



# Antisense Transcription Regulates the Expression of the Enterohemorrhagic *Escherichia coli* Virulence Regulatory Gene *ler* in Response to the Intracellular Iron Concentration

Toru Tobe<sup>1\*</sup>, Hilo Yen<sup>2</sup>, Hiroki Takahashi<sup>3</sup>, Yoko Kagayama<sup>4</sup>, Naotake Ogasawara<sup>4</sup>, Taku Oshima<sup>4</sup>

**1** Department of Biomedical Informatics, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan, **2** Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan, **3** Medical Mycology Research Center, Chiba University, Chiba, Chiba, Japan, **4** Graduate School of Information Science, Nara Institute of Science and Technology, Ikoma, Nara, Japan

## Abstract

Enteric pathogens, such as enterohemorrhagic *E. coli* (EHEC) O157:H7, encounter varying concentrations of iron during their life cycle. In the gastrointestinal tract, the amount of available free iron is limited because of absorption by host factors. EHEC and other enteric pathogens have developed sophisticated iron-responsive systems to utilize limited iron resources, and these systems are primarily regulated by the Fur repressor protein. The iron concentration could be a signal that controls gene expression in the intestines. In this study, we explored the role of iron in LEE (locus for enterocyte effacement) virulence gene expression in EHEC. In contrast to the expression of Fur-regulated genes, the expression of *LEE* genes was greatly reduced in *fur* mutants irrespective of the iron concentration. The expression of the *ler* gene, the LEE-encoded master regulator, was affected at a post-transcription step by *fur* mutation. Further analysis showed that the loss of Fur affected the translation of the *ler* gene by increasing the intracellular concentration of free iron, and the transcription of the antisense strand was necessary for regulation. The results indicate that *LEE* gene expression is closely linked to the control of intracellular free iron homeostasis.

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\* Email: torutobe@sahs.med.osaka-u.ac.jp

## Introduction

Enteric pathogens that infect the mammalian gut use specific traits, referred to as virulence factors, to grow on the intestinal surface and sometimes pass through the epithelial barrier to reach deeper tissues. To activate the expression of virulence factors at the appropriate time and to target niches, pathogens often sense the chemical and/or physical conditions of the intestinal environment. The intestines contain a variety of environmental factors that are altered by ingested food, the metabolic activity of the microflora, the location in the intestine and the physiological conditions of the host. One or several of these factors are thought to be signal(s) that regulate the expression of virulence genes by enteropathogens.

Iron is essential for living systems but is toxic at high levels. Many enzymes require iron as a cofactor for catalytic activity [1]. However, free iron accelerates the production of hydroxyl radicals by the Fenton reaction [2]. Bacteria possess several specific systems to capture and take up iron, and the associated genes are strictly regulated by the iron concentration through an iron-interacting DNA binding protein [3]. Fur, the ferric uptake regulator, is a repressor of genes involved in iron utilization [4]. At low concentrations of iron, Fur not bound by iron (apo-Fur) is released

from the operators of target genes, and the promoters become active. In addition to iron utilizing systems, Fur and iron regulate a variety of genes involved in respiration, acid resistance, oxidative stress responses, and virulence [5]. The expression of virulence genes is known to be regulated by the iron concentration in a variety of enteric bacteria, such as *Vibrio* spp, *Pseudomonas aeruginosa*, *Yersinia* spp, *Salmonella* spp, and pathogenic *Escherichia coli* [6]. In enterohemorrhagic *E. coli* (EHEC), *stx* (Shiga-toxin) genes are directly regulated by Fur, and their expression is activated when the iron concentration decreases [7,8].

Enterohemorrhagic *E. coli* (EHEC) are human pathogens that cause a wide range of symptoms from watery diarrhea to bloody diarrhea and hemolytic uremic syndrome (HUS) [9]. Colonization of the intestinal mucosa by EHEC causes lesions known as A/E lesions, which are characterized by intimate attachment of the bacteria to the host cell surface and the disruption of the brush borders [10]. The virulence factors necessary for A/E lesion induction are primarily encoded by the chromosomal region called LEE (locus for enterocyte effacement) [11,12]. LEE also encodes the transcriptional regulator Ler, which activates the transcription of other *LEE* genes. The expression of *LEE* genes is regulated in response to changes in the environmental conditions, and some

responses are regulated at the *LEE1* promoter, which is a promoter in the *LEE1* operon, which contains the *ler* gene [13]. In EHEC, one of the regulatory proteins necessary for the activation of the *LEE1* promoter is Pch, which is encoded by *pch* genes at other chromosomal loci [14,15]. Both Ler and Pch coordinate the regulation of virulence genes, including *LEE* genes and non-*LEE* effector genes, in response to changes in environmental conditions [13], and play important roles in integrating the virulence regulon into the backbone regulatory systems in *E. coli* [16–19].

In this study, we investigated the response of *LEE* genes to changes in the iron concentration and the role of the Fur regulator in *LEE* gene expression. To elucidate the mechanism of Fur-dependent expression, we also examined how bacteria with different genetic backgrounds respond to changes in the intracellular free iron concentration. Finally, we identified a novel regulatory mechanism mediated by the transcription of the anti-sense strand.

## Results

### Repression of LEE-encoded virulence factor production by iron

EHEC infection targets the intestinal mucosa, where there is little free iron. The expression of virulence factors involved in adherence and colonization is activated during bacterial growth in intestinal environment. A low concentration of iron could be an environmental signal that affects the expression of virulence genes in EHEC. To test this hypothesis, we compared the production of LEE-encoded virulence factors between cultures grown in medium containing various concentrations of iron. The expression of *LEE* genes was elevated in EHEC grown in Dulbecco's modified Eagle medium (DMEM), which contains only 0.25 μM Fe(NO<sub>3</sub>)<sub>3</sub>, compared with that in bacteria grown in LB medium, which contains ~7.6 μM Fe [20]. Increasing the Fe(NO<sub>3</sub>)<sub>3</sub> concentration from 4 to 25 μM in DMEM did not inhibit the growth of EHEC and instead stimulated growth, resulting in a higher density in the stationary phase (Fig. 1A). The addition of iron to DMEM decreased the production of LEE-encoded proteins (Fig. 1B). The levels of EspB and Tir were reduced compared with those in bacteria grown in unmodified DMEM, which contains a relatively low amount of Fe(NO<sub>3</sub>)<sub>3</sub>. To further examine the effect of the iron concentration on LEE-encoded protein production, iron was depleted from DMEM by adding an iron-specific chelator, α,α'-dipyridyl (Dip), and the production of EspB in EHEC was examined. As shown in Fig. 1A and 1B, the depletion of iron from DMEM by adding α,α'-dipyridyl slightly increased the levels of EspB and Tir produced in EHEC. These results strongly suggest that the expression of *LEE* genes is affected by the iron content of the medium.

The production of both EspB and Tir was affected by changing the iron concentration in the medium, suggesting that the transcription of the regulatory gene in LEE, *ler*, could be affected. Ler is the first gene in the *LEE1* operon. The promoter activity of the *LEE1* operon was monitored using fusions with the *lux* operon of *Photobacterium luminescens*. The promoter activity was decreased with increasing iron concentration in the medium (Fig. 1C). When an iron chelator was added in the medium, the promoter activity was increased compared with that in bacteria grown in the unmodified medium (Fig. 1C). These results are consistent with the above results for the production of LEE-encoded proteins and suggest that the expression of all *LEE* genes is affected by the iron concentration in the medium.

### Fur is required for full expression of *LEE* genes

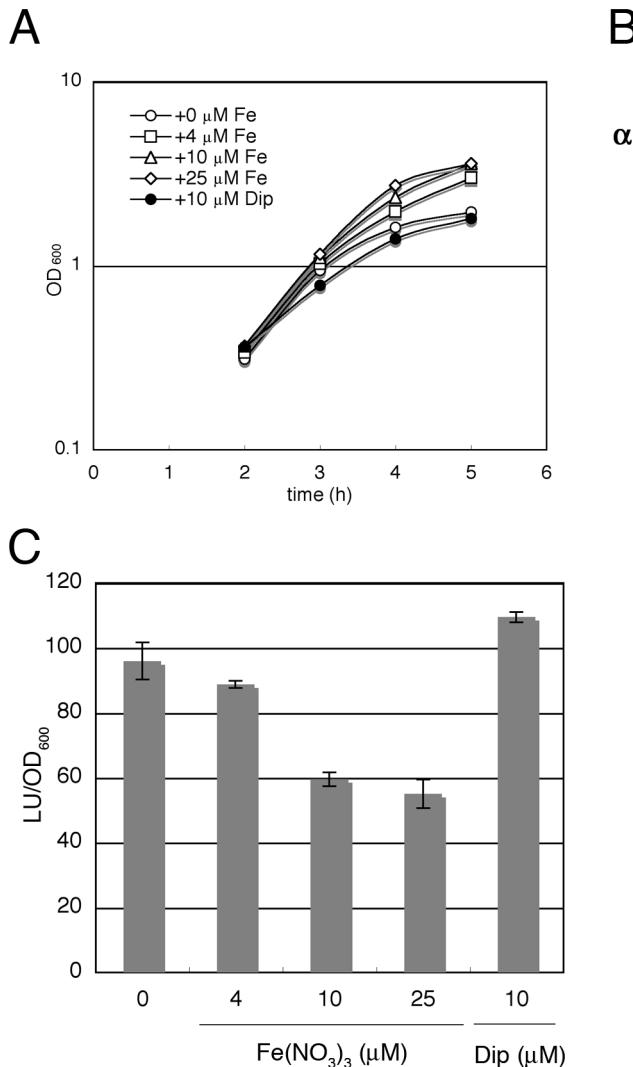
The ferric uptake regulator Fur is a key regulator of iron metabolism [21]. In the presence of iron at relatively high concentrations, Fe<sup>2+</sup>-bound Fur binds as a dimer to iron-responsive promoter regions and represses promoter activity [4]. In contrast, under low-iron conditions, Fe<sup>2+</sup>-free Fur is released from these binding sites, and gene expression is de-repressed. To examine the involvement of Fur in the iron-responsive regulation of *LEE* genes, a *fur*-deletion mutant of EHEC was created, and the production level of the LEE-encoded protein EspB was compared with that in the parental strain during growth in DMEM containing a low (0.25 μM) or high (10.25 μM) concentration of Fe(NO<sub>3</sub>)<sub>3</sub>. As shown in Fig. 2, the deletion of the *fur* gene reduced the production of EspB under both the low and high iron conditions. Even in DMEM without added iron, the level of EspB was remarkably reduced in the *fur* mutant compared with the level in the wild type. The introduction of the *fur* gene on a multi-copy number plasmid restored the production of EspB in the mutant under both conditions (Fig. 2).

Because the concentration of iron in DMEM is low, it is unlikely that Fur represses genes belonging to the Fur regulon [5]. Using GeneChip, the transcript level of each gene was compared between the wild-type and *fur* mutant EHEC. In EHEC grown in DMEM, there were no significant differences in the transcription levels of genes of the Fur regulon between the wild type and the *fur* mutant (Fig. 3A). This result indicates that the genes of the Fur regulon in wild type EHEC are de-repressed and fully expressed when the bacteria are grown in DMEM. To confirm the response of Fur-regulated promoters, the promoter activity of the *fepA* gene was measured using an operon fusion with the luciferase gene. As expected, the promoter activity in wild-type EHEC was much higher when grown in DMEM than when grown in LB, and the activity was not further enhanced in the *fur* mutant (Fig. 3B). In contrast, in LB, which contains approximately 7.6 μM iron, the transcript levels of genes of the Fur regulon were markedly increased in the *fur* mutant compared with those in the wild type. This result indicates that the Fur regulon genes are repressed by Fur, as observed in *E. coli* K-12 when grown in LB medium. The transcription of Fur regulon genes in wild-type EHEC was not fully repressed in DMEM containing an additional 10 μM of Fe (final concentration at 10.25 μM) (Fig. 3A). The *fepA* promoter activity in EHEC grown in DMEM containing an additional 10 μM of Fe was repressed by Fur but was higher than that of bacteria grown in LB (Fig. 3B). These results indicate that the addition of 10 μM Fe was not sufficient to fully repress Fur-regulated genes in EHEC grown in DMEM, which may contain components with iron-chelator activity.

In contrast, the transcription levels of *LEE* genes in the *fur* mutant were reduced compared with those in the wild type when grown in DMEM or DMEM plus 10 μM Fe (Fig. 3C). A decrease in the transcript levels in the *fur* mutant was also observed when EHEC was grown in LB (Fig. 3C). These results are consistent with the observations for EspB protein production, which was decreased in the *fur* mutant grown in both iron-poor and iron-rich medium. Therefore, it is likely that Fur can affect *LEE* gene expression even at low concentrations of iron, at which Fur is unable to repress the expression of genes of the Fur regulon, in a manner independent of its repressor activity.

### Fur regulates the expression of genes that regulate *LEE* gene expression

Because expression of *LEE* genes is regulated coordinately at the transcription step, Fur could affect the production of regulator(s) of



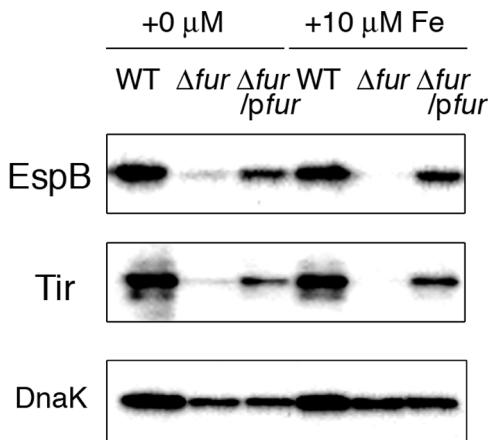
**Figure 1. Effect of a high concentration of iron on the growth and expression of LEE-encoded virulence factors.** EHEC O157 Sakai was grown in DMEM containing an additional Fe(NO<sub>3</sub>)<sub>3</sub> or  $\alpha,\alpha'$ -dipyridyl (Dip). A. Effect of various concentrations of iron in the medium on the growth of wild-type EHEC O157 Sakai. The growth of the cultures was monitored by measuring the OD<sub>600</sub>. B. Effect of various concentrations of iron on the expression levels of EspB and Tir. Proteins in whole lysates were detected by immunoblotting with specific antibodies. C. Effect of various concentrations of iron on LEE1 promoter activity. The LEE1 promoter activity in wild-type EHEC O157 Sakai in the exponential phase of growth was monitored using the P<sub>LEE1</sub>-lux operon fusion.

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LEE genes, such as Ler and Pch. To elucidate the involvement of Fur in the expression of the *ler* and *pch* genes, an EHEC strain possessing a FLAG-tagged *pchA* gene but neither *pchB* nor *pchC* was compared with the isogenic *fur* mutant. The bacteria were grown in DMEM without any additional iron, and Ler, PchA-FLAG and EspB were detected by immunoblotting using specific antibodies against each protein. As shown in Fig. 4A, the amount of EspB protein in the *fur* mutant was reduced compared with that in the parental strain. The production of both regulators, Ler and PchA, was also markedly reduced in the *fur* mutant (Fig. 4A). Because the promoter of the LEE1 operon, which includes the *ler* gene, is positively regulated by PchA, the decreased expression of the *pch* gene in the *fur* mutant could be a cause of the reduced expression of Ler and the LEE genes. To test this hypothesis, we examined the expression of LEE genes in EHEC expressing PchA constitutively from the P<sub>lac</sub>-*pchA*-FLAG gene. When PchA-FLAG was expressed in the *pch* mutant of EHEC, EspB protein was

produced at a level comparable to that in the wild type. In the *fur* mutant, the EspB expression level was much lower than that in the wild type, even though the same level of PchA protein was expressed (Fig. 4B). Furthermore, the expression of *ler* was monitored in EHEC possessing the *ler*-FLAG gene and the P<sub>lac</sub>-*pchA* gene. Although the amounts of Ler in both strains were increased by the expression of *pchA* from the *lac* promoter, the level in the wild type was much higher than that in the *fur* mutant (Fig. 4C).

Next, *ler* with the *lac* promoter (P<sub>lac</sub>-*ler*-FLAG) was introduced into the *fur* mutant, and the effect of the constitutive expression of Ler was examined. When Ler was produced in the *fur* mutant at the same level as in the wild type, the expression of EspB in the *fur* mutant reached the same level as that in the wild type (Fig. 4D). This result suggests that the *fur* mutation did not affect the activity of the Ler protein but did affect the expression of the *ler* gene. Next, we examined the promoter activity of the LEE1 operon in



**Figure 2. Expression of LEE-encoded virulence factors in the *fur* mutant.** Wild-type (WT), fur mutant ( $\Delta$ *fur*) and the complemented strain ( $\Delta$ *fur*/*pfur*) of EHEC O157 Sakai were grown in DMEM with/without 10  $\mu$ M Fe( $\text{NO}_3$ )<sub>3</sub> to the logarithmic growth phase. EspB and Tir were detected by immunoblotting.

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the *fur* mutant using the  $P_{LEE1}$ -luciferase fusion (Fig. 4D). As shown in the previous experiment, the promoter activity was reduced in DMEM containing an additional 10  $\mu$ M of Fe( $\text{NO}_3$ )<sub>3</sub> and in LB compared with the activity in unmodified DMEM, whereas, the promoter activity in the *fur* mutant was the same as that in the wild type irrespective of the iron concentration. These results clearly indicate that Fur is not necessary for the activation of the *LEE1* promoter and that the reduction in the promoter activity at high iron concentrations is not the mechanism of Fur-mediated regulation.

#### The sequence downstream of the *ler* gene is necessary for Fur-dependent expression

Because the promoter of the *LEE1* operon was not affected by Fur, post-transcriptional regulation could be the target of the Fur-dependent expression of *LEE* genes. Furthermore, when the *ler* gene with only the coding sequence was expressed from the *lac* promoter, EspB production became independent of Fur, suggesting that the sequence around the *ler* gene is necessary for Fur-dependent expression. To test the hypothesis, a plasmid carrying  $P_{lac}$ -*ler* with the downstream sequence was introduced into the *ler* mutant and *ler*/*fur* double mutant, and the expression levels of EspB were compared. In EHEC expressing Ler from *ler* with the downstream sequence, the expression of EspB was reduced by the presence of the *fur* mutation, whereas the same level of expression as that in the *fur*-positive strain was observed in the *fur* mutant when the protein was expressed from the *ler* gene without the downstream sequence (Fig. 5A and B). To further explore the role of the *ler* downstream sequence in *ler* expression, the expression levels of MBP fusion proteins from various MBP-*ler* fusion constructs were examined in the *fur* mutant. When MBP was fused with the *ler* C-terminus without the downstream sequence, the expression of the MBP-Ler fusion protein was not affected by the *fur* mutation (MBP fusion plasmid 2: MBP-Ler11). In contrast, the expression of the same MBP-Ler fusion protein from the MBP-Ler fusion construct with the additional downstream sequence was reduced by the *fur* mutation (Fig. 5C and D, plasmid 3: MBP-Ler11DWN). Next, we attempted to identify the region necessary for Fur-dependent expression. The deletion of the N-terminal 198 bp (from ATG) of the *ler* coding sequence had no effect on

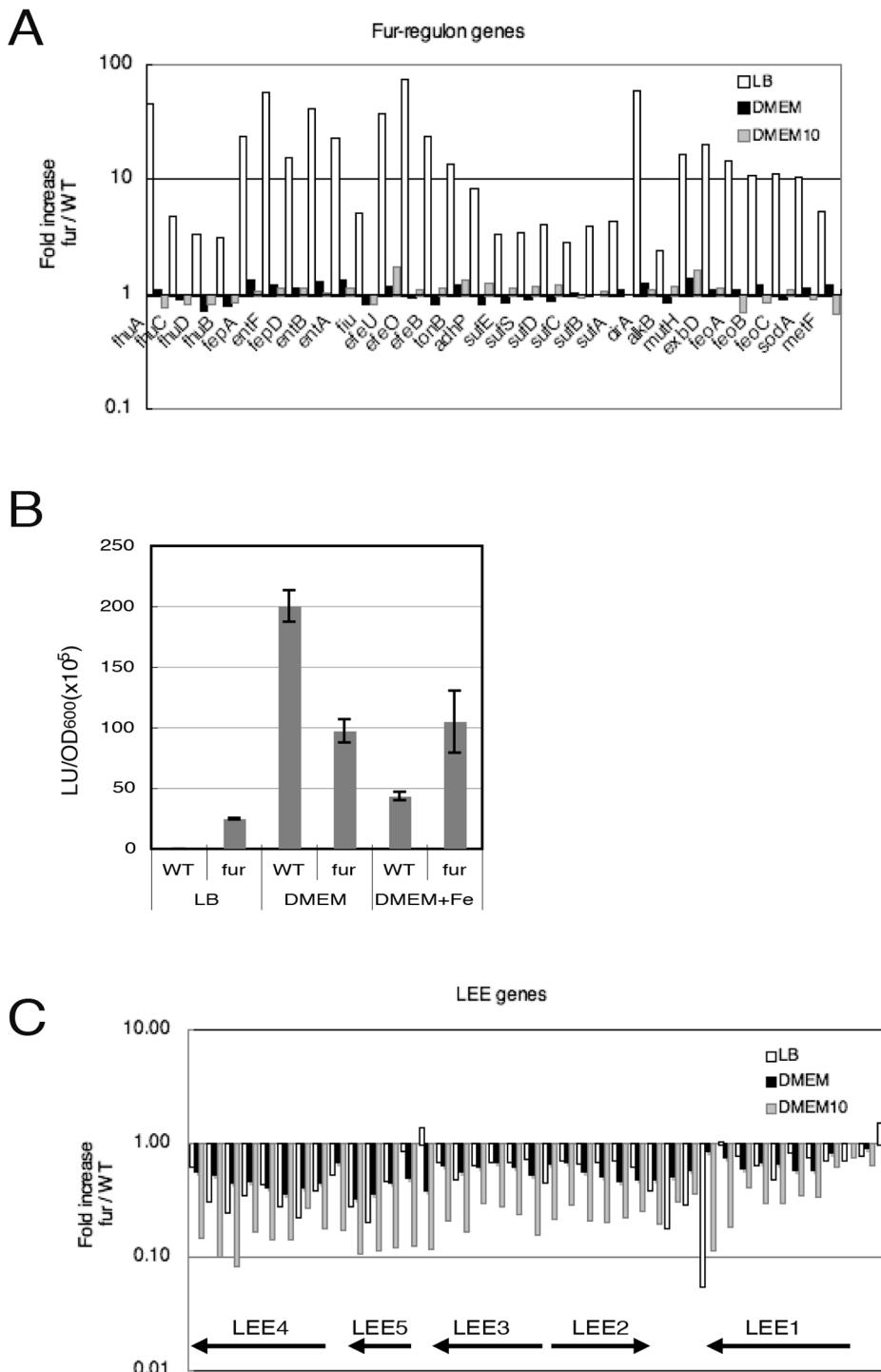
reduced expression, but the deletion of 1–288 bp of the coding sequence resulted in the expression of the MBP-Ler fusion protein in the *fur* mutant at almost the same level as in the *fur*-positive strain (Fig. 5C and D, compare plasmids 4 and 5). Furthermore, to explore the necessity of the translation of the *ler* portion of the fusion, a stop codon was placed at the junction of the MBP and *ler* coding sequences in plasmid 4. The resulting plasmid lost *fur*-dependent expression (Fig. 5D and E, plasmid 7). These results suggest that at least two sequence regions, the sequence from 198 to 288 relative to the ATG of *ler* and the 233 bp downstream of the stop codon of *ler*, are involved in the Fur-dependent expression of *ler*. Furthermore, the translation of bp 198–288 relative to the ATG of *ler* was markedly affected by the absence of the *fur* gene.

#### Post-transcriptional regulation of *ler* by antisense RNA

One possible mechanism for the regulation of the translation of *ler* is competition for translational processing by transcripts from the opposite strand. Because the downstream sequence of *ler* is necessary for the observed regulation, we assessed the promoter activity in this region. The DNA fragment spanning from +620 (from ATG of *ler* gene) to +198 was placed upstream of the luciferase operon, and the luciferase activity in wild-type and *fur* mutant EHEC O157 was quantified. Promoter activity from the opposite direction was detected. To explore role of transcription from the opposite strand in the *fur*-dependent expression of the *ler* gene, deletion derivatives of the downstream region (upstream of the opposite strand) were fused to create MBP-Ler gene fusions or luciferase operon fusions (Fig. 6, A). The promoter activity was decreased by shortening the fragment and by the deletion of 192 bp (U3) (Fig. 6, B). To confirm the transcript from opposite strand, the 5'-end specific sequence was amplified with 5' RACE method (see Materials and Methods) using a primer for the opposite strand in the *ler* coding region. A single band around 180 bp was detected from TAP (Tobacco acid pyrophosphatase)-treated RNA of wild type or *fur* mutant, but not from TAP-untreated RNA (Fig. 6, C). Therefore, the transcript from the opposite promoter is designated the *arl* RNA (antisense regulator of *ler* RNA). The production of MBP-Ler fusions from the plasmids 66DWN, U1 and U2 was reduced in the *fur* mutant, whereas MBP-Ler production from U3 was not affected by the *fur* mutation (Fig. 6, D). The promoter activities were almost the same in *fur*-positive strains and the *fur*-negative strain, indicating that the promoter activity for the *arl* RNA was not affected by the *fur* mutation. These results indicate that the transcription of the *arl* RNA on the opposite strand is necessary for the reduction in Ler production caused by the *fur* mutation.

#### Intracellular iron is involved in the repression of LEE expression

The iron ion concentration in DMEM is low, and at this concentration, Fur does not bind to its target sites and Fur regulon genes are expressed, as shown above. It is unlikely that the binding of Fur to chromosomal targets directly activates *pch* promoters or the *LEE1* promoter. Furthermore, the binding of Fur to the promoter regions of *pch* genes and the *LEE1* operon was not observed even in media containing high concentrations of iron, such as LB; such binding was also absent in DMEM (data not shown). Because a high concentration of iron in the medium decreases the expression of *LEE* genes and because the *fur* mutation causes a reduction in the production of the cytoplasmic iron-binding protein ferritin, we hypothesized that intracellular free iron ions could suppress the expression of *LEE* genes. To test this hypothesis, we expressed the iron-binding protein ferritin in EHEC and compared the production of LEE-encoded virulence

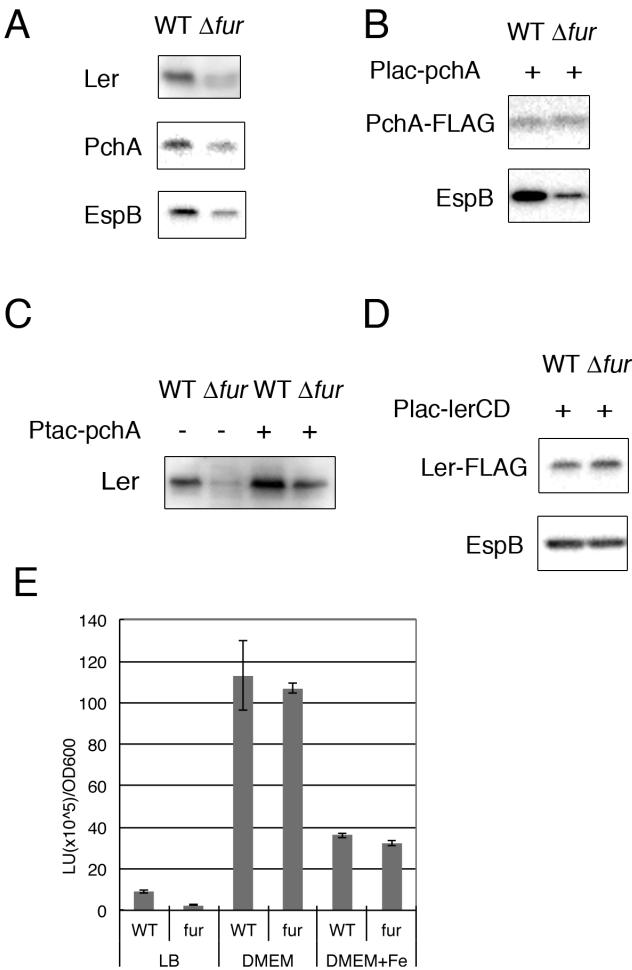


**Figure 3. Responses of Fur regulon genes and LEE genes.** Transcriptomic analysis was performed with wild-type and the *fur* mutant EHEC O157 Sakai grown in LB or DMEM with/without 10 µM Fe(NO<sub>3</sub>)<sub>3</sub>, and the ratio of the transcript level for each gene in the *fur* mutant to the transcript level in the wild type was obtained. A. Ratios of the transcript levels of genes in the Fur regulon. B. Promoter activity of the *fepA* gene in wild-type and *fur* mutant bacteria grown in LB or DMEM with/without 10 µM Fe(NO<sub>3</sub>)<sub>3</sub>. The promoter activity was measured using a luciferase-operon fusion. C. Ratios of the transcript levels of LEE genes in EHEC grown in LB or DMEM with/without 10 µM Fe(NO<sub>3</sub>)<sub>3</sub>. The ratios were obtained from the transcriptome data.

transcriptome data.  
doi:10.1371/journal.pone.0101582.g003

factors. As shown in Figure 7A, the expression of *ftnA* by the  $P_{lac}$ -*ftn* gene on a plasmid greatly increased the level of EspB protein in the *fur* mutant, whereas the levels in the wild-type bacteria were not altered by the introduction of the  $P_{lac}$ -*ftn* gene. The Fur protein

can reduce the concentration of iron ion in the cytoplasm because Fur binds iron, and thus, we examined the effect of a Fur mutant protein lacking the ability to bind iron on *LEE* gene expression. The histidine residue in the iron-binding site [22] was replaced by



**Figure 4. Expression of regulators of LEE genes in the fur mutant of EHEC.** A. Ler, PchA and EspB expression in the fur mutant. EHEC O157 Sakai pchA-FLAG or the corresponding fur mutant was grown in DMEM. Ler and EspB were detected using specific antibodies, and PchA was detected using an anti-FLAG antibody. B. The effect of constitutive expression of PchA in the fur mutant. Wild-type or fur mutant EHEC O157 Sakai harboring  $P_{lac}$ -pchA-FLAG was grown in DMEM containing IPTG (2  $\mu$ M). PchA-FLAG and EspB were detected using anti-FLAG and anti-EspB antibodies, respectively. C. Effect of PchA overexpression on ler expression in the fur mutant. The EHEC O157 Sakai ler-FLAG strain and the corresponding fur mutant harboring the  $P_{lac}$ -pchA plasmid were grown in DMEM containing IPTG (5  $\mu$ M). Ler-FLAG was detected using an anti-FLAG antibody. D. Rescue of EspB expression in the fur mutant by the  $P_{lac}$ -ler plasmid. EHEC  $\Delta$ ler and  $\Delta$ ler $\Delta$ fur mutants harboring the plasmid carrying  $P_{lac}$ -lerCD were grown in DMEM containing IPTG (5  $\mu$ M). E. Promoter activity of the LEE1 operon in the fur mutant. Wild-type or fur mutant EHEC O157 Sakai harboring pLux- $P_{LEE1}$  were grown in LB or DMEM with/without 10  $\mu$ M Fe(NO<sub>3</sub>)<sub>3</sub>. The promoter activity was measured using the luciferase activity (LU/OD<sub>600</sub>). The averages of three independent experiments are shown with the SEM.

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isoleucine. The introduction of wild-type Fur into the fur mutant rescued the production of EspB, whereas the mutant Fur (FurH89I) could not increase the level of EspB in the fur mutant (Fig. 7, B). These results suggest that the intracellular concentration of free iron is a critical regulator of LEE gene expression. To further confirm the relationship between LEE gene expression and the intracellular free iron concentration, the overexpression and deletion of the  $\gamma$ hB gene were examined. A Fur-regulated sRNA,

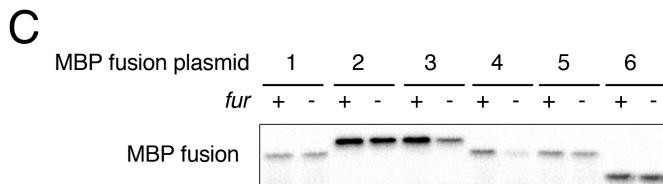
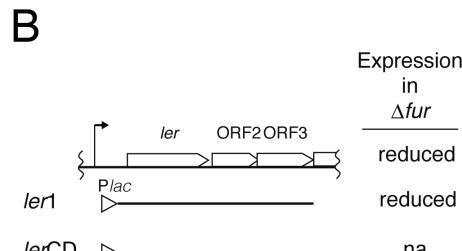
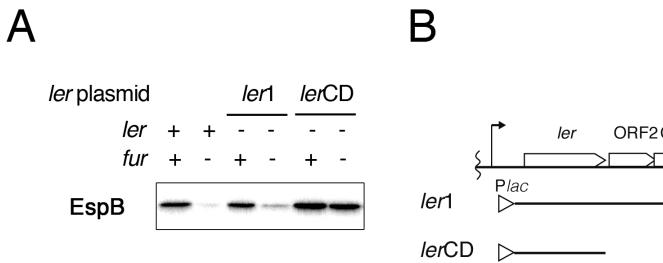
RyhB, regulates the expression of iron uptake genes and affects the intracellular iron concentration [23,24]. Although the expression levels of EspB in the of  $\gamma$ hB deletion mutant were not altered compared with those in the wild-type bacteria, the deletion of the  $\gamma$ hB gene from the fur mutant increased the expression level of EspB (Fig. 7, C). Furthermore, the production of the MBP-Ler fusion protein from Ler66DWN was examined in this same set of strains. As shown for EspB, the amount of MBP-Ler in the fur  $\gamma$ hB double KO mutant was higher than that in the fur mutant (Fig. 7, D).

Using a streptonigrin sensitivity assay, we compared the intracellular concentrations of free iron in these strains. The growth of wild-type bacteria in DMEM containing 8  $\mu$ g/ml streptonigrin was reduced to 87.9 $\pm$ 0.4% of that without streptonigrin, but the growth of the fur mutant was more sensitive (reduced to 85.4 $\pm$ 0.8%). The introduction of the  $\gamma$ hB mutation in the fur mutant rescued sensitivity, resulting in a level comparable to that in the wild type (Table 1). These results strongly suggest that the intracellular concentration of free iron is closely associated with the expression of LEE genes.

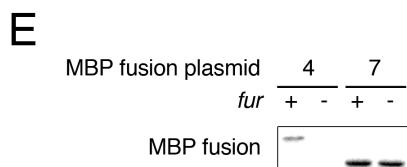
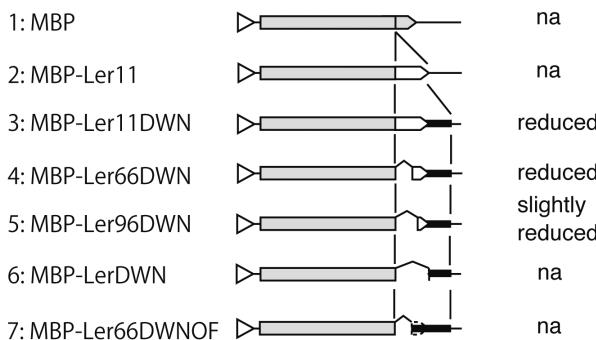
## Discussion

We found that the virulence genes of EHEC are regulated by the intracellular iron concentration. The identified regulatory mechanism targets the translation of the ler gene, which encodes the global virulence regulator Ler. Transcription of the antisense strand from a promoter downstream of ler is necessary for the bacteria to respond to changes in the intracellular concentration of free iron. Thus, we propose that the regulation of LEE and related virulence genes is closely linked to intracellular iron homeostasis.

The transcriptional regulator Fur plays a central role in bacterial iron homeostasis. The expression of genes related to iron uptake and utilization in EHEC is repressed by Fur during growth in medium containing high concentration of iron, such as LB, as shown for other bacteria. Although Fur affects the expression of LEE genes, the molecular mechanism of the Fur-regulated expression of LEE genes is different from the mechanism regulating Fur regulon genes. The expression of LEE-encoded virulence factors was greatly reduced by the deletion of the fur gene regardless of the concentration of iron in the medium. This result suggests that the mechanism of Fur-regulated LEE gene expression is not the same as that of genes belonging to the Fur regulon. The loss of the Fur regulator disrupts intracellular iron homeostasis through the deregulation of iron transport and utility systems. Previous reports have shown that the intracellular free iron concentration is elevated in fur mutants [24,25]. We examined the expression of LEE genes under a variety of conditions that affect the intracellular free iron concentration. The overexpression of *ftnA*, which encodes the cytoplasmic iron-storage protein ferritin, increased the expression of EspB in the fur mutant. The introduction of a  $\gamma$ hB mutation into the fur mutant, which has been shown to reduce the iron concentration [24], rescued espB and ler expression. In addition, a streptonigrin sensitivity assay indicated that the free iron concentration in EHEC was higher in the fur mutant and that this level was reduced to a level comparable with that in wild-type bacteria by the additional introduction of a  $\gamma$ hB mutation. These results strongly suggest that increases in the intracellular free iron concentration reduce the expression of LEE genes. Furthermore, these results suggest that Fur contributes to the expression of LEE genes through an indirect mechanism. This hypothesis was further supported by the observation that gene expression could be rescued by the introduction of the fur gene on a plasmid into the fur mutant,



Expression  
in  
 $\Delta fur$



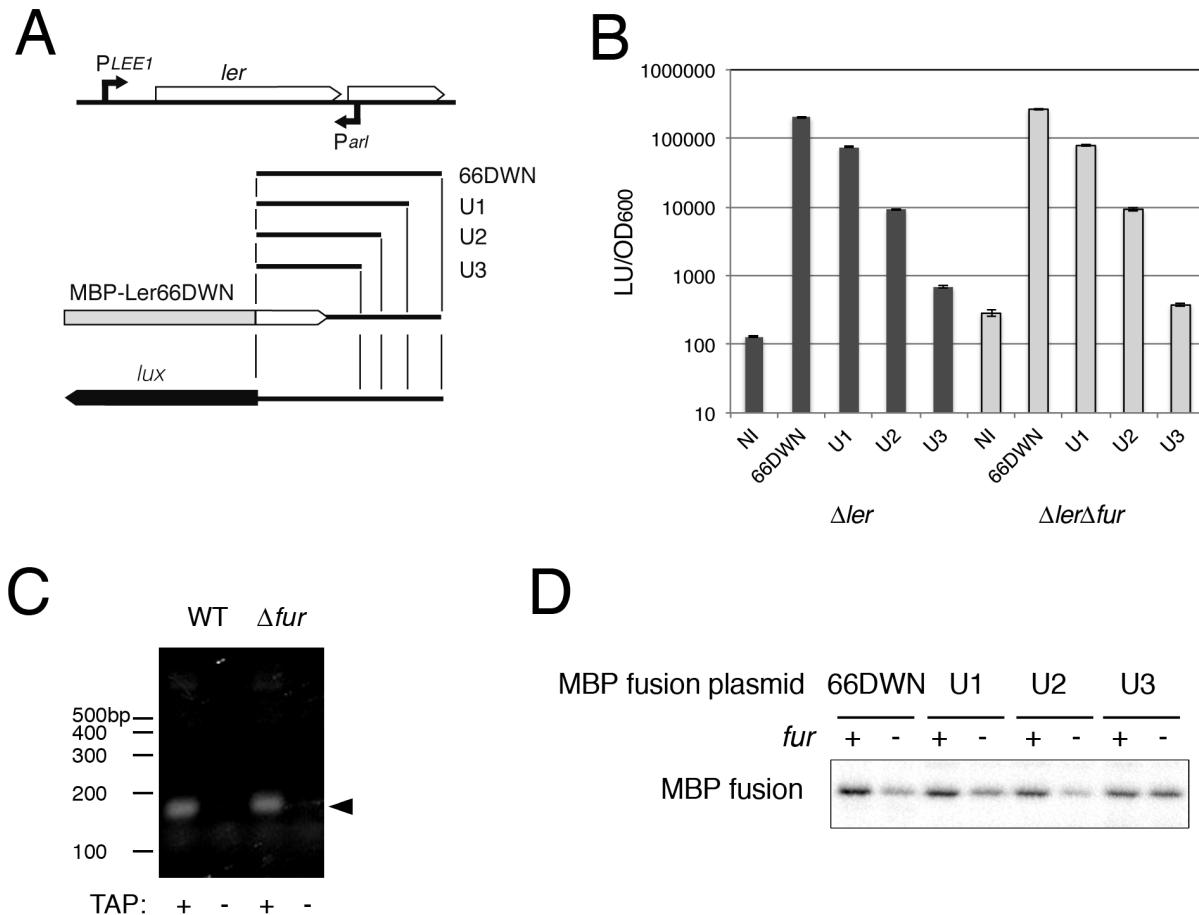
**Figure 5. Necessity of the *ler* downstream sequence for Fur-dependent expression.** A. Expression of EspB in a *fur* mutant harboring  $P_{lac}\text{-}ler$  with or without the downstream sequence.  $P_{lac}\text{-}ler$  fusion with (*ler1*) or without (*lerCD*) the downstream sequence was introduced into the EHEC Sakai  $\Delta ler$  or  $\Delta ler\Delta fur$  mutant. The expression of EspB was detected by immunoblotting using an anti-EspB antibody. B. Structure of the  $P_{lac}\text{-}ler$  fusions used in A and the EspB expression levels. C. Production of MBP-Ler fusion proteins in the *fur* mutant. Plasmids carrying a variety of MBP-Ler fusion constructs were introduced into the EHEC  $\Delta ler$  and  $\Delta ler\Delta fur$  mutants. MBP and MBP-Ler fusion proteins were detected by immunoblotting using an anti-MBP antibody. D. Structure of the MBP-Ler fusion genes used and the levels of fusion protein production. Gray boxes and white boxes represent MBP and part of the Ler protein, respectively. Thick lines indicate the downstream sequence of *ler*. Open triangles indicate the *tac* promoter. E. Production of MBP fusions with in-frame or out-of frame fusion. Plasmids carrying MBP with the *ler* downstream sequence connected in-frame or out-of frame were introduced into the EHEC  $\Delta ler$  and  $\Delta ler\Delta fur$  mutants.

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whereas a plasmid expressing a mutant Fur that is unable to bind iron could not rescue gene expression. Thus, these results indicate that the expression of *LEE* genes is closely associated with intracellular free iron homeostasis.

The expression of *LEE* genes is regulated by the transcription factor Ler, which is encoded by the *ler* gene, the first gene in the *LEE1* operon. The *LEE1* operon promoter is activated by other transcription factors, including Pch proteins, which are encoded by phage-like genomes. Our search for the target of regulation in Fur-dependent expression revealed that a post-transcription step in *ler* expression was the point of regulation rather than the promoter activity of the *LEE1* operon. Although transcriptomic analysis

showed that the transcription level of the *LEE1* operon was reduced in the *fur* mutant, the effect was not apparent for upstream genes of the operon. The transcript level of *ler* gene, which is the first gene in the *LEE1* operon, decreased only 19–34% with the introduction of the *fur* mutation. It is likely that the decrease in the transcript levels for downstream genes in the *LEE1* operon is caused by the degradation of transcripts rather than reduced transcription levels. Furthermore, the activity of the *LEE1* promoter in the *fur* mutant was almost the same as that in wild-type bacteria when measured using a luciferase fusion plasmid. These results suggest that transcription initiation from the *LEE1* promoter was not the target of the regulatory mechanism. This



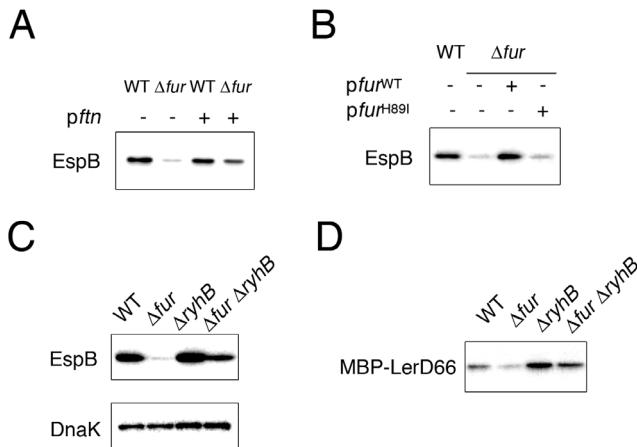
**Figure 6. Role of transcription from the antisense strand in *ler* expression.** Deletion derivatives of the MBP-*ler*66DWN fusion gene or luciferase operon fusions were created by inserting fragments with various deletions (U1, U2 and U3) (A). B. Promoter activity from the opposite strand. pLux-*ler*66DWN and deletion derivatives were introduced into the EHEC Sakai  $\Delta ler$  and  $\Delta ler\Delta fur$  mutant, and the promoter activity was measured using the luciferase activity. C. Production of arl RNA. 5' end of arl RNA was amplified by 5' RACE method. Arrowhead indicates PCR products. D. Production of MBP-*Ler*66DWN fusion proteins from the deletion derivatives. Plasmids expressing MBP-*Ler*66DWN fusions with various lengths of the downstream fragment were introduced into the  $\Delta ler$  and  $\Delta ler\Delta fur$  mutants, and the production of the MBP-*Ler*66DWN fusion was detected by immunoblotting with anti-MBP.

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hypothesis was confirmed by the observation that the expression of *ler* from the *P<sub>lac</sub>-ler1* plasmid, which contains the *ler* gene with its downstream sequence, was Fur dependent. Therefore, it is likely that interference with a post-transcriptional step of the expression of an upstream gene resulted in the degradation of the downstream transcripts of *LEE1*.

Although expression of *ler* from a DNA fragment corresponding to only the coding sequence can overcome the effect of the *fur* mutation, the *ler* gene with the downstream sequence exhibited Fur-dependent expression. By constructing variety of MBP-*Ler* fusion genes, we showed that the translation of *ler* was necessary for the reduction in the *fur* mutant. Finally, we found that the opposite strand of the downstream sequence has promoter activity using luciferase fusion plasmids. The elimination of this promoter activity by deleting part of the downstream sequence resulted in the loss of Fur-dependent expression of MBP-*Ler*, indicating that the transcription of the opposite strand of the downstream sequence of the *ler* gene is necessary for the repression of *ler* translation in the *fur* mutant. Transcriptome analysis of the transcription start site in the EHEC O157 Sakai strain revealed transcription from 26 bp downstream of the *ler* stop on the antisense strand (manuscript in preparation). Interference with the

translation of the C-terminal part of *ler* could be mediated by formation of a *ler* mRNA-antisense RNA complex, which is accelerated by a high concentration of free iron, or superoxide and hydroxyl radicals produced by free iron. Therefore, the antisense transcript, designated the arl RNA, plays a major role in the regulation of *ler* gene expression in response to changes in the intracellular iron concentration (Fig. 8). Non-coding RNAs have been identified in many bacteria, and some of them have been shown to be key regulators of gene expression [26]. Most of them are trans-encoded RNAs with partial antisense sequences complementary to target RNAs. In addition, recent comprehensive genome-wide analyses have revealed the presence of many cis-encoded antisense RNAs that are perfectly complementary to target RNAs [27–31]. The regulatory action of these RNAs can occur at the levels of transcription, mRNA stability or translation [32]. The expression of *ler* is regulated in response to disruptions of iron homeostasis through the antisense arl RNA. Our analysis showed that the translation of at least one-third of the *ler* mRNA is necessary and sufficient for the action of the antisense arl RNA. In addition, the introduction of a translational stop codon at junction of the MBP-*ler* fusion abrogated *fur*-regulated expression, and this effect was mediated by the arl RNA. Furthermore, the transcript

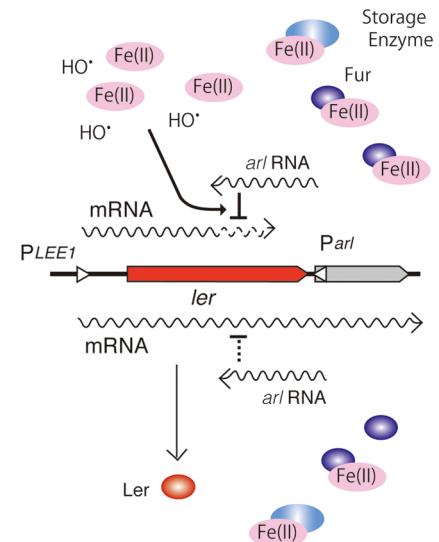


**Figure 7. Effect of iron binding proteins and *ryhB* mutation on Fur-dependent expression.** A. Effect of the overexpression of the ferritin gene. Wild-type or *fur* mutant EHEC harboring pGEM-*ftnA* was grown in DMEM. EspB was detected by immunoblotting using an anti-EspB antibody. B. Role of the iron-binding capacity of Fur. The *fur* mutant expressing wild-type Fur or a Fur (H89I) mutant was grown in DMEM. C. Effect of *ryhB* mutation on Fur-dependent expression. The EspB expression levels in wild-type, *fur* mutant, *ryhB* mutant, and *fur ryhB* mutant EHEC were monitored by immunoblotting using an anti-EspB antibody. D. Effect of *ryhB* on the expression of MBP-Ler fusion proteins. Wild-type or mutant EHEC harboring plasmid encoding MBP-LerD66DW was grown in DMEM, and the production of MBP-Ler fusion proteins was detected by immunoblotting using an anti-MBP antibody.

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level of *ler* in the *fur* mutant was lower than that in wild-type bacteria, as shown by transcriptome analysis. These results suggest that *arl* RNA affects both the stability of the RNA and the completion of *ler* translation. It is likely that the degradation of *ler* mRNA by formation of double-strand RNA with *arl* RNA in the downstream region results in the premature termination of translation. Thus, we propose a novel mechanism for *ler* expression in which the expression of this gene is regulated at the level of translation in response to the iron concentration through interference by a transcript from the antisense strand.

Sensing and response to changes in iron concentration could be important to enteric pathogens. Indeed, number of bacterial virulence factors has been shown to be regulated by the concentration of iron [6]. These virulence determinants include T3SS encoded by SPI1 of *Salmonella enterica*. Fur is involved in positive regulation of *hilA* gene, which encodes a master regulator of SPI1, through activation of *hilD*, encoding a positive regulator for *hilA* [33]. In *Salmonella*, Fur directly targets the *hilD* promoter



**Figure 8. A schematic model of the iron-sensitive regulation of *ler* gene.** Antisense RNA (*arl* RNA) is transcribed from downstream of *ler*. In the presence of high concentration of free iron in the cell, translation or stability of *ler* mRNA, transcribed from *LEE1* promoter, is reduced by the action of *arl* RNA, which is enhanced by iron or hydroxyl radical. While, in normal growth conditions, where intracellular free iron is low because iron is bound to iron-bound proteins such as storage enzymes, and Fur, inhibitory effect of *arl* RNA on *ler* expression is much reduced, resulting in the expression of *ler* and downstream genes.

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and activates its transcription [34]. While, though expression of LEE encoding T3SS is repressed by high concentration of iron and Fur affect the response, the molecular mechanism implicated in the regulation of LEE in EHEC is dissimilar to that of SPI1 in *Salmonella*. Thus, the regulation at post-transcriptional level through the action of antisense RNA could be evolved as an alternative regulatory mechanism to respond to iron concentration.

## Materials and Methods

### Bacterial strains, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. The deletion mutation for *fur* and *ryhB* were introduced into EHEC O157 Sakai using the method of Datsenko and Wanner [35]. Bacteria were grown in LB or DMEM with the addition of other reagents as described in the text. The pLux-*P<sub>LEE1</sub>* and pLux-*P<sub>fepA</sub>* plasmids were constructed by inserting a DNA fragment containing the *LEE1* promoter or the *fepA* promoter, which were isolated by PCR with primers LEE1-375-SalII and LEE1-D-NcoI and PfepA-U-Sal and PfepA-D-Bam, respectively, into pLux [36]. The pMAL-*ler* plasmid and its derivatives and the pLux-*ler*D66DW plasmid and its derivatives were constructed by inserting the DNA sequence of the *ler* gene together with the downstream sequence, which was isolated by PCR with appropriate primer set, into pMAL-c2x or pLux. The primers used for the isolation of specific DNA fragments are listed in Table 3.

### Analysis of proteins in whole-cell lysates

Bacteria were collected from cultures by centrifugation, and the cell pellet was dissolved in SDS sample buffer. The concentration of each sample was normalized to the OD<sub>600</sub> of the culture, and

**Table 1.** Streptonigrin sensitivity.

Growth percentage against no addition		
	SNG (4 µg/ml)	SNG (8 µg/ml)
WT	90.6±0.4	87.9±0.4
<i>fur</i>	89.8±0.5	85.4±0.8
<i>ryhB</i>	90.3±0.6	88.1±0.7
<i>fur ryhB</i>	92.8±1.1	89.5±0.2

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the samples were analyzed by immunoblotting after SDS-polyacrylamide (12% or 10%) gel electrophoresis (SDS-PAGE) and transfer to an Immobilon membrane (Millipore). The proteins were detected with primary antibodies specific for EspB, Tir [37], DnaK (Calbiochem) and FLAG (Sigma) and horseradish peroxidase-conjugated secondary antibodies, followed by visualization with an ECL detection kit (Amersham Biosciences).

### Promoter activity assay

Bacteria harboring promoter-luciferase fusion plasmids were grown in LB or DMEM after dilution (100-fold) of the overnight culture in LB. At the sampling time points, 800 µl was removed to measure the OD<sub>600</sub>, and 100 µl was taken to measure the luminescence intensity using a TD-20/20 luminometer (Turner Biosystems). The luciferase activity was calculated by dividing the

luminescence intensity by the OD<sub>600</sub>. The average and standard error were calculated from the results of three experiments.

### 5'RACE

Total RNA was extracted and purified from EHEC strains with RNeasy Protect Mini Kit (QIAGEN). Rapid amplification of cDNA ends (RACE) was performed with First-Choice RLM-RACE Kit (ambion) using manufacturer's manual with some modifications. 5 µg of DNase I-treated RNA was treated with TAP (Tabacco Acid Pyrophosphatase) or left untreated, and then 5' RACE Adapter was ligated. cDNA was synthesized from Adapter-attached RNA with random decamer. 5'-end of *arl* was amplified by PCR with primers (5' RACE Outer Primer and ler-N66-BamHI), and products were visualized after electrophoresis in Gel-Red containing agarose gel.

**Table 2.** Bacterial strains and plasmids used in this study.

	Description	Reference
Strain		
Sakai (RIMD 0509952)	Wild type EHEC O157:H7	[39]
SKI1748	Sakai <i>Δfur</i>	This study
SKI1163	Sakai <i>pchA-FLAG DpchB DpchC</i>	[19]
SKI1172	Sakai <i>ler-FLAG</i>	[19]
SKI0352	Sakai <i>Δler</i>	[17]
SKI1877	Sakai <i>ΔlerΔfur</i>	This study
SKI1736	Sakai <i>ΔryhB</i>	This study
SKI1801	Sakai <i>Δfur ΔryhB</i>	This study
Plasmid		
pLux	promoter-less lux operon	[36]
pLux-PLLEE1	LEE1 promoter - lux operon fusion	This study
pLux-PfepA	fepA promoter-lux operon fusion	This study
pWKS-pchA-FLAG	Plac-pchA-FLAG	[19]
pTB101-pchA	Ptac-pchA	[19]
pWKS-ler-CD	Plac-ler coding sequence	This study
pWKS-ler1	Plac-ler with downstream sequence	This study
pMAL-c2a	plasmid for MBP fusion construction	New England BioLabs
pMAL-ler11	MBP-Ler fusion without downstream sequence	This study
pMAL-ler11DWN	MBP-ler fusion with downstream sequence	This study
pMAL-ler66DWN	deletion derivative of pMAL-ler11	This study
pMAL-ler96DWN	deletion derivative of pMAL-ler11	This study
pMAL-ler96DWN	deletion derivative of pMAL-ler11	This study
pMAL-lerDWN	MBP with only downstream sequence	This study
pMAL-ler66DWNNOF	same as pMAL-ler66DWN but stop codon at junction	This study
pMAL-ler66U1	deletion derivative of pMAL-ler66DWN	This study
pMAL-ler66U2	deletion derivative of pMAL-ler66DWN	This study
pMAL-ler66U3	deletion derivative of pMAL-ler66DWN	This study
pGEM-ftnA	ftnA clone	This study
pGEM-fur	fur clone	This study
pGEM-fur(H89I)	fur(H89I) clone	This study
pLux-ler66DWN	opposite direction of ler-downstream sequence to lux operon	This study
pLux-ler66U1	deletion derivative of pLux-ler66DWN	This study
pLux-ler66U2	deletion derivative of pLux-ler66DWN	This study
pLux-ler66U3	deletion derivative of pLux-ler66DWN	This study

doi:10.1371/journal.pone.0101582.t002

**Table 3.** Primers used for construction of plasmids.

LEE1-375-Sall	CTTCGTCGACGTGCTGGCTGTAGCTTATGTC
LEE1-D-Ncol	TGAGCCATGGGCTGCGGCCTACGCCGACCAGG
PfepA-D-Bam	TGTTGGATCCACCGCGAATATACTAACATCTGCGG
PfepA-U-Sal	TGTAGTCGACAGACATCAGTACCTGCAATTG
ler-N11-BamHI	TCCTGGATCCGAAATAATTACATACAACAAGTC
ler-N66-BamHI	TCCTGGATCCGCTGATGATGGACTCGCTC
ler-N96-BamHI	GGAAGGATCCCAGCCACGCTGGCTAAAGAAG
ECs4587-D-Xhol	GAACCTCGAGCTATTATTATAATCTGATTCGCA
ler-STOP-BamHI	AGGTGGATCTAACATGAAATAATTAAATGATAACGATAAC
asL1-U1	GTGTGTCGACCGTAACCTTATCAAAGGGTG
asL1-U2	CTTGGTCGACCAATAAAAACATTGCGGCTTC
asL1-U3	GTTGGTCGACATCTCCAGCTCAGTTATCG

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### Streptonigrin sensitivity assay

The streptonigrin sensitivity of each strain was measured by comparing the growth in DMEM with or without streptonigrin, as previously described [38]. Briefly, bacteria were collected from overnight cultures in LB, washed with DMEM, and then resuspended in DMEM. DMEM containing DMF (dimethyl formamide) or streptonigrin was inoculated using a bacterial suspension (to be  $OD_{600}=0.1$ ) and incubated at 37°C with

### References

- Andrews SC, Robinson AK, Rodriguez-Quinones F (2003) Bacterial iron homeostasis. *FEMS Microbiol Rev* 27: 215–237.
- Imlay J, Linn S (1988) DNA damage and oxygen radical toxicity. *Science* 240: 1302–1309.
- Guerinot ML (1994) Microbial iron transport. *Annu Rev Microbiol* 48: 743–772.
- Hantke K (2001) Iron and metal regulation in bacteria. *Curr Opin Microbiol* 4: 172–177.
- McHugh JP, Rodriguez-Quinones F, Abdul-Tehrani H, Svistunenko DA, Poole RK, et al. (2003) Global Iron-dependent Gene Regulation in *Escherichia coli*. *Journal of Biological Chemistry* 278: 29478–29486.
- Litwin CM, Calderwood SB (1993) Role of iron in regulation of virulence genes. *Clin Microbiol Rev* 6: 137–149.
- Calderwood SB, Mekalanos JJ (1987) Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the fur locus. *Journal of Bacteriology* 169: 4759–4764.
- De Grandis S, Ginsberg J, Toone M, Climie S, Friesen J, et al. (1987) Nucleotide sequence and promoter mapping of the *Escherichia coli* Shiga-like toxin operon of bacteriophage H-19B. *Journal of Bacteriology* 169: 4313–4319.
- Kaper JB, Nataro JP, Moseley HL (2004) Pathogenic *Escherichia coli*. *Nat Rev Microbiol* 2: 123–140.
- Knutton S, Lloyd DR, McNeish AS (1987) Adhesion of enteropathogenic *Escherichia coli* to human intestinal enterocytes and cultured human intestinal mucosa. *Infect Immun* 55: 69–77.
- Elliott SJ, Wainwright LA, McDaniel TK, Jarvis KG, Deng YK, et al. (1998) The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. *Mol Microbiol* 28: 1–4.
- Perna NT, Mayhew GF, Posfai G, Elliott S, Donnenberg MS, et al. (1998) Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. *Infect Immun* 66: 3810–3817.
- Mellies JL, Barron AMS, Carmona AM (2007) Enteropathogenic and Enterohemorrhagic *Escherichia coli* Virulence Gene Regulation. *Infect Immun* 75: 4199–4210.
- Iyoda S, Watanabe H (2004) Positive effects of multiple pch genes on expression of the locus of enterocyte effacement genes and adherence of enterohaemorrhagic *Escherichia coli* O157:H7 to HEp-2 cells. *Microbiology* 150: 2357–2371.
- Porter ME, Mitchell P, Free A, Smith DGE, Gally DL (2005) The LEE1 Promoters from both Enteropathogenic and Enterohemorrhagic *Escherichia coli* Can Be Activated by PerC-Like Proteins from Either Organism. *J Bacteriol* 187: 458–472.
- Tobe T, Ando H, Ishikawa H, Abe H, Tashiro K, et al. (2005) Dual regulatory pathways integrating the RcsC-RcsD-RcsB signalling system control enterohaemorrhagic *Escherichia coli* pathogenicity. *Mol Microbiol* 58: 320–333.
- Nakanishi N, Abe H, Ogura Y, Hayashi T, Tashiro K, et al. (2006) ppGpp with DksA controls gene expression in the locus of enterocyte effacement (LEE) pathogenicity island of enterohemorrhagic *Escherichia coli* through activation of two virulence regulatory genes. *Mol Microbiol* 61: 194–205.
- Nakanishi N, Tashiro K, Kuwara S, Hayashi T, Sugimoto N, et al. (2009) Regulation of virulence by butyrate sensing in enterohemorrhagic *Escherichia coli*. *Microbiology* 155: 521–530.
- Abe H, Miyahara A, Oshima T, Tashiro K, Ogura Y, et al. (2008) Global Regulation by Horizontally Transferred Regulators Establishes the Pathogenicity of *Escherichia coli*. *DNA Res* 15: 25–38.
- Lee JY, Passalacqua KD, Hanna PC, Sherman DH (2011) Regulation of Petrobactin and Bacillibactin Biosynthesis in *Bacillus anthracis* under Iron and Oxygen Variation. *PLoS ONE* 6: e20777.
- Hantke K (1981) Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. *Mol Gen Genet* 182: 288–292.
- Ahmad R, Brandsdal BO, Michaud-Soret I, Willlassen N-P (2009) Ferric uptake regulator protein: Binding free energy calculations and per-residue free energy decomposition. *Proteins: Structure, Function, and Bioinformatics* 75: 373–386.
- Jacques J-F, Jang S, Prévost K, Desnoyers G, Desmarais M, et al. (2006) RyhB small RNA modulates the free intracellular iron pool and is essential for normal growth during iron limitation in *Escherichia coli*. *Molecular Microbiology* 62: 1181–1190.
- Salvail H, Lanther-Bourbonnais P, Sobota JM, Caza M, Benjamin J-AM, et al. (2010) A small RNA promotes siderophore production through transcriptional and metabolic remodeling. *Proceedings of the National Academy of Sciences* 107: 15223–15228.
- Keyer K, Imlay JA (1996) Superoxide accelerates DNA damage by elevating free-iron levels. *Proceedings of the National Academy of Sciences of the United States of America* 93: 13635–13640.
- Gottesman S, Storz G (2010) Bacterial Small RNA Regulators: Versatile Roles and Rapidly Evolving Variations. *Cold Spring Harbor Perspectives in Biology* 3.
- Sharma CM, Hoffmann S, Darfeuille F, Reignier J, Findeisz S, et al. (2010) The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature* 464: 250–255.
- Lasa I, Toledo-Araña A, Dobin A, Villanueva M, de los Mozos IR, et al. (2011) Genome-wide antisense transcription drives mRNA processing in bacteria. *Proceedings of the National Academy of Sciences* 108: 20172–20177.
- Koo JT, Alleyne TM, Schiano CA, Jafari N, Lathem WW (2011) Global discovery of small RNAs in *Yersinia pseudotuberculosis* identifies *Yersinia*-specific small, noncoding RNAs required for virulence. *Proc Natl Acad Sci U S A* 108: E709–E717.
- Kröger C, Dillon SC, Cameron ADS, Papenfort K, Sivasankaran SK, et al. (2012) The transcriptional landscape and small RNAs of *Salmonella enterica*

shaking for 18 h. Growth was measured by the  $OD_{600}$ , and the percentage inhibition of growth was calculated with respect to the growth of corresponding control cultures. The average percentage and standard error were determined from the results of three experiments.

### Transcriptome analysis

Total RNA from EHEC cells was isolated with an RNeasy kit (QIAGEN) using the method recommended by the manufacturer. The RNA was further purified after treatment with RNase-free DNase I (Takara). The Affymetrix GeneChip *E. coli* Genome 2.0 array was used to compare the transcriptomes of wild-type and *fur* mutant EHEC O157 Sakai. The processing of the extracted RNA, cDNA labeling, hybridization and slide scanning were performed according to manufacturer's instructions (<http://www.affymetrix.com>). Two independent experiments for each condition were performed. The data corresponding to EHEC O157 Sakai were extracted and normalized based on the total signal intensity. The ratio of the transcript levels for each gene was calculated as an average of the ratios for four combinations of two experiments for each condition.

### Author Contributions

Conceived and designed the experiments: TT. Performed the experiments: TT HY TO YK. Analyzed the data: TT HT TO. Contributed reagents/materials/analysis tools: NO TO. Contributed to the writing of the manuscript: TT.

- serovar Typhimurium. Proceedings of the National Academy of Sciences 109: E1277–E1286.
31. Soutourina OA, Monot M, Boudry P, Saujet L, Pichon C, et al. (2013) Genome-Wide Identification of Regulatory RNAs in the Human Pathogen *Clostridium difficile*. PLoS Genet 9: e1003493.
  32. Sesto N, Wurtzel O, Archambaud C, Sorek R, Cossart P (2013) The excludon: a new concept in bacterial antisense RNA-mediated gene regulation. Nat Rev Microbiol 11: 75–82.
  33. Ellermeier JR, Slauach JM (2008) Fur Regulates Expression of the *Salmonella* Pathogenicity Island 1 Type III Secretion System through HilD. J Bacteriol 190: 476–486.
  34. Teixidó L, Carrasco B, Alonso JC, Barbé J, Campoy S (2011) Fur Activates the Expression of *Salmonella enterica* Pathogenicity Island 1 by Directly Interacting with the *hilD* Operator *In Vivo* and *In Vitro*. PLoS ONE 6: e19711.
  35. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proceedings of the National Academy of Sciences 97: 6640–6645.
  36. Burton NA, Johnson MD, Antczak P, Robinson A, Lund PA (2010) Novel aspects of the acid response network of *E. coli* K-12 are revealed by a study of transcriptional dynamics. J Mol Biol 401: 726–742.
  37. Abe H, Tatsuno I, Tobe T, Okutani A, Sasakawa C (2002) Bicarbonate ion stimulates the expression of locus of enterocyte effacement-encoded genes in enterohemorrhagic *Escherichia coli* O157:H7. Infect Immun 70: 3500–3509.
  38. Nachin L, El Hassouni M, Loiseau L, Expert D, Baras F (2001) SoxR-dependent response to oxidative stress and virulence of *Erwinia chrysanthemi*: the key role of SulC, an orphan ABC ATPase. Mol Microbiol 39: 960–972.
  39. Hayashi T, Makino K, Ohnishi M, Kurokawa K, Ishii K, et al. (2001) Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. DNA Res 8: 11–22.