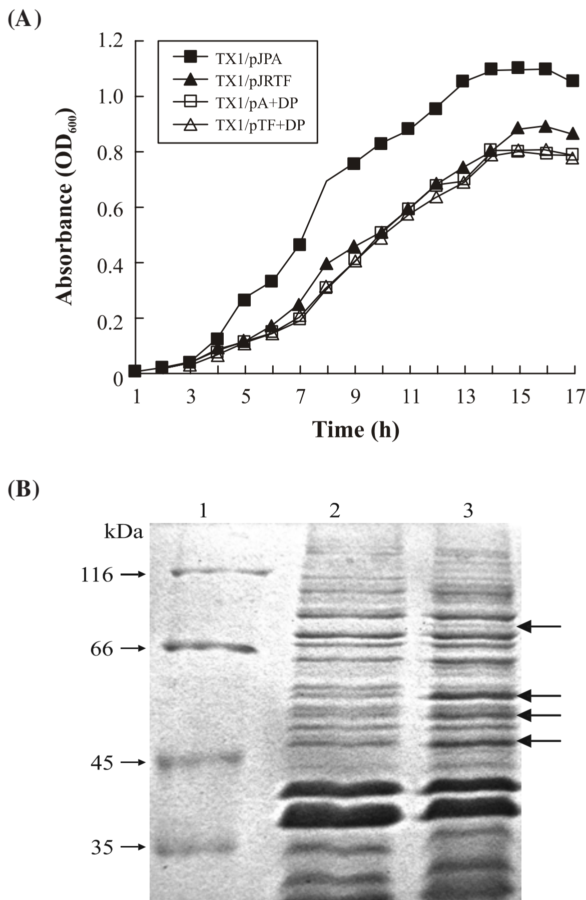

Fig. 3. Effect of overexpression of *fur_{Et}* on bacterial growth (A) and outer membrane protein production (B). (A) TX1/pJRA and TX1/pJRTF were grown in LB medium supplemented with or without 100 μM 2,2'-dipyridyl (DP). Aliquots were taken every hour for the measurement of absorbance at OD_{600}. (B) Outer membrane proteins of TX1/pJRA (lane 2) and TX1/pJRTF (lane 3) were separated on 12% SDS-polyacrylamide gel. The protein bands in which TX1/pJRTF differed from TX1/pJRA are indicated by arrows. Lane 1, molecular weight markers.

Overexpression of *fur_{Et}* attenuates bacterial virulence

Since TX1 is a fish pathogen, we determined whether overexpression of *fur_{Et}* had any effect upon bacterial virulence. For this purpose, TX1/pJRA and TX1/pJRTF were cultured to an OD_{600} of 0.5 in LB medium; the cell pellets were washed and resuspended in phosphate-buffered saline (PBS) to 1×10^7 cfu/ml. Japanese flounder (~17 g) were divided randomly into four groups (42 fish/group); two groups were injected intraperitonealy (i.p.) with 100 μl of TX1/pJRA suspension prepared above while the other two groups were i.p. injected with 100 μl of TX1/pJRTF suspension prepared above. The animals were monitored for mortality in the 14 days post infection. By the end of the monitored period the accumulated mortality of the fish infected with TX1/pJRTF (57%) was significantly ($P<0.001$; Chi-square test) lower than that of fish infected with TX1/pJRA (90.5%). To determine whether there was any difference in dissemination between TX1/pJRA and TX1/pJRTF, the kidney and liver of the infected fish were removed under sterile conditions at 1, 2, 3, and 4 days post infection; the organs were homogenized in PBS with glass homogenizers and plated on LB plates supplemented with tetracycline (marker for TX1) and ampicillin (marker for pJRA and pJRTF). After 24 h growth at 30°C, the colonies that appeared on the plates were counted and their identities were verified by PCR and sequencing of the PCR products. The result showed that the numbers of TX1/pJRTF recovered from the organs at all time points were significantly ($P<0.01$; two-tailed *t*-test) lower than those of TX1/pJRA (data not shown). Taken to-

gether, these results indicated that overexpresion of fur_{Et} mitigates bacterial virulence. This could be accounted for by the above observed growth defect caused by fur_{Et} over-expresssion or/and by the possibility that, like the Fur proteins of other bacterial species (Ratledge and Dover, 2000; Sha and Chopra, 2001), Fur_{Et} may be involved in the regulation of cellular factors that contribute to the development/maintenance of bacterial virulence; upsetting the temporal production of these factors by overexpression of fur_{Et} presumably affects some vital physiological aspects that are fundamental to bacterial infection and survival.

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