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Regulation of the *Edwardsiella tarda* Hemolysin Gene and *luxS* by EthR

Wang, Fang[1,2,3], Min Zhang[1,2], Yong-hua Hu[1,2], Wei-wei Zhang[1,2], and Li Sun[1]*

[1]*Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, P.R. China*
[2]*Graduate University of the Chinese Academy of Sciences, Beijing 100049, P.R. China*
[3]*Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, P.R. China*

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***Edwardsiella tarda* is a pathogen with a broad host range that includes human and animals. The *E. tarda* hemolysin (Eth) system, which comprises EthA and EthB, is a noted virulence element that is widely distributed in pathogenic isolates of *E. tarda*. Previous study has shown that the expression of *ethB* is regulated by iron, which suggests the possibility that the ferric uptake regulator (Fur) is involved in the regulation of *ethB*. The work presented in this report supports the previous findings and demonstrates that *ethB* expression was decreased under conditions when the *E. tarda* Fur (Fur_{Et}) was overproduced, and enhanced when Fur_{Et} was inactivated. We also identified a second *ethB* regulator, EthR, which is a transcription regulator of the GntR family. EthR represses *ethB* expression by direct interaction with the *ethB* promoter region. In addition to *ethB*, EthR also modulates, but positively, *luxS* expression and AI-2 production by binding to the *luxS* promoter region. The expression of *ethR* itself is subject to negative autoregulation; interference with this regulation by overexpressing *ethR* during the process of infection caused (i) drastic changes in *ethB* and *luxS* expressions, (ii) vitiation in the tissue dissemination and survival ability of the bacterium, and (iii) significant attenuation of the overall bacterial virulence. These results not only provide new insights into the regulation mechanisms of the Eth hemolysin and LuxS/AI-2 quorum sensing systems but also highlight the importance of these systems in bacterial virulence.**

Keywords: *Edwardsiella tarda*, hemolysin, *luxS*, RNAi, virulence

**Corresponding author*
Phone: +86-532-82898834; Fax: +86-532-82898834;
E-mail: lsun@ms.qdio.ac.cn

Present address: Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Road, Qingdao 266071, P. R. China

Edwardsiella tarda is a Gram-negative bacterium of the family *Enterobacteriaceae*. It is an opportunistic pathogen with a broad host range that includes human, animals, and fish [1, 22, 29]. As a human pathogen, *E. tarda* is known to cause acute gastroenteritis and is associated with meningitis, septicemia, and wound infections. As a fish pathogen, *E. tarda* can infect many different species, including the economically important turbot, flounder, salmon, catfish, eel, seabream, and mullet. *E. tarda* is the etiological agent of edwardsiellosis, a serious systematic disease of cultured fish that has caused heavy stock mortalities in aquaculture industries worldwide. Several virulence-associated factors and systems have been identified in *E. tarda*, which include dermatotoxins [34], chondroitinase [16], catalase [30], hemolysins [13, 15], and type III and type VI secretion systems [23, 33, 41].

Bacterial quorum sensing is a process of cell–cell communication *via* the production of and response to small signaling molecules called autoinducers. Of the autoinducers that have been identified, the type II autoinducer (AI-2) is synthesized *via* several steps, involving an enzyme called LuxS, which catalyzes the conversion of S-ribosylhomocysteine to homocysteine and 4,5-dihydroxy-2,3-pentanedione, the latter being the precursor of AI-2 [28]. In addition to its role in quorum sensing as an AI-2 synthase, LuxS also plays a part in cellular metabolism by participating in the activated methyl cycle [35, 38]. LuxS has been identified in diverse bacteria and known to affect multiple aspects of cellular life including pathogenicity. Our recent study showed that the LuxS-mediated signal transduction pathway is involved in pathogenesis of *E. tarda* [39].

A previous study by Hirono *et al.* [13] has demonstrated that the *eth* cluster, which codes for the Eth hemolysin system of *E. tarda*, consists of two genes, *ethA* and *ethB*. *ethA* encodes the hemolysin, whereas *ethB* encodes the activation/secretion apparatus that is required for the maturation and translocation of EthA. The expression of

ethB is negatively regulated by iron, probably through the ferric uptake regulator (Fur), a transcription regulator that is activated by iron [11]. In this report, we describe the results of further study on the regulation of *ethB* and demonstrated that EthR, a GntR-like protein, is a potent *ethB* regulator that also modulates the expression of *luxS*.

Materials and Methods

Bacterial Strains

The bacterial strains used in this study are listed in Table 1. All strains were grown in Luria–Bertani broth (LB) medium [27] at 37°C (for *Escherichia coli*) or 28°C (for *Edwardsiella tarda*). Appropriate antibiotics were supplemented at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 25 μg/ml; tetracycline, 15 μg/ml. When indicated, X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) was supplemented at the concentration of 40 μg/ml.

Plasmid Construction

The plasmids and the primers used in this study are listed in Table 1. To construct pBER, *ethR* was amplified by PCR with primers EF3/ER3, and the 723-bp PCR products were inserted into pBT at the SmaI site. To construct pJER, pBER was digested with SwaI, and the 1.5-kb DNA fragment was inserted into pJRA at the EcoRV site. To construct pJFUR, pTF was digested with SwaI, and the 1.3-kb DNA fragment was inserted into pJRA at the EcoRV site. To

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

Strain or plasmid or primer	Relevant characteristics[a]	Source or reference
Bacterial strains		
E. coli		
DH5α	F⁻(Φ80d *lacZ* Δ*M15*) Δ(*lacZYA-argF*) U169 *recA1 endA hsdR17* (*rk*⁻, *mk*⁻) *supE44 thil gyrA relA1*	Takara (China)
NCK	F⁻λ⁻ Δ *lacX74 rpsL galOP308 fur*	[36]
TOP10	*mcrA*Δ(*mrr-hsdRMS-mcrBC*)Φ80Δ*lacZ* Δ*M15* Δ*lacX74 deoR recA1 endA1*	Tiangen (China)
TOP10λRS65T	As TOP10 but lysogenic for λRS65T (*tetAPO-cat-lacZ YA* Cm^r)	[31]
E. *tarda*		
TX1	Tc^r; fish pathogen	[39]
Plasmids		
p178	Ap^r; cloning vector	[39]
pBT	Ap^r; expression plasmid carrying P_{trc}	[39]
pBER	Ap^r; pBT expressing *ethR*	This study
pTF	Ap^r; pBT expressing fur_{Et}	[36]
pSC7	Kn^r; promoter probe plasmid	[39]
pSC318	Kn^r; pSC7 carrying P_{ethB}–*tetR* fusion	This study
pSC13	Tc^r; promoter probe plasmid	[36]
pD329	Tc^r; pSC13 carrying *ethR* promoter–*lacZ* fusion	This study
pSC17	Kn^r; promoter probe plasmid	[39]
pSC288	Tc^r; carrying *luxS* promoter fused to the luciferase gene	This study
pET258	Kn^r; expression plasmid	[40]
pJRA	Ap^r; pDN18 derivative	[39]
pJER	Ap^r; pJRA expressing *ethR*	This study
pJEBP	Ap^r; pJRA carrying P_{ethB}–*lacZ* fusion	This study
pJFUR	Ap^r; pJRA expressing fur_{Et}	This study
pJRI	Ap^r; pJRA expressing *ethR* antisense RNA	This study
Primers	Sequences (5'-> 3')[b]	
322F4	ATTTCTATGCGCACCCGTT	
322R4	GCGCTCATGAGCCCGAA	
EF3	GATATCGATTAGGATCGACTAGCC (EcoRV)	
EF10	GATATCGCCGTGAAAACCCGTT (EcoRV)	
EF24	GAGGAATATAGATATGCATACCCGA	
EHBF7	AAATAGCGCACCAGCCTTCTAT	
EHBR13	ATTATTCCCCCGGGCGAC	
ER3	GATATCCGTCGAGTGTTAGGCTC (EcoRV)	
ER8	GATATCTAGTCGATCCTAATCGG (EcoRV)	
ER29	GCTGGCCTATCTTCTCAGCAAGA	

[a]Ap^r: ampicillin resistant; Cm^r: chloramphenicol resistant; Kn^r: kanamycin resistant; Tc^r: tetracycline resistant.
[b]Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends.

construct pJRI, the 723-bp DNA encoding the antisense *ethR* (generated by PCR with primers ER3/EF3) was inserted into pBT at the SmaI site, yielding pBERI, which was digested with SwaI, and the 1.5-kb DNA fragment was inserted into pJRA at the EcoRV site, resulting in pJRI. pSC318 was constructed by inserting the 318-bp DNA (amplified by PCR with primers EHBF7/EHBR13) immediately upstream of the translation start of *ethB* into pSC7 at the EcoRV site. pD329 was constructed by inserting the 329-bp DNA (amplified by PCR with primers EF10/ER8) upstream of the translation start of *ethR* into pSC13 at the SwaI site. To construct pJEBP, the 318-bp DNA (amplified by PCR as above) upstream of the translation start of *ethB* was inserted into p178 at the SwaI site, yielding p178EBP, which was digested with SmaI, and the 3.3-kb DNA fragment was inserted into pJRA at the EcoRV site. To construct pSC288, the 288-bp *luxS* promoter was amplified by PCR with primers F10/R9 [39], and the PCR products were inserted into pSC17 at the SwaI site.

Expression and Purification of the Recombinant EthR

The coding sequence of *ethR* was ligated into pET258 between the NdeI–XhoI sites, resulting in pEHR. BL21(DE3) (Tiangen, China) was transformed with pEHR. The recombinant EthR was purified from BL21(DE3)/pEHR by using nickel–nitrilotriacetic acid beads as described previously [40].

Quantitative Real-Time PCR (qRT–PCR)

Total RNA was extracted from fish tissues and from cells grown in appropriate medium to an optical density at 600 nm (OD_{600}) of 0.8 by using the SV total RNA isolation system (Promega). To verify that the RNA was free of DNA contamination, PCR amplifications were performed using the total RNA (at least 10 times of that used in subsequent qRT–PCR) as the template and the primers intended for all qRT–PCR as primers. The results showed that all PCR amplifications were negative.

qRT–PCR was carried out in an ABI 7300 Real-time Detection System (Applied Biosystems) by using the SYBR ExScript qRT–PCR Kit (Takara, China). Each assay was performed in triplicate with the 16S rRNA as controls. Dissociation analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The comparative CT method ($2^{-\Delta\Delta CT}$ method [19]) was used to analyze the mRNA level. All data are given in terms of relative mRNA expressed as means plus or minus standard errors (SE) of the means. Statistical analyses were performed by using the two-tailed *t*-test.

Electrophoretic Mobility Shift Assay

The DNA to be examined was labeled with carboxyfluorescein at the 5' end. The labeled DNA (150 ng) was mixed with various concentrations of EthR and incubated at 25°C for 30 min in 20 μl of binding buffer (10 mM Tris–Bis, 4 mM KCl, 0.1 mM $MnCl_2$, 0.5 mg/ml BSA, and 15% glycerol). The samples were then electrophoresed in a nondenaturing 8% polyacrylamide gel. As a negative control, a 246-bp DNA fragment (generated by PCR with primers 322F4/322R4) of pBR322 was also included in the assay.

β-Galactosidase Assay

This was performed according to the method of Miller [21]. Briefly, cells were cultured in LB medium to OD_{600} of 1; 1 ml of the cell culture was lysed by the addition of 25 μl of chloroform and 12 μl of SDS (10%), followed by vortexing for 10 s. Fifty μl of the lysed cells was transferred to 450 μl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol), followed by the addition of 100 μl of *o*-nitrophenyl β-D-galactopyranoside (Sigma) (4 mg/ml). The mixture was incubated at room temperature for 2 min to 1 h, and the reaction was stopped by the addition of 400 μl of 1 M sodium carbonate. Absorbance was measured at 420 and 550 nm, and β-galactosidase units were calculated according to Miller [21].

Luciferase Assay

Cells were grown in LB medium to mid-logarithmic phase. The cells were lysed and assayed for luciferase activity by using the Luciferase Assay System (Promega). Light production was measured by using a Glomax luminometer (Promega).

AI-2 Assay

AI-2 assay was performed as described by Surette and Bassler [32]. Briefly, overnight cultures of bacteria grown in LB medium at 28°C were diluted 1:100 in fresh LB medium; 2 ml of cell culture was taken every 30 min, from which the cell-free supernatant was obtained by centrifugation and then filtering through a 0.22-μm filter (Millipore). For measurement of bioluminescence induction, overnight culture of the *V. harveyi* strain BB170 [American Type Culture Collection (ATCC)] grown in AB medium at 28°C was diluted 1:5,000 in fresh AB medium supplemented with 10% cell-free supernatant (as prepared above) of the tested strains or with the growth medium (as the control). The growth was continued and light production was measured by using a Glomax luminometer (Promega).

Bacterial Conjugation

pJRA and its variants were introduced into the *E. coli* strain S17-1λpir (Biomedal, Spain) by transformation. Conjugation between the transformants and TX1 was performed exactly as described previously [39].

Animal Model Study

To examine bacterial dissemination, TX1/pJER and TX1/pJRA were cultured to an OD_{600} of 0.6 in LB medium, washed, and resuspended in phosphate-buffered saline (PBS) to 4×10^6 CFU/ml. Japanese flounder (*Paralichthys olivaceus*; average weight, 13 g) were divided randomly into three groups (5 fish/group) and each group was injected intraperitoneally (i.p.) with 100 μl of PBS, 100 μl of TX1/pJER (prepared as above), and 100 μl of TX1/pJRA (prepared as above), respectively. The livers of the fish were taken at 2, 24, 48, and 72 h post-infection. The organs were homogenized in PBS, and the homogenates were plated on LB agar plates supplemented with ampicillin (marker for pJRA and pJER) and tetracycline (marker for TX1). After incubating at 28°C for 48 h, the colonies that appeared on the plates were enumerated. The nature of these colonies was verified by PCR analysis using primers specific to pJER, pJRA, and TX1. The PCR products were subsequently analyzed by DNA sequencing. Statistical analysis was performed by using the Students *t*-test.

To examine general bacterial virulence, Japanese flounder were divided randomly into two groups (30 fish/group). Each group was injected i.p. with the same dose of TX1/pJER or TX1/pJRA as described above. The animals were monitored for mortality for 14 days post-infection, and the accumulated mortalities were calculated

at the end of the monitored period. Statistical analysis was performed by using the Chi-squared test.

Database Search and Nucleotide Sequence Accession Number
Nucleotide and amino acid sequence searching was conducted using the BLAST programs at the NCBI (National Center for Biotechnology Information). The nucleotide sequence of *ethR* has been deposited in the GenBank database under Accession No. EU478438.

Results

The *E. tarda* Fur (Fur_{Et}) is Involved in the Regulation of *ethB* Expression

To examine whether Fur_{Et} had any effect on the expression of *ethB*, the conjugative plasmid pJFUR, which constitutively expresses fur_{Et}, was introduced into the pathogenic *E. tarda* strain TX1 by conjugation. Overexpression of fur_{Et} in TX1/pJFUR was confirmed by qRT–PCR analysis, which showed that fur_{Et} expression in TX1/pJFUR was 17.1-fold more than that in TX1/pJRA. In contrast, *ethB* expression in TX1/pJFUR was 2.3-fold less than that in TX1/pJRA (Fig. 1A), suggesting that Fur_{Et} had an inhibitory effect on the expression of *ethB*. Since Fur is a metalloprotein that requires iron for biological activity, we determined *ethB* expression in the presence of the iron chelator 2,2'-dipyridyl, which inactivates Fur by deleting iron from the medium. The result showed that the presence of 2,2'-dipyridyl caused a 2.8-fold increase in *ethB* expression in TX1/pJFUR (Fig. 1A). In line with this observation, the β-galactosidase assay showed that TX1 harboring pJEBP, which carries the putative *ethB* promoter [13] P_{ethB}, fused to a promoterless *lacZ* reporter gene, produced 3-fold more β-galactosidase activity in the presence than in the absence of 2,2'-dipyridyl, suggesting that Fur_{Et} repressed transcription from P_{ethB}. Taken together, these results demonstrated that *ethB* expression is regulated by Fur_{Et} in a negative manner.

Identification of EthR

Preliminary studies in our laboratory showed that *ethB* expression *in vitro* (*i.e.*, in LB medium at 28°C) was more than 8-fold less than that *in vivo* (*i.e.*, inside the liver and kidney of the animal that had been infected with TX1). Since, as demonstrated above, inactivation of Fur_{Et} caused less than 3-fold change in *ethB* expression, we suspected the existence of other yet unidentified *ethB* regulator(s) that may repress *ethB* expression under *in vitro* conditions. To screen for the potential *ethB* repressor, we utilized a genetic system called PSDT (Positive Selection of DtxR Targets), which was initially developed for the positive selection of diphtheria toxin repressor (DtxR) target sites [31]. To suit our purpose in this study, the original PSDT system was modified by replacing the plasmid pSC6 with pSC318, in which P_{ethB} directs the expression of the tetracycline resistance protein repressor *tetR*. pSC318 was introduced, *via* transformation, into the *E. coli* reporter strain TOP10λRS65T, which is the *E. coli* strain TOP10 lysogenized with a lambda phage that carries the promoterless reporter gene *cat* (encoding the chloramphenicol acetyltransferase) fused to the promoter of *tetA* (encoding the tetracycline resistance protein A). Since TetR is a stringent repressor of *tetA*, the expression of *cat* (*i.e.*, chloramphenicol resistance) is controlled by TetR, which in turn is controlled by P_{ethB}. Therefore, in this system, the expression of *cat* (or chloramphenicol resistance) can be regulated by elements that regulate the activity of P_{ethB}.

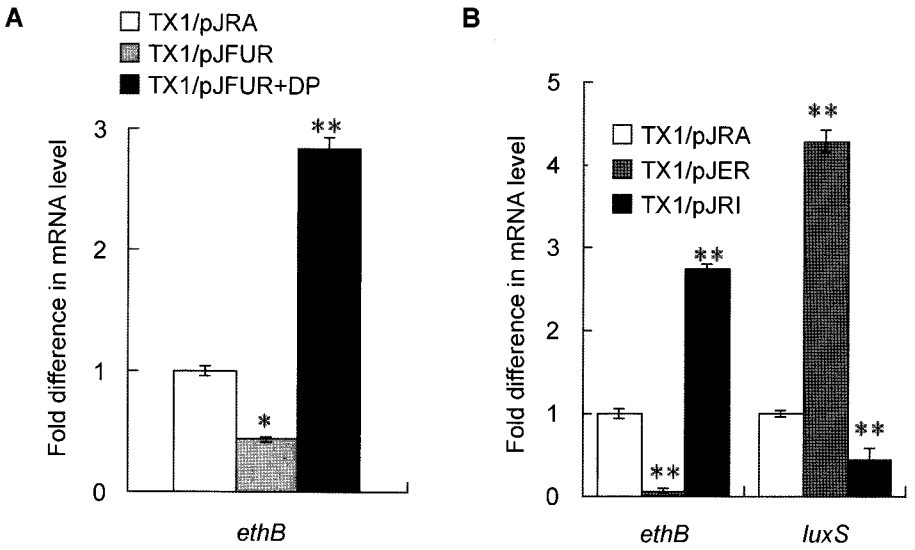

Fig. 1. Effect of Fur_{Et} on the expression of *ethB* (**A**) and effect of EthR on the expressions of *ethB* and *luxS* (**B**).
qRT–PCR was performed using total RNA prepared from cells grown in LB medium to an OD_{600} of ~0.7 in the presence of 50 μM $FeCl_3$ or 200 μM 2,2'-dipyridyl (DP). The mRNA levels of *ethB* and *luxS* were normalized to that of the 16S rRNA. For each gene, the expression level in TX1/pJRA was set as 1. Data are the means of three to four independent experiments and presented as the means±SE. **, $P<0.001$; *, $P<0.01$.

For the selection of *ethB* repressors, a Sau3A1 genomic DNA library of TX1 was cloned into pBR322 at the BamHI site, and the resulting recombinant plasmids were introduced, *via* transformation, into TOP10λRS65T/pSC318. The transformants were selected on LB agar plates supplemented with ampicillin (marker for pBR322 derivatives), kanamycin (marker for pSC318), and chloramphenicol based on the rationale that the presence of a negative regulator of P_{ethB} expressed from the recombinant pBR322 should block *tetR* expression and thus allow the expression of *cat* from TOP10λRS65T.

One chloramphenicol-resistant clone so obtained was found to harbor a recombinant pBR322 carrying a DNA insert that contains a 693-bp open reading frame (ORF). Sequence analysis revealed that *orf693* encodes a putative protein that shares the highest (97%) sequence identity with the GntR family transcription regulator of *E. ictaluri* (GenBank Accession No. ABX88885). An N-terminal DNA-binding motif (residues 14 to 77) and a C-terminal effector-binding domain (residues 106 to 227) typical of the GntR family of transcription regulators [4, 25] were identified in ORF693 by using the NCBI Conserved Domain Search server. Based on these data, ORF693 was considered a member of the GntR family and tentatively named EthR (*ethB* regulator).

EthR Negatively Regulates *ethB* Expression

To investigate the effect of EthR on the expression of *ethB*, *ethR* expression in TX1 was disrupted by antisense RNA interference. This was performed by introducing the conjugative plasmid pJRI, which constitutively expresses the *ethR* antisense RNA (corresponding to the region between positions 704 and −19 relative to the translational start), and the control plasmid pJRA into TX1. Expression of the *ethR* antisense RNA in the transconjugant TX1/pJRI was confirmed by qRT–PCR (data not shown). In addition, to examine the effect of *ethR* overexpression, the conjugative plasmid pJER, which constitutively expresses *ethR*, was also introduced into TX1 by conjugation. TX1/pJRI, TX1/pJER, and TX1/pJRA were then analyzed for the expression of *ethB* and *ethR* by qRT–PCR. The results showed that the level of *ethR* expression increased 19.6-fold in TX1/pJER and decreased 3-fold in TX1/pJRI compared with that in TX1/pJRA, suggesting that in TX1/pJER, *ethR* expression was indeed heightened whereas in TX1/pJRI, *ethR* expression was attenuated. In contrast to the expression pattern of *ethR*, the expression of *ethB* decreased 14.7-fold in TX1/pJER and increased 2.8-fold in TX1/pJRI compared with that in TX1/pJRA (Fig. 1B), suggesting that the expression of *ethB* was repressed by EthR. To examine whether EthR could interact directly with the *ethB* promoter, electrophoretic mobility shift analysis was performed, which showed that EthR bound specifically to the *ethB* promoter region (Fig. 2A). Taken together, these results demonstrated that EthR regulates *ethB* expression by direct interaction with the *ethB* promoter region.

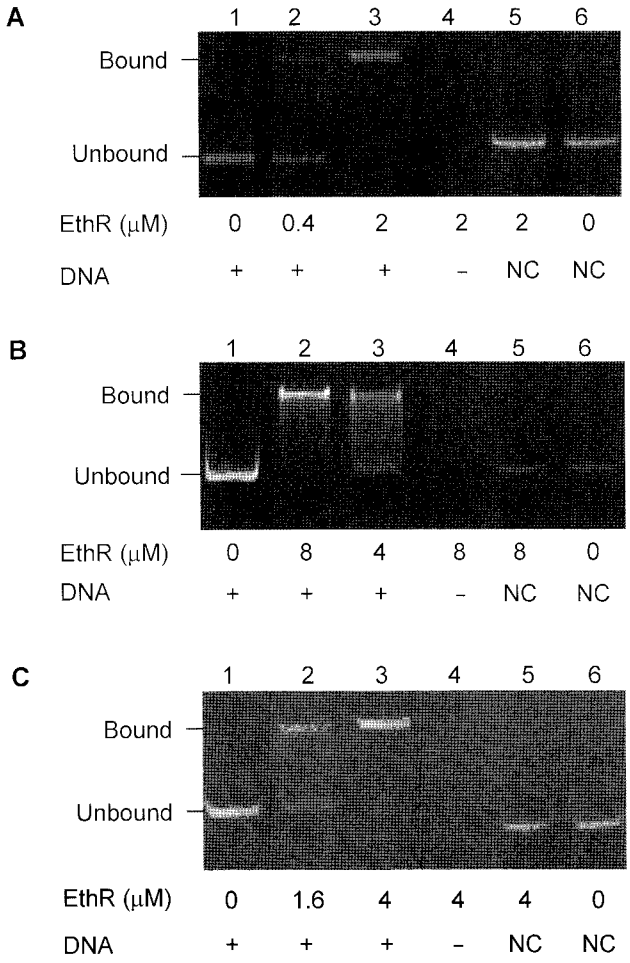

Fig. 2. Specific binding of EthR to the promoter regions of *ethB* (**A**), *luxS* (**B**), and *ethR* (**C**).
Electrophoretic mobility shift assay was performed in the binding buffer containing the purified recombinant EthR and a carboxyfluorescein-labeled DNA fragment (DNA), which is a 188-bp DNA harboring P_{ethB} in **A**, a 288-bp DNA harboring the *luxS* promoter in **B**, and D329 in **C**. The DNA used in the negative control (lanes 5 and 6 of all panels) is a 246-bp sequence derived from pBR322. NC, negative control DNA.

EthR Modulates *luxS* Expression in a Positive Manner

In a recent study of *luxS* expression and AI-2 production in TX1, we found that the AI-2 activity of this bacterium is controlled, at least in part, at the level of *luxS* expression [39]. In a related study, we noticed that the AI-2 activities of TX1/pJER and TX1/pJRI were ~2-fold higher and ~3-fold lower than that of TX1/pJRA, respectively, which suggested the possibility that EthR positively modulated *luxS* expression. To investigate whether or not this was the case, qRT–PCR was carried out to examine the expression of *luxS* in TX1/pJER, TX1/pJRI, and TX1/pJRA. The

result showed that the level of *luxS* expression was 4.3-fold higher in TX1/pJER and 2.1-fold lower in TX1/pJRI than that in TX1/pJRA (Fig. 1B), suggesting that EthR exerted a positive effect on the expression of *luxS*. To determine whether EthR could directly affect the activity of the *luxS* promoter, DH5α harboring pSC288, which carries the *luxS* promoter fused to a promoterless luciferase reporter gene, was transformed with pBER, which expresses *ethR*. Subsequent luciferase assays showed that the luciferase activity of DH5α/pSC288/pBER was 2-fold higher than that of DH5α/pSC288 harboring the control plasmid pBT, suggesting that EthR could activate transcription from the *luxS* promoter. In line with this observation, the result of electrophoretic mobility shift assay indicated that EthR could bind specifically to the *luxS* promoter region (Fig. 2B). Taken together, these results demonstrated that EthR positively modulates *luxS* expression, probably *via* direct interaction with the *luxS* promoter region.

EthR Negatively Regulates its Own Expression

To examine whether EthR could regulate transcription from its own promoter, the 329-bp DNA (named D329) immediately upstream of the translational start of *ethR* was cloned into the promoter probe plasmid pSC13, resulting in plasmid pD329, in which D329 was fused to the promoterless *lacZ* reporter gene. The *fur*-defective *E. coli* strain NCK was transformed with pD329 and the transformants appeared blue on Xgal medium, suggesting that D329 contained a functional promoter, which was likely the promoter of *ethR*. NCK/pD329 was then transformed separately with pBER (expressing *ethR*) and the control plasmid pBT; the transformants were assayed for β-galactosidase production, which showed that the β-galactosidase activity of NCK/pD329/pBER was 23-fold less than that of NCK/pD329/pBT, suggesting that EthR inhibited transcription from its own promoter in D329. Consistently, the results of electrophoretic mobility shift analysis demonstrated that EthR could bind specifically to D329 (Fig. 2C).

To further investigate whether EthR autoregulation held true in TX1, qRT–PCR was carried out to examine the expression of the chromosomal *ethR* in TX1/pJER and TX1/pJRA by using primers EF24 and ER29, which cover the sequences from positions −41 to −17 and 51 to 70 relative to the translation start of *ethR*, respectively. Since pJER contains only the coding region plus the putative ribosome binding site of *ethR* and has no EF24 sequence, when qRT-PCR was conducted to analyze *ethR* expression in TX1/pJER, positive PCR products could only be produced from the mRNA transcribed from the chromosomal *ethR* and not from pJER. The result showed that the chromosomal *ethR* expression was 6.5-fold less in TX1/pJER than that in TX1/pJRA.

Taken together, these results demonstrated that EthR regulates its own expression in a negative manner.

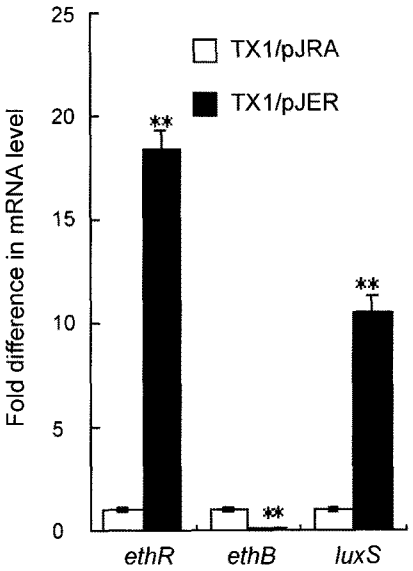

Fig. 3. Effect of *ethR* overexpression on *ethB* and *luxS* expression during infection.

qRT–PCR was performed using total RNA extracted from the kidneys of the fish infected with TX1/pJRA or TX1/pJER. The mRNA levels of *ethR*, *ethB*, and *luxS* were normalized to that of the 16S rRNA. For each gene, the expression level in TX1/pJRA was set as 1. Data are the means of three independent experiments and presented as the means±SE. **, $P<0.001$.

Overexpression of *ethR* During Infection Has Multiple Effects

Effect on *ethB* and *luxS* expression. Since the Eth hemolysin is known to be a virulence factor of *E. tarda*, we compared the expression levels of *ethB* under conditions of *in vitro* growth and *in vivo* growth (inside the animal host). For this purpose, qRT–PCR was carried out using total RNA extracted from TX1/pJRA grown in LB medium at 28°C (*in vitro*) and from the kidney and liver of Japanese flounder infected with TX1/pJRA (*in vivo*). The results showed that *ethB* expression *in vivo* was 8.2-fold higher than that *in vitro*, suggesting that the *eth* system was highly activated during infection. To examine the effect of *ethR* overexpression during infection, qRT–PCR was performed to analyze *ethB* and *luxS* expression using RNA extracted from the kidney of the fish infected with TX1/pJER and TX1/pJRA, respectively. The results showed that *ethB* expression decreased 17.8-fold in TX1/pJER-infected fish compared with that in TX1/pJRA-infected fish (Fig. 3). In contrast, *luxS* expression in TX1/pJER-infected fish was 10.6-fold higher than that in TX1/pJRA-infected fish (Fig. 3). Hence, it appeared that overexpression of *ethR* drastically altered the expression patterns of *ethB* and *luxS* in the host environment during infection.

Effect on bacterial dissemination and survival in tissues. To investigate whether there was any difference in tissue dissemination and survival abilities between TX1/pJER and

TX1/pJRA, Japanese flounder were administered *via* i.p. injection with TX1/pJER, TX1/pJRA, and PBS, respectively. The livers of the fish were taken at 2, 24, 48, and 72 h post-infection and examined for bacterial recovery. The result showed that the amounts of TX1/pJER recovered from the organs were significantly ($P<0.001$) lower than those of TX1/pJRA during the entire infection period (Fig. 4). No colonies emerged on the plates applied with tissue homogenates of the fish that had been mock-infected with PBS.

To examine whether the difference in bacterial recovery from the TX1/pJER- and TX1/pJRA-infected fish was due to selective loss of pJER under survival pressures, the *in vivo* retention levels of pJER and pJRA were determined. For this purpose, the livers were taken from TX1/pJER- and TX1/pJRA-infected fish at 24, 48, and 72 h post-infection and homogenized in PBS; the homogenates were plated on both LB agar plates supplemented with tetracycline alone (for the selection of TX1, TX1/pJRA, and TX1/pJER) and LB plates supplemented with tetracycline plus ampicillin (for the selection of TX1/pJRA and TX1/pJER). The results showed that, with the organs derived from TX1/pJER-infected fish, the numbers of TX1/pJER that appeared on the tetracycline plus ampicillin plates were 9.7–11.2% lower than those of TX1 that appeared on the tetracycline plates; PCR analysis using TX1- and pJER-specific primers indicated that 88.8% (71/80) of the TX1 colonies on the tetracycline plates were TX1/pJER. With the organs derived from the fish infected with TX1/pJRA, the numbers of TX1/pJRA that emerged on the tetracycline plus ampicillin plates were 8.1–9.9% lower than those of TX1 that emerged on the tetracycline plates; 90% (72/80) of the TX1 colonies on the tetracycline plates were verified to be TX1/pJRA. Hence, the plasmid loss rate of TX1/pJER was at most 3.1% higher than that of TX1/pJRA during infection. This difference in plasmid stability was apparently far too low to account for the 53- to 2,190-fold difference (Fig. 4) in the amounts of TX1/pJER and TX1/pJRA recovered from the infected fish. Taken together, these results demonstrated that overexpression of *ethR* drastically vitiates the dissemination and survival ability of TX1.

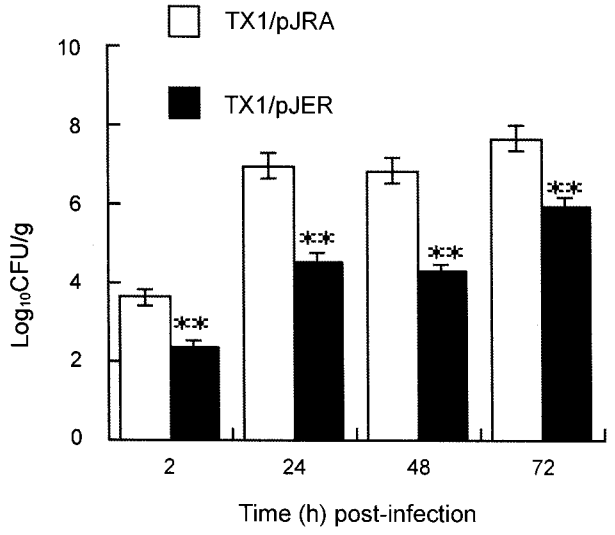

Fig. 4. Effect of *ethR* overexpression on bacterial dissemination and survival in liver.

Japanese flounder were i.p. injected with either TX1/pJRA or TX1/pJER; the livers of the infected fish were taken at different time points post-infection. The organs were homogenized in PBS and plated on selective LB plates. The plasmid-bearing TX1 that appeared on the plates were enumerated. **, $P<0.001$.

Effect on general bacterial virulence. To examine the effect of *ethR* overexpression on the overall bacterial virulence, Japanese flounder were administered *via* i.p. injection with the same dose of TX1/pJRA or TX1/pJER. The fish were monitored for mortality for 14 days post-infection and the accumulated mortalities were calculated at the end of the monitored period. The results showed that the accumulated mortality of the fish infected with TX1/pJER (30%) was significantly ($P<0.001$) lower than that of the fish infected with TX1/pJRA (97.5%), suggesting that overexpression of *ethR* significantly attenuated the overall bacterial virulence of TX1.

DISCUSSION

Studies by Hirono *et al.* [13] have demonstrated that the expression of *ethB* is negatively modulated by iron, which suggests the possibility that Fur_{Et} is involved in the regulation of *ethB*. Consistent with this observation, we found that overexpression of fur_{Et} reduced the expression of *ethB*, whereas inactivation of Fur_{Et} enhanced *ethB* expression. Compared with Fur_{Et}, EthR is likely a more potent regulator of *ethB*, since overexpression of *ethR* caused a more drastic change in *ethB* expression than that caused by overexpression of fur_{Et}. Although initially discovered as a repressor of the *Bacillus subtilis* gluconate operon [7, 24], GntR-like proteins have now been found to exist in a wide range of bacterial species and regulate diverse cellular aspects that include metabolism [5, 6, 10, 18, 23, 26], iron assimilation [9], taurine utilization [37], phosphate transport [3], swarming [17], cellular development [14], and antibiotic production [12]. However, to our knowledge, regulation of hemolysin expression by proteins of the GntR family, as was observed in our study with EthR, has not been documented before. Lin *et al.* [20] has reported that LuxZ, a member of the GntR family, enhanced the bioluminescence of the *lux* operon of *Photobacterium leiognathi*, but it is not clear as to the exact genetic mechanism involved. In our study, we found that EthR exhibited a positive effect on *luxS* expression and AI-2 activity, which was consistent with

our earlier observation that the AI-2 activity of *E. tarda* correlates largely with *luxS* expression. It is interesting that EthR could act as both an activator and a repressor, depending on the specific target promoter. As far as the three EthR targets identified in this study are concerned, *ethB* appears to be most sensitive to EthR whereas *luxS* is relatively the least sensitive, as judged by the results of qRT–PCR and electrophoretic mobility shift analyses. However, this does not imply that EthR functions principally as an *ethB* regulator, for the actual effect of EthR on *luxS* expression and AI-2 production is large.

Participation of GntR family transcription regulators in bacterial virulence has been reported previously, in which GntR-like proteins are found to modulate the expression of genes concerned in interactions with host cells [2, 8]. It is noteworthy that in our study, both of the non-self targets of EthR happen to be virulence-associated. The Eth hemolysin has long been known as a virulence factor that interacts directly with the host. The LuxS/AI-2 quorum sensing system has recently been demonstrated to be involved in bacterial pathogenicity *via* the regulation of certain virulence elements. The discovery in our study that expression of both *ethB* and *lux*S is controlled by EthR made it clear that the Eth hemolysin and the LuxS/AI-2 systems are expressed under dual or multiple controls – the former by EthR and Fur/iron, the latter by EthR, cAMP–CRP, and other growth conditions [39]. These findings, together with the observation that aberrant expression of *ethB* and *lux*S in response to *ethR* overexpression was associated with drastic attenuation in the overall bacterial virulence, suggest strongly that the Eth hemolysin and the LuxS/AI-2 systems play important roles in the course of TX1 infection. It is possible that, in addition to *ethB* and *lux*S, other genes may be subject to EthR regulation; in this case, interference with the expression of *ethR* would disrupt physiological processes affected not only by the Eth hemolysin and the LuxS systems but also by other yet unidentified factors that may contribute, directly or indirectly, to pathogenicity.

In conclusion, our study demonstrated that EthR modulates the expressions of *ethB*, *luxS*, and *ethR* itself; disruption of the regulated expression of *ethR* significantly attenuates bacterial virulence. Our results extend the range of the known cellular processes involving GntR-like transcription regulators and also highlight the importance of the Eth hemolysin and the LuxS/AI-2 systems in the development/maintenance of bacterial virulence.

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