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Regulation of *metE*$^{+}$ mRNA expression by FnrS small RNA in *Salmonella enterica* serovar Typhimurium

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ABSTRACT

Methionine is critical for variety of metabolic processes in biological organisms, acting as a precursor or intermediate for many final products. The last step for the synthesis of methionine is the methylation of homocysteine, which is catalyzed by MetE. Here, we use *Salmonella enterica* serovar Typhimurium LT2 to study the regulation of the *metE*$^{+}$ gene by an anaerobically induced small non-coding RNA-FnrS, the expression of which is strictly dependent on the anaerobic regulator-FNR. The MetE-HA protein was expressed at an increased level in the *fnrS*$^{-}$ and *hfq*$^{-}$ deficient strains under anaerobic conditions. The Hfq protein is predicted to stabilize the binding between small RNA(s) and their target mRNA(s). A transcriptional (op) and translational (pr) *metE::lacZ* fusion gene were separately constructed, with the *metE*$^{+}$-promoter fused to a *lacZ* reporter gene. In an anaerobic environment, the *metE*::*lacZ* (pr) fusion gene and reverse transcription-PCR identified that FnrS and/or FNR negatively regulate *metE*$^{+}$ mRNA levels in the rich media. Analysis of FnrS revealed a sequence complementary to the 5′ mRNA translational initiation region (TIR) of the *metE*$^{+}$ gene. Mutation(s) predicted to disrupt base pairing between FnrS and *met*E^{+} TIR were constructed in *fnr*S, and most of those resulted in the loss of repressive activity. When compensatory mutation(s) were made in *metE*$^{+}$ 5′ TIR to restore base pairing with FnrS, the repressive regulation was completely restored. Therefore, in this study, we identified that in anaerobic phase, there is a repression of *metE*$^{+}$ gene expression by FnrS and that base-paring, between both expressive transcripts, plays an important role for this negative regulation.

1. Introduction

Methionine is important in central metabolism; it is essential to initiation and elongation of translation, and its derivative *S*-adenosylmethionine (SAM) serves as a universal methyl donor (Hondorp and Matthews, 2009). Additionally, methionine is an intermediate compound involved in many biosynthetic pathways. The last step in the formation of methionine involves the methylation of homocysteine. The methyl group is transferred from 5-methyltetrahydrofolate to homocysteine by one of two transmethylase enzymes (Hondorp and Matthews, 2009). This reaction is catalyzed either by a vitamin B_{12}-dependent transmethylase, the *metH*$^{+}$ gene product, or by a vitamin B_{12}-independent transmethylase, the *metE*$^{+}$ gene product. However, in the absence of exogenously supplied vitamin B_{12} (cobalamin), MetE will be the only transmethylase of methionine biosynthesis, making *metE*$^{+}$ an essential gene. Therefore *metE*$^{+}$ is an essential gene for methionine biosynthesis (Hondorp and Matthews, 2009).

MetE was often discussed in relation to oxidative stress, caused by the oxidation of the cysteine 645 of MetE (Hondorp and Matthews, 2009); furthermore, when the bacteria transition from anaerobic to aerobic conditions, the MetE protein becomes one of the abundant proteins in the cell (Cheeseman et al., 1983; Gyaneshwar et al., 2005). The MetE gene is regulated through the methionine levels of the cell (Partridge et al., 2006). In *E. coli*, it was noted that *metE*$^{+}$ is regulated by the aerobic regulated protein-FNR under anaerobic conditions. However, since there is no FNR-binding site in *metE*$^{+}$ 5′ upstream region, at that time, the real mechanisms of regulation was unclear (Constantinidou et al., 2006).

Recently, non-coding RNAs have emerged as important components of regulatory circuits. A major class consists of trans-encoded gene regulatory RNAs that act by an antisense mechanism to activate or repress translation of target mRNAs. Many of these sRNAs are made in response to changes in environmental conditions. All the trans-acting base-pairing RNAs characterized thus far in *E. coli* and *Salmonella*

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associate with and require the RNA chaperone Hfq, which acts to stabilize most of the base-pairing RNAs (Durand and Storz, 2010; De Lay et al., 2013). Some well-studied regulatory examples of sRNAs that bind Hfq and regulate target mRNAs by base pairing include sRNA MicC with its target mRNA *ompC*$^+$ (Rothfork et al., 2004), sRNA SgrS with its target *ptsG*$^+$(Kawamoto et al., 2006), and other sRNA examples include DsrA, RprA, RyhB, DicF, OxyS and GcvB (Gottesman, 2002,2004; Storz et al., 2004; Pulvermacher et al., 2009). It was reported before that trans-acting small RNAs, which bind to the 5′-UTR of the mRNA target could have two different functions (Gottesman and Storz, 2011). One is hindering the ribosome binding site, resulting in the repression of translation. Another is preventing the formation of an inhibitory secondary structure of the mRNA itself such that activation of translation could occur. Repression of translation could be leading to destabilization of the corresponding mRNA. Activation of translation, however, could be leading to stabilization of the individual mRNA. Also, sRNA binding might directly affect mRNA levels *via*either directly cutting by RNase III or RNase E or masking the intrinsic RNase cleavage site for the stable mRNA transcripts (Storz et al., 2011; De Lay et al., 2013; Papenfort and Vanderpool, 2015).

FnrS was identified as an anaerobically induced small non-coding RNA in *Escherichia coli* (Boysen et al., 2010). Its expression is strictly dependent on the anaerobic transcriptional fumarate and nitrate reductase regulator (FNR) (Boysen et al., 2010; Durand and Storz, 2010). Global proteomic and transcriptomic profiling have shown that the expression of multiple genes can be negatively regulated by FnrS (Boysen et al., 2010; Durand and Storz, 2010). Accordingly, it was demonstrated that FNR negatively regulates genes such as *sodA*$^+$, *sodB*$^+$, *cycD*$^+$*C*$^+$ and *metE*$^+$ and thereby FnrS functions to adjust gene expression for adaptation to anaerobic growth (Boysen et al., 2010; Durand and Storz, 2010). Recently, it has been reported that FnrS sRNA regulates its target genes by post-transcriptional regulation in *Neisseria gonorrhoeae* (Tanwer et al., 2017). Also, the secondary structure of FnrS has been predicted and mapped *in vivo* by DMS (dimethylsulfate) probing (Durand and Storz, 2010). Our earlier study demonstrated that FnrS can regulate *metE*$^+$ gene expression *via*adjusting mRNA levels during anaerobic growth of *Salmonella* Typhimurium (Chien et al., 2015). However, there is no genetic evidence to indicate that *metE*$^+$ is down-regulated by FnrS *via*base pairing between 5′ mRNA of *metE*$^+$ and FnrS.

In this work, we focused on the regulation of *Salmonella* Typhimurium *metE*$^+$ gene expression by a highly conserved, anaerobically induced FnrS (Fig. 1A and 1B) and demonstrated that the down-regulation of the *metE*$^+$ gene expression was mediated by base pairing between FnrS and the 5′ upstream TIR of *metE*$^+$ mRNA.

2. Materials and methods

2.1. Bacterial strains, plasmids and phages

The *E. coli*, *S. typhimurium*, plasmids and phages used in this study are listed in Table 1. *Salmonella* Typhimurium LT2 as the wild type strain was used for cloning the *metE*$^+$ and *fnrS*$^+$ genes. *E.coli* MC4100 and DJ480 as well as their derivatives were used as host strains. All the primers used in PCR-cloning were listed in supplementary data. Plasmids, used in this study, included pBAD33 (Guzman et al., 1995) for *metE*$^+$ gene expression. Plasmid pRS414 and pRS415 (Simons et al., 1987) respectively are for protein fusion and operon fusion. The pBR-P_{Lac} plasmid (Guiller et al., 2006) is used for expressions of FnrS and its derivatives. For pBAD33-*metE*$^+$, in a PCR, the forward primer with *Kpn*I site covering 182 bps upstream of translational start site, and the reverse primer containing the stop codon with *Hin*dIII site and the whole length 2265 bps of *metE*$^+$ gene were cloning into pBAD33. For pBAD33-*metE*$^+$*-ha*, of which *metE*$^+$ C-termini carries HA tag, the similar forward primer as the above and the *Hin*dIII HA-reverse primer were used for cloning into pBAD33 *Kpn*I-*Hin*dIII site. A λ*metE::lacZ* transcriptional (op) or translational (pr) fusion was constructed by synthesis of DNA fragments with *Eco*RI sites at bp-254 upstream of translational start site and with *Bam*HI site at bp-38 downstream of translational start site. Following digestion of *Eco*RI and *Bam*HI, the DNA fragments were gel-purified and ligated into *Eco*RI and *Bam*HI sites of plasmids pRS414 (protein fusion, pr) or pRS415 (operon fusion, op). In a transcriptional fusion (operon fusion), the upstream *metE*$^+$ gene encoding region and downstream *lacZ*$^+$ reporter gene are translated from their own TIR. In a translational fusion (protein fusion), a fusion protein is translated from the TIR upstream of *metE*$^+$ to make a fusion protein containing *lacZ*$^+$ reporter gene in-frame fused to the remaining part of the upstream *metE*$^+$. These two plasmids carrying *metE*::*lacZ* fusions were then crossed over with λRS45 phage. The two plasmids were transformed into XL-1 blue, and the resultant transformants (Ap^r) were selected on ampicillin plates. The transfomant cells were then infected with the lambda derivative λRS45 and selected for blue plaques on X-gal plates. These resultant phages contained the *metE::lacZ* transcriptional (op) or translational (pr) fusion genes transferred into λRS45 phage. Each phage lysate was used to infect various strains of *E. coli* and the resultant single phage lysogen was selected. A single phage lysogen was identified by measurement of β-galactosidase levels of five different lysogens, and those with the lowest expression value were selected as the single lysogen. One-step PCR method was used to generate pBR-P_{LAC}-*fnrS*$^+$. Basically, the forward primer (LT2 *fnrS* Fw) with *Aat*II site and *fnr*S Rv primer with *Eco*RI site were used to amplify the *S.* Typhimurium chromosomal DNA covering the *fnr*S$^+$ entire length of 118 bps and the resulting DNA fragment was cloned into pBR-P_{LAC} *Aat*II/*Eco*RI sites. The pBR- P_{LAC}-*fnr*SΔloop3 and pBR- P_{LAC} -*fnr*SΔloop2, 3, were constructed by using the LT2*fnrS* Fw primer and the target site reverse primers. LT2 *fnrS*Δloop3 and LT2 *fnrS*Δloop2, 3, were separately PCR-amplified and the resulting PCR fragments in series cloned into pBR-P_{LAC} *Aat*II/*Eco*RI sites. The correct insert DNA fragment was confirmed by DNA-sequencing.

2.2. Mutagenesis in *fnrS*$^+$ and *metE*$^+$ upstream TIR region

Using plasmids pRS414-*metE*$^+$ upstream nucleotides and pBR-P_{LAC}-*fnrS*$^+$ as the template, separately; the two-step PCR mutagenesis methods were used to create nucleotide changes in the *metE*$^+$ mRNA upstream region and *fnrS*$^+$ gene. Two oligonucleotides of nearly 20 ~ 40 bases complementary to each other and carrying the mutation (s) in the middle of oligonucleotide, were used in PCR reactions with the primers to clone the wild-type fragment. For *fnrS* mutants, I(U57A/U58 G/U59A), II(C47A/U48A/U49 G), III(G4C/G5 T), 41(U41 G), 43(U43 G) and 54(C54 G), the final PCR products were cloned into pBR-P_{LAC} *Aat*II and *Eco*RI sites. For *metE-2* (A-2 T) (A-3C) and *metE54* (G-8C), the final PCR products were again cloned into pRS414 and pRS415. After that, the resultant plasmids were infected by λRS45 and the similar procedures as described above were used to select the single phage lysogens for both construction.

2.3. Growth conditions

The bacteria were grown in Luria-Bertani (LB) medium or M63 minimal medium (with 0.4% glucose as the carbon source.) at 37 °C. When needed, ampicillin (100 μg/ml), kanamycin (30 μg/ml) and chloramphenicol (17 μg/ml) were added. Supplements were added as the following final concentrations when needed: 80 μg/ml L-Methionine, 1 μg/ml B_1. To induce *metE*$^+$*-ha* gene expression from pBAD33-*metE*$^+$*-ha*, arabinose was added to the final concentration of 0.02%. For anaerobic experiments, cells were first grown aerobically until OD_{600} near 0.4, the bacterial cells were collected by centrifugation and the pellets were re-suspended in the fresh medium as well as then the mixtures were incubated anaerobically with different time intervals. When anaerobically grown cells were shifted back to aerobic conditions, the cells were firstly collected by centrifugation, treated with the

Fig. 1. The upstream sequences of the *Salmonella* Typhimurium LT2 *metE*$^+$ gene and the FnrS sequences and its predicted secondary structure, using Mfold (http::/unafold.rna.albany.edu). (A) The -35, -10 promoter and the SD sequence of the *metE*$^+$ are marked by a filled line. The +1 is marked as the transcriptional start site and Met is the initial amino acid of the MetE protein. The *metE::lacZ* fusion plasmid includes that the *metE*$^+$ promoter region is fused with the *lacZ* reporter gene by compatible *Eco*RI-*Bam*HI sites. The black spots under the *metE*$^+$ upstream sequences are the putative complementary sequences for FnrS binding. (B) The nucleotide from 5′ end till position 46 is a stem-loop 1, the nucleotide 62 to 87 is a stem-loop 2, the nucleotide 95 to 114 is a stem-loop 3. The black dots indicate that the nucleotides of FnrS likely base-paired with the sequences of the *metE*$^+$ 5′ mRNA.

fresh medium and regrown yet again under aerobic conditions for 30 min. For FnrS repression assays, the series of *fnrS*$^+$ and its derivative mutants were induced from the pBR-P_{LAC} plasmid by the addition of IPTG, with the final concentration 1 mM, for 30 min.

2.4. Construction of the λmetE::lacZ fusion gene into different genetic background

The 5′-UTR of *Salmonella* Typhimurium *metE*$^+$ wild-type and the mutants were cloned into pRS414 and pRS415 to form *lacZ* protein fusion (pr) and operon fusion (op), separately. After transferring the *metE*::*lacZ* fusion gene into the λRS45, the resulting phages, two types of λ*metE*::*lacZ* phages, (pr) fusion or (op) fusion, were separately lysogenized into a *lacZ*-deficient strain (DJ480 or DJ480Δ*met*E). The newly formed lysogens were isolated. The β-galactosidase activity was then measured for selection of the each single phage lysogen.

2.5. P1 transduction and other general methods

The mutant strain was constructed by introducing antibiotic cassette to replace wild-type target gene using P1 transduction, and then eliminated the antibiotic cassette by pCP20 plasmid (Cherepanov and Wackernagel, 1995). For plasmid constructions, *E.coli* XL-1 blue competent cells were used. Plasmid purification was performed according to the instructions of manufacturers (Qiagene or Viogene).

2.6. β-galactosidase assays

All strains were grown until log phase and the cells were lysed in 800 μl of Z-buffer with 30 μl of 0.1% SDS and 40 μl of chloroform. All of the β-galactosidase activity was determined in triplicate for each assay. The activity was calculated by Miller units (Miller, 1992).

Table 1
Bacteria, plasmids and phages used in this study.

Strains	Description	Source or reference
Salmonella enterica serovar strain Typhimurium LT2	Wild-type	ATCC 700720
Escherichia coli		
MC4100	F-*ara*D139D(*argF-lac*)U169 *rpsL*150 *relA*1 *flhD*5301 *deoC*1 *fruA*25 *rbsR22*	
C600	F-*e14*$^-$ *mcrA*1 *thr-1 thiE*-1 *thr*-1 *leuB*6 *lacY*1 *fhuA*21 *glnX*44 *rfbD*1	
XL1-blue	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi relA*1 *lac*$^-$ [F' *proAB*$^+$ *lacI*^{q} *lacZ* △M15 Tn10 (*ter*^{R})]	
BW25113	△*lacZ*4787(::*rrnB-3*) *hsdR*514 D(*araD-araB*)567D(*rhaD-rhaB*)568 *rph*-1	Barry L. Wanner
JW3805	BW25113 D*metE*::kan	(Baba et al., 2006)
JW1328	BW25113 D*fnr*::kan	(Baba et al., 2006)
JW4130	BW25113 D*hfq*::kan	(Baba et al., 2006)
DJ480	D*lacX*74 (lambda-based *lacZ* fusions)	Ding Jin (NCI)
YF 10001	MC4100 D*metE*	This study
YF 10002	MC4100 D*metE* D*hfq*::FTR	This study
YF 10003	MC4100 D*metE* D*fnrS*::FTR	This study
CW10002	DJ480 λ*metE*::*lacZ* (pr)	This study
CW10003	DJ480 D*fnrS*::kan λ*metE*::*lacZ* (pr)	This study
CW10004	DJ480 λ*metE*::*lacZ* (op)	This study
CW10005	DJ480 D*fnrS*::kan λ*metE*::*lacZ* (op)	This study
CW10006	DJ480 D*metE*::kan λ*metE*::*lacZ* (pr)	This study
CW10007	DJ480 D*metE*::FRT D*fnrS*::kan λ*metE*::*lacZ* (pr)	This study
CW10008	DJ480 D*metE*::FRTD*fnr*::kan λ*metE*::*lacZ* (pr)	This study
CW10009	DJ480D*metE*::FRT D*fnrS*::FRT D*fnr*::kan λ*metE*::*lacZ* (pr)	This study
CW10014	DJ480 D*fnrS*::kan λ*metE*2::*lacZ* (op)	This study
CW10015	DJ480 D*fnrS*::kan λ*metE*54::*lacZ* (op)	This study
Plasmids		
pBAD33	*ori* (pACYC18) *araC* P_{BAD} *cm*^{r}	(Guzman et al., 1995)
pBR-P_{LAC}	*ori* (pMB1) P_{LAC} promoter based expression vector *amp*^{r}	(Guillier et al., 2006)
pRS414	*bla*-Tl$_4$ *Eco*R I-*Sma* I-*Bam*H I *lacZ* (pr)	(Simons et al., 1987)
pRS415	*bla*-Tl$_4$ *Eco*R I-*Sma* I-*Bam*H I *lacZ* (op)	(Simons et al., 1987)
pCP20	*FLP*$^+$ l*cI*857$^+$ lP_R Rep^{ts} *amp*^{r} *cm*^{r}	(Cherepanov and Wackernagel, 1995)
pYF11	pBAD33-184bp 5'-UTR-*metE* $^+$-*ha*	This study
pYF16	pRS414-P_{metE}::*lacZ* fusion gene	This study
pYF17	pRS415-P_{metE}::*lacZ* fusion gene	This study
pYF18	pBR-P_{LAC}-*fnrS*$^+$	This study
pYF19	pBR-P_{LAC}-*fnrS* Dloop 2	This study
pYF20	pBR-P_{LAC}-*fnrS* Dloop 2 ~ 3	This study
pCW3	pBR-P_{LAC}-*fnrS* I (U57A/U58G/U59A)	This study
pCW4	pBR-P_{LAC}-*fnrS* II (C47A/U48A/U49G)	This study
pCW5	pBR-P_{LAC}-*fnrS* III (G4C/G5T)	This study
pCW6	pBR-P_{LAC}-*fnrS* 41 (U41G)	This study
pCW7	pBR-P_{LAC}-*fnrS* 43 (U43G)	This study
pCW8	pBR-P_{LAC}-*fnrS* 54 (C54G)	This study
Phages		
lRS45	imm21 *ind*$^+$ *bla*'-*lacZ*$_{SC}$	(Simons et al., 1987)
P1*via*		
l*metE*::*lacZ* (op)	carrying *metE*$^+$ promoter with *lacZ*$^+$	This study
l*metE*::*lacZ* (pr)	carrying *metE*$^+$ promoter with '*lacZ*	This study
l*metE*-2::*lacZ* (op)	(A-2T)(A-3C) relative to (+1) ATG	This study
l*metE*54::*lacZ*(op)	(G-8C) relative to (+1) ATG	This study

2.7. Western blot assays

Gel electrophoresis was performed by 8% SDS-PAGE. For validating sample loadings in Western blot assays, the equal amount of samples, normalized by OD_{600}, were loaded and electrophoresed by 8% SDS-PAGE as performed and stated before (Chang et al., 2016, 2019). After electrophoresis, the proteins were electro-blotted onto nitrocellulose membranes. Then the anti-HA as the primary antibody and goat anti-mouse HRP as the secondary antibody, were used to detect the MetE-HA expression levels. The MetE-HA expression levels were analyzed by Image J (version 1.52a) from the National Institutes of Health (NIH) (Rasband, 2011). The methods used in Image J as described previously (Hsieh et al., 2011). The measurement of each sample was performed. The amounts of MetE-HA in Western blot assays were calculated using the sample taken in the control as 100%, and the relative levels of MetE-HA in different strains were determined.

2.8. RNA isolation, RT-PCR and qRT-PCR experiments

The bacterial cells were grown to $OD_{600} = 0.4 \sim 0.8$ under indicated conditions. At each time point, total RNA in each bacteria was extracted by RNeasy Mini kits (Qiagene). The residual DNA was removed by an additional clean-up, using DNase I according to the manufacturer's instruction. A Nano-Drop 1000 instrument (Thermo Fisher Scientific) was used for measuring the concentration of RNA. For detection of the levels of *metE::lacZ* mRNA in various bacterial strains, an equal amount of each extracted RNA (4 μg) was subjected to RT-PCR via the OneStep RT-PCR kit (Qiagene) with the specific primers and the *rrsA* (16S rRNA) gene was also reverse-transcribed under similar conditions, as an internal control.

The quantitative real-time PCR was performed using the real-time PCR machine of ABI (StepOne Plus™). The SYBER-Green master mix (ABI) and specific primers for *metE*::*lacZ* mRNA gene expression were used according to the manufacturer's instructions. All the reactions were performed in triplicate. The *rrsA* rRNA was used as the endogenous reference control and the relative gene expression was determined by the $2^{-\Delta\Delta C}$T relative quantification methods (Livak and Schmittgen, 2001).

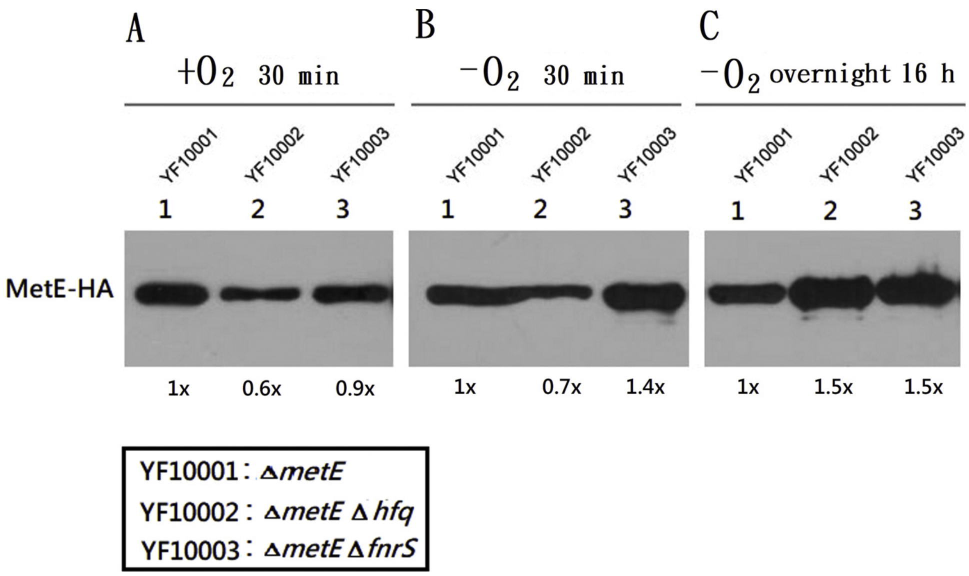

Fig. 2. Effects of FnrS and Hfq on MetE-HA expression levels. The wild-type (YF10001), the *hfq* mutant strain (YF10002), and *fnrS* mutant strain (YF10003) were transformed with pBAD33-*metE*$^+$-*ha* (pYF11). All the above three strains were devoid of the chromosomal *metE*$^+$ gene. The resultant transformants were separately cultured in LB media plus 0.02% L-arabinose and chloramphenicol (17 μg/ml) at 37°C to the OD_{600} = 0.5 and then shifted the cultures under (A) aerobic, (B) anaerobic, both for 30 min and (C) anaerobic overnight (16 h). The Western blot analyses were used for the detection of the MetE-HA protein in all various strains.

3. Results

3.1. MetE expression levels in the wild-type, hfq- and fnrS- deficient strains under aerobic and anaerobic conditions

To measure the MetE expression under aerobic or anaerobic conditions from the wild-type, *fnrS*- and *hfq*- deficient strains, the pBAD33-5′-UTR-*metE*$^+$-*ha* was transformed into the wild-type (YF10001), *hfq*- (YF10002) and *fnrS*-(YF10003)- deficient strains, separately. Notably, all the above bacterial strains were devoid of *metE*$^+$ gene in their chromosomes. We then measured MetE protein expression through Western blotting after induction by 0.02% arabinose from the above transformant strains. All the logarithmic bacterial cells were first shifted from anaerobically to aerobically about 30 min. The results showed that the similar expressional levels of MetE-HA were observed among all strains under the aerobic condition (Fig. 2A, the left panel). Yet, when all the logarithmic bacterial cells were shifted from aerobic to anaerobic conditions at about 30min, MetE-HA expression level in the *fnrS*- mutant was about 1.4-fold higher than that in the wild-type (Fig. 2B). Moreover, MetE-HA expression levels of the *hfq*- and *fnrS*- deficient strains are significantly about 1.5-fold higher as compared to those of the wild type strain for a longer anaerobic state (16h) (Fig. 2C, the right panel).

3.2. Down-regulation of metE::lacZ (pr) gene expression by FnrS and/or FNR under anaerobic condition and a RT-PCR analysis as well as a real-time quantitative PCR analysis for the gene expression

The transcriptional (operon fusion, op) and translational (protein fusion, pr) fusion genes of *metE*::*lac*Z were constructed separately to monitor the regulation of *Salmonella* Typhimurium *metE*$^+$ gene by fusion with a reporter gene, *lacZ*$^+$. In each one, the *metE*$^+$ promoter is in front of the *lacZ*$^+$. After transferring the *metE::lacZ* fusion genes into the λRS45, the resulting phages λ*metE::lacZ* (op) and λ*metE::lacZ* (pr) were separately lysogenized into DJ480 and DJ480Δ*met*E. The newly single phage lysogens were each isolated, and the β-galactosidase activity was measured for each single phage lysogen in LB media. The two different types of the fusions, λ*metE::lacZ* (op) and λ*metE::lacZ* (pr), both had significant expression levels (Fig. 3A and the later results). To further identify that *fnrS*$^+$ has effects on *metE::lacZ* gene expression, the wild-type strain DJ480Δ*met*E λ*metE::lacZ* (pr), CW10006, and its derivative *fnrS*-deficient strain (CW10007), as well as other mutant strains, Δ*fnr* (CW10008) and Δ*fnrS*Δ*fnr* (CW10009),were each grown in LB medium to OD_{600} near 0.4 under aerobic conditions. Each logarithmic bacterial cells were collected and re-suspended in fresh media and were incubated anaerobically for 180 min, separately. The *metE::lacZ* (pr) gene expression increased over time in the *fnrS*-deficient strain upon a shift of the bacterial cells under anaerobic conditions (Fig. 3A). In contrast, the expression of the wild-type λ*metE::lacZ* (pr) strain was relatively repressed under anaerobic conditions (Fig. 3A). Accordingly, *metE*$^+$ gene expression was down regulated by FNR. In *E. coli,* it was shown that *fnrS*$^+$ gene was positively regulated by the regulation of FNR under anaerobic conditions. Therefore, we examined that the function of the FNR is to associate with the FnrS using the Δ*fnr* deficient mutant (CW10008) and the Δ*fnrS*Δ*fnr* double deficient strain (CW10009). The anaerobic induction of the *metE::lacZ* (pr) gene expression was measured among these two different mutant strains. For the LB media incubation, as compared to the wild-type strain, the strains Δ*fnr* and Δ*fnrS* Δ*fnr*, all carrying λ*metE::lacZ*,(pr), showed relative higher β-galactosidase levels under anaerobic conditions (Fig. 3A).

To additionally identify that the anaerobic induction of FnrS leads to the down regulation of *Salmonella* Typhimurium *metE::lacZ* mRNA levels, reverse-transcription PCR experiments were used to quantify the fusion mRNA levels of the wild-type and the various mutant strains. Total RNA was isolated each from the wild-type cells and the deficient strains (Δ*fnrS*, Δ*fnr* and Δ*fnrS*Δ*fnr*). Using the *metE::lacZ* and *rrsA* (16S rRNA) specific primers separately, 250 ng of total RNA was amplified by the one-step RT-PCR method. The *rrsA* (16S rRNA) was equally used as an internal control for the detection of mRNA expression. The *rrsA* rRNA were equally expressed between the wild-type and the mutant strains (Fig. 3B). Similarly with the results of the β-galactosidase assays, the expression of *metE::lacZ* mRNA levels from the *fnrS* or/and *fnr* mutants were relative higher than that of the wild-type strain (Fig. 3B).

To quantify the fusion mRNA levels from different bacterial strains, the real time qPCR was used to measure the ratio of gene expression between the wild-type and Δ*fnrS* mutant strain. An equal amount of the cDNA (1 ng or 10 ng) from two samples was in series used as the target gene (*metE::lacZ*) and reference gene (*rrsA*) for real-time PCR. The cycle threshold method (C_T method) (Livak and Schmittgen, 2001) was adopted for calculation of the *metE::lacZ* gene expression levels between the wild-type and Δ *fnrS* mutant strain. As a result, both assays, with different cDNA concentration (1 ng or 10 ng), showed the similar results. The *metE::lacZ* mRNA levels of *Δ fnrS* mutant have almost 3.5- or 4- fold increased as compared to those from the wild-type (Fig. 3C).

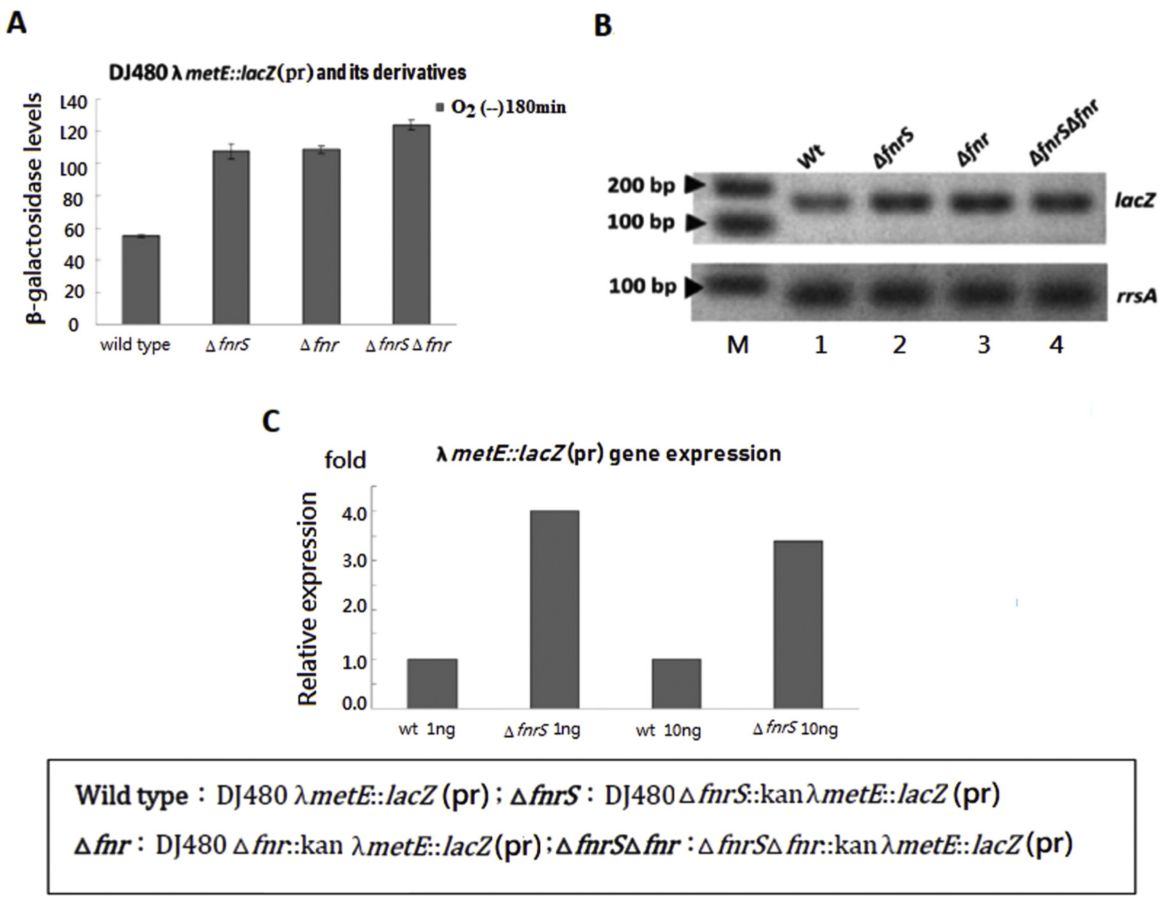

Fig. 3. Effects of FnrS and/or FNR on *metE::lacZ* (pr) gene expression under anaerobic condition. RT-PCR as well as real-time PCR analyses of their mRNA expression levels. The bacteria were cultured in LB medium at 37°C under the anaerobic for 3 h. (A) The β-galactosidase analyses of *metE::lacZ* (pr) were performed in the wild-type, *ΔfnrS*, *Δfnr* and *ΔfnrS Δfnr* strains. (B) To process the RT-PCR analysis, the total RNAs were extracted after 3 h of anaerobic incubation, using the *rrsA* gene (16sRNA) as the internal control. Using the 250 ng of the total RNA to perform the 35 PCR cycles of the One-Step RT-PCR, an equal amount of each PCR products (cDNA) was run on 2% agarose gel electrophoresis. (C) Using the 1 ng and 10 ng of cDNA to process the Quantitative-PCR, the relative quantitative ratio was through the quantitative methods, $2_T^{-\Delta\Delta C}$.

3.3. The effects on metE::lacZ (pr) and metE$^+$-ha gene expression by different regions of FnrS

To identify if a specific region of FnrS could regulate *metE*$^+$ gene expression, we examined the expression levels of the *metE::lacZ* (pr) upon overexpression by FnrS and its derivative mutants. Firstly, we constructed different loop-deficient mutants (Δloop 2–3 and Δloop 3) of FnrS by direct deletion mutagenesis of pBR- P_{LAC}-*fnr*S$^+$. Then, FnrS and its derivatives were in series constructed under the pBR-P_{LAC} promoter control and the expression of all the downstream genes can be induced by IPTG. Using CW10003 [Δ*fnr*S, λ*metE*::*lac*Z (pr)] as the host, after transformation, the resultant transformants were each measured for β-galactosidase activity, separately. Upon induction, the levels of β-galactosidase from the *metE::lacZ* (pr) gene expression of an each bacteria, with the two loop-deletion FnrSΔloop2–3 or the one loop deletion FnrSΔloop3, were higher than that of the FnrS$^+$ control (Fig. 4A). Only the bacterial cells, in which the pBR-P_{LAC}-*fnrS*$^+$ plasmids were expressing the full length FnrS, showed decreased β-galactosidase activity. The bacteria cells carrying pBR-$P_{Lac,}$ with higher β-galactosidase levels, were used as a negative control.

We also measured the amount of MetE-HA in different transformants, of which all carried pBAD33-5′-UTR-*metE*$^+$-*ha* and pBR-P_{LAC} or its derivatives including pBR- P_{LAC}-*fnrS*$^+$, and different deletion mutants (*i.e.* pBR-P_{LAC}-*fnrS* Δloop 2 ∼ 3 or pBR- P_{LAC}- *fnrS* Δloop3). Basically, YF10003 (Δ*metE*, Δ*fnrS*) was used as the host cells. The bacterial transformants carrying pBR-P_{LAC} and pBAD33-5′-UTR-*metE*$^+$-*ha*, after an arabinose (0.02%) and an IPTG (1 mM) induction, were used as a positive control (Fig. 4B, lane 1 in both panels). The MetE-HA could be detected in both aerobic and anaerobic states. However, after similar induction of the transformants that carry pBR-P_{LAC}-*fnrS*$^+$ and pBAD33-5′-UTR-*metE*$^+$-*ha,* the two different state bacterial strains showed decreased MetE-HA levels about 37% and 40%, respectively; as compared to those in the control strains (Fig. 4B, lane 2 in both panels; compared to lane 1). Both bacterial cells, carrying pBR-P_{LAC}-*fnrS*Δloop2 ∼ 3 or pBR-P_{LAC}-*fnrS*Δloop3, showed higher levels of MetE-HA as indicated (Fig. 4B, lane 3 and 4 in both panels, as compared to lane 1). These data also revealed that the loop 3 of FnrS could have effects on the down-regulation of the *metE*$^+$ gene expression.

3.4. Effects on the metE::lacZ (op or pr) and the metE-ha gene expression by FnrS point mutants

The Target RNA program (http://snowwhite.wellesley.edu/targetRNA) (Tjaden et al., 2006) was used to predict the base-paring between FnrS and *metE*$^+$ mRNA 5′TIR (Fig. 5A). It showed that the FnrS loop1-loop2 is complementary to the translational initiation region of the *metE*$^+$ gene (Fig. 5A). To test whether the base-pairing in the predicted regions (loop 1 ∼ loop 2) of FnrS were required for the regulation of *metE*$^+$ gene expression, we constructed six mutants including the 3 previously known mutants and three new mutation(s) among the loop 1 and loop 2 of FnrS. All six mutants were cloned into the pBR-P_{LAC} of which the FnrS mutants could be overexpressed under P_{LAC} promoter by

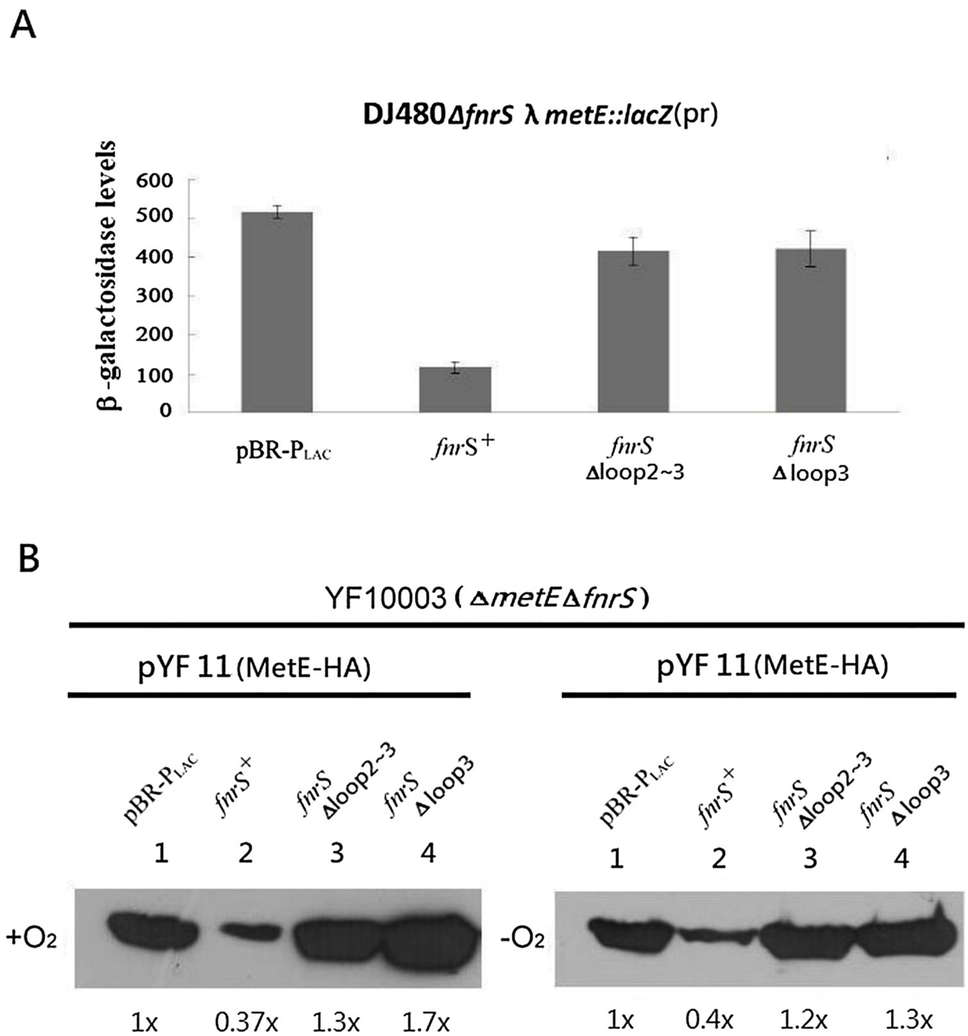

Fig. 4. Effects of FnrS and its derivative deletion mutants on λ*metE::lacZ* (pr) and *metE*$^+$*-ha* gene expression levels. (A) The β-galactosidase levels of λ*metE::lacZ* (pr) in the bacterial cells carrying pBR-P$_{LAC}$, pBR-P$_{LAC}$-*fnrS*$^+$, pBR-P$_{LAC}$-*fnrS*Δloop2–3 and pBR-P$_{LAC}$-*fnrS*Δloop1, in series. (B) The correlation between the different regions of FnrS structure and MetE-HA expression levels was identified in the Δ*fnrS* Δ*metE* mutant strain (YF10003). The expressions of pBAD33-*metE*$^+$*-ha* were induced by 0.02% arabinose and the expressions of pBR-P$_{LAC}$-*fnrS*$^+$ and its derivatives were induced by 1 mM IPTG. The Western blot analyses were used to detect the MetE-HA expression in LB medium with addition of appropriate antibiotics and above bacterial growth were under aerobic (O$^+$, the left panel) or anaerobic condition (O$^-$, the right panel).

IPTG induction. After transformation of DJ480Δ*fnrS* λ*metE::lacZ* (pr) and DJ480Δ*fnrS* λ*metE::lacZ (*op) with pBR-P$_{LAC}$ and its derivatives in series, the resultant transformants were measured of their β-galactosidase activity, separately. As shown in Fig. 5B and Fig. 5C, the bacterial cells carrying pBR-P$_{LAC}$ were shown the highest β-galactosidase activity. The other two higher β-galactosidase activity occurred in the bacteria carrying pBR-P$_{LAC}$-*fnrS* II (C47A/U48A/U49 G) or pBR-P$_{LAC}$-*fnrS* 54 (C54 G) as compared to that of the control pBR-P$_{LAC}$-*fnrS*$^+$. The bacterial cells, carrying either pBR-P$_{LAC}$-*fnrS* I ((U57A/U58 G/U59A) or pBR-P$_{LAC}$-*fnrS* 43 (U43 G), showed the subtle increased β-galactosidase activity. However, the bacterial cells carrying either pBR-P$_{LAC}$-*fnrS* III (G4C/G5 T) or pBR-P$_{LAC}$-*fnrS* 41 (U41 G) showed decreased β-galactosidase activity.

We also measured the amounts of MetE-HA in different transformants, of which all the bacterial cells carried pBAD33-5′-UTR-*metE*$^+$*-ha* and pBR-P$_{LAC}$ or its derivatives including pBR- P$_{LAC}$-*fnrS*$^+$, and different pBR-P$_{LAC}$-*fnrS* point mutants as described above. After the bacterial cells were induced by 0.02% arabinose and 0.1 mM IPTG for 3 h, Western blot assays were used to measure the MetE-HA levels by using the HA-tag antibody. As usual, the bacterial cells with pBR-P$_{LAC}$ have the highest amount of MetE-HA (Fig. 5D, lane 1). The bacterial cells showed the decreased amount of MetE-HA, while they carried the pBR-P$_{LAC}$-*fnrS*$^+$ (Fig. 5D, lane 2). In contrast, the bacterial cells carrying pBR- P$_{LAC}$-*fnrS* I (U57A/U58 G/U59A), pBR-P$_{LAC}$-*fnrS II* (C47A/U48A/U49 G), pBR- P$_{LAC}$-*fnrS* 43 (U43 G) or pBR-P$_{LAC}$-*fnrS*54 (C54 G), all showed increased amounts of MetE-HA (Fig. 5D, lane 3, 4, 7 and 8). Both bacterial cells, which carried either pBR-P$_{LAC}$-*fnrS* III (G4C/G5 T) or pBR-P$_{LAC}$-*fnrS* 41(U41 G), have the most decreased amount of MetE-HA (Fig. 5D, lane 5 and 6).

3.5. Effects of mutations in metE::lacZ that restore base pairing to mutant FnrS for the target gene repression over again

To identify that there is base-pairing between the 5′mRNA *metE* TIR region and the FnrS small RNA, two different point mutation(s) were constructed in the *metE-2*(A-2 T)(A-3C)*::lacZ* (op) and *metE54*(G-8C)*::lacZ* (op) and both fusion genes were transferred into λRS45. Two resultant phages were then lysogenized into DJ480Δ*fnrS*. These two lysogenic bacterial cells, CW10014 and CW10015, were also separately transformed with pBR-P$_{LAC}$ and pBR-P$_{LAC}$-*fnrS*$^+$. Subsequently, the lysogen carrying λ*metE-2* (A-2 T) (A-3C)*::lacZ* (op) was transformed with pBR-P$_{LAC}$-*fnrS* II. Also, the other lysogen carrying *metE54* (G-8C)*::lacZ* (op) was transformed with pBR-P$_{LAC}$-*fnrS* 54. Both bacteria, carrying the pBR-P$_{LAC,}$ have the higher β-galactosidase levels. Again, upon an IPTG induction, FnrS appeared to lose an ability to repress the *metE-2*(A-2 T)(A-3C)*::lacZ* (op) or *metE54*(G-8C)*::lacZ* (op) fusion gene expression in the bacterial cells, in which both cells carried pBR-P$_{LAC}$-*fnrS*$^+$ and showed higher β-galactosidase levels (Fig. 6A and 6B, lane 2 as compared to lane 1). In contrast, the bacterial cells carried the complementary mutation(s) in either λ*metE-2::lacZ* or λ*metE54::lacZ* fusions, the repression of each gene expression was observed with each of their corresponding FnrS mutants (Fig. 6A and 6B, lane 3). Consequently, these two bacteria have the lower β-galactosidase levels.

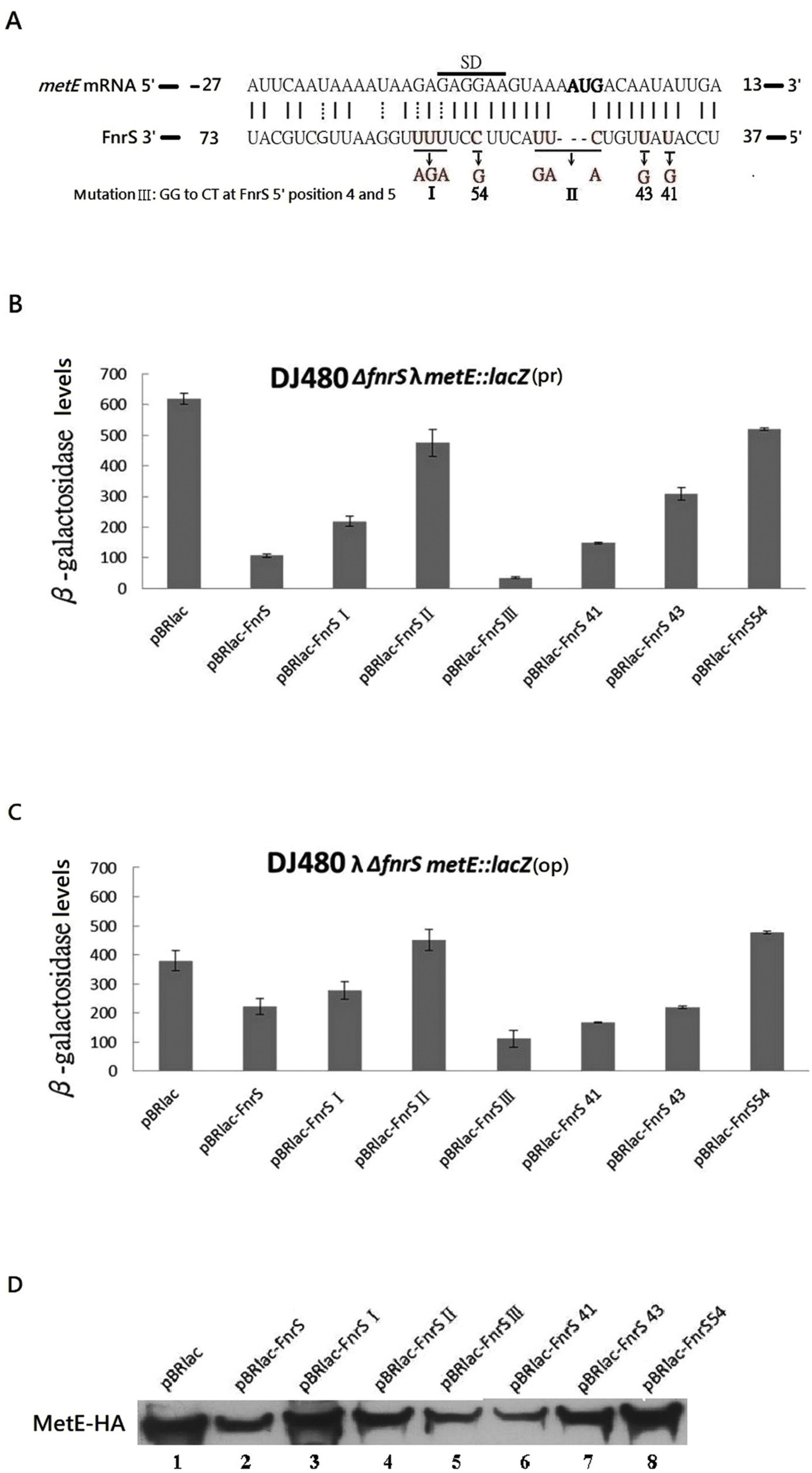

Fig. 5. The effect on $metE^{+}$ gene expression by FnrS point mutants. (A) The point mutations in FnrS were underlined as indicated. (B) β-galactosidase levels of λ*metE::lacZ* (pr) in different bacterial strains which carry pBR-P_{LAC}, pBR- P_{LAC}-$fnrS^{+}$ and its various derivatives, separately. (C) β-galactosidase levels of λ*metE::lacZ* (op) in different bacterial strains which carry pBR-P_{LAC}, pBR-P_{LAC}-$fnrS^{+}$ and its various derivatives, separately. (D) The Western blot analyses were used to detect the MetE-HA expression levels under the regulation by different FnrS point mutants. The various bacterial cells were each cultured in the rich media under the aerobic condition and when the bacterial cells were grown to the logarithm growth phase, IPTG, with 1 mM final concentration, was added to induce the gene expression under the pBR-P_{LAC} promoter control.

4. Discussion

In this study, we have used genetic analyses to identify that in *Salmonella* Typhimurium, $metE^{+}$ gene expression could be repressed by FnrS in an anaerobic environment. With Western blot analyses, we demonstrated that MetE-HA protein showed increased expression in both *hfq*⁻ and *fnrS*⁻ mutants under anaerobic conditions after an overnight incubation (16 h) (Fig. 2). Only the *fnrS*⁻ mutant showed higher MetE-HA levels under anaerobic conditions for a short time incubation (30 min) (Fig. 2). Therefore, it is likely that FnrS directly regulates $metE^{+}$ gene expression in anaerobic state.

The bacterial cells expressing the MetE-HA protein could

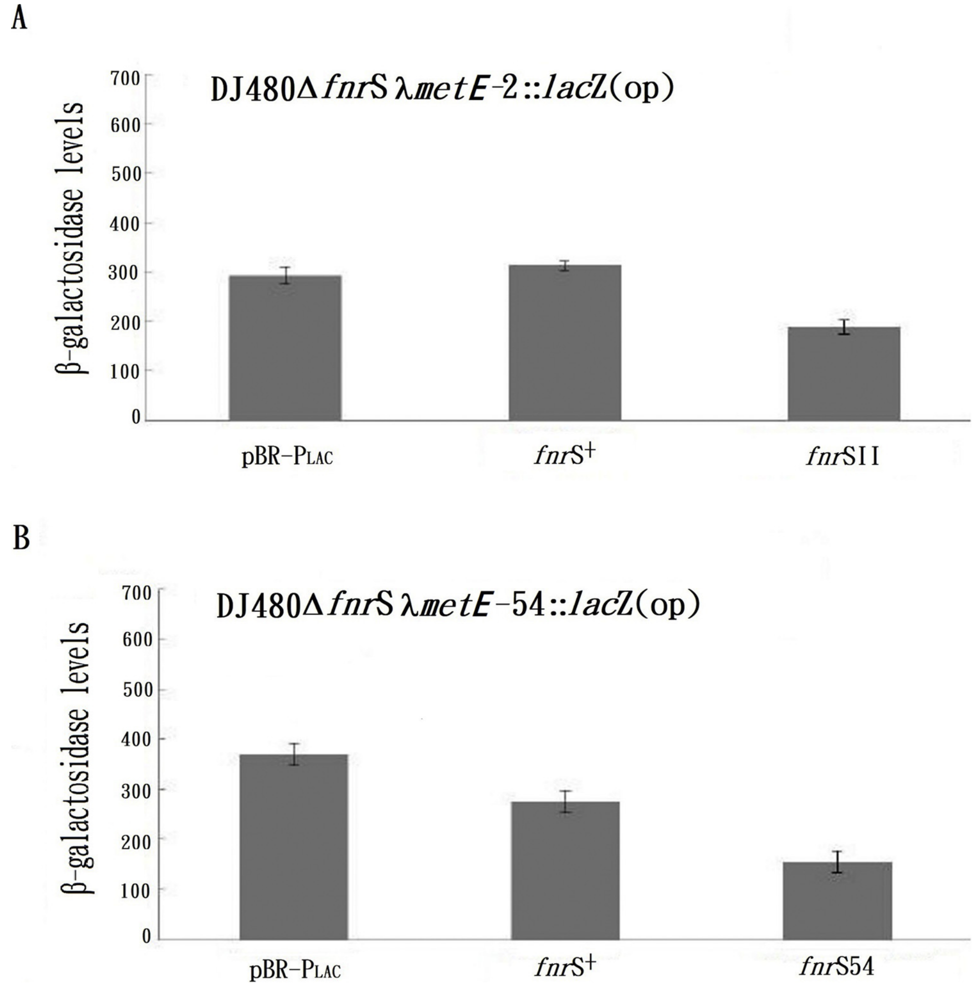

Fig. 6. The complementary strain of λ*metE::lacZ* derivatives to the FnrS point mutant(s). The bacteria were cultured in the rich media under the aerobic condition until the log growth phase, using the 1 mM IPTG to induce the pBR-P_{LAC} promoter. (A) β-galactosidase assays of *metE*-2::*lacZ* (op) in different bacterial strains, which carries pBR-P_{LAC}, pBR-P_{LAC}-*fnrS*$^+$ or pBR-P_{LAC}-*fnrS* II (B) β-galactosidase assays of *metE*-54::*lacZ* (op) in different bacterial strains, which carries pBR-P_{LAC}, pBR-P_{LAC}-*fnrS*$^+$ or pBR-P_{LAC}-*fnrS*54.

complement the deletion of *metE*$^+$ gene in the chromosome of *E. coli.* The MetE-HA transformant cells, which lacked the chromosomal *metE* gene, can grow on glucose minimal media without addition of L-methionine (data not shown). Therefore, the addition of HA-tag to the C-terminal end of MetE protein does not have an effect on its normal activity. Also, our previous studies demonstrated that MetE-HA levels were increased in Δ*fnrS*, Δ*fnr*, Δ*hfq*, Δ*fnrS*Δ*fnr*, and Δ*fnrS*Δ*hfq* mutants under a longer anaerobic incubation, as compared to that of the wild-type (Chien et al., 2015). Similarly, using the reverse-transcription PCR assays, their correspondent *metE*$^+$-*ha* mRNA levels were increased in the above mutants while also compared to that of the wild-type (Chien et al., 2015). Therefore, Hfq protein is likely involved in the regulation of *metE*$^+$ gene expression under the anaerobic state. As noted, Hfq protein can stabilize the base-pairing between the target mRNA and small RNA (Soper and Woodson, 2008; De Lay et al., 2013).

Using the *metE::lacZ* (pr) fusion gene, we also identified that as compared to itself in the wild-type, the β-galactosidase levels were increased in Δ*fnrS*, Δ*fnr* and Δ*fnrS*Δ*fnr* mutants (Fig. 3A). Similarly, using the reverse-transcription PCR analytic methods, the *metE::lacZ* (pr) mRNA levels were increased in Δ*fnrS*, Δ*fnr* and Δ*fnrS*Δ*fnr* mutants as compared to that of the wild-type (Fig. 3B). The real-time qPCR assay was also adopted to calculate the ratio of *metE::lacZ* (pr) mRNA between Δ*fnrS* mutant and wild-type. Using the $2^{-\Delta\Delta CT}$ relative quantification methods (Livak and Schmittgen, 2001), the *metE::lacZ* (pr) mRNA in Δ*fnrS* mutant was about 3.5～4 fold higher than that in wild-type (Fig. 3C). Therefore, the *metE::lacZ* mRNA levels could be negatively regulated by FnrS due to the post-transcriptional regulation in an anaerobic condition. Meanwhile, FNR protein can positively regulate *fnrS*$^+$ gene expression under anaerobic conditions. FnrS was not produced in the FNR mutant and that led to the higher gene expression of *metE::lacZ* fusion gene.

In addition, the overproduction of FnrS$^+$ could repress the expression of *metE::lacZ* (pr) and MetE-HA (Fig. 4). However, the deletion of the loop 2～3 and loop 3 of FnrS, led to the elevated β-galactosidase levels and MetE-HA amounts (Fig. 4). Since using the TargetRNA program, the base-pairing, between FnrS and 5′*metE*$^+$ TIR mRNA, were predicted within the loop 1～loop 2 of FnrS. Mostly, the deletion of loop 3 and loop 2～3 of FnrS would alter the configuration of itself and that resulted in losing its normal repressing activity. Similarly, the deletion of loop 1 in FnrS would also result in an alteration of its secondary structures. We also constructed the *met*E$^+$-*ha*$^+$ 5′ upstream deletion mutants, and the results showed that the MetE-HA levels were still regulated by the overproduction of FnrS (data not shown). These results indicated that the FnrS target sites were in the *met*E$^+$ TIR region as predicted by the TargetRNA program.

Furthermore, using FnrS mutants with the point mutation(s) in the loop 1 and 2 of FnrS, some of them altered their repressing activity for *metE::lacZ* and MetE-HA gene expressions. As shown, FnrS I (U57A/U58 G/U59A), FnrS II (C47A/U48A/U49 G), FnrS43 (U43 G) and FnrS54 (C54 G) all lost their repressive activity for *metE*$^+$ gene expression (Fig. 5). Through further exploration, these mutations in FnrS were shown to disrupt the base-paring between 5′ mRNA TIR and FnrS. In contrast, FnrS III (G4C/G5 T) and FnrS 41 (U41 G) resulted in more repressing activity. Since in FnrS III, 5 T would base-pair with its 40A and in FnrS 41, 41 G would somewhat base-pair with its 6U (seen in Fig. 1B). It is likely that the extra base-pairing forms a more stable stem-loop structure of FnrS that could enhance its repressive activity toward *metE*$^+$ gene expression.

Using the point mutation(s) in FnrS and altering their complementary nucleotides in *metE*$^+$ 5′ TIR mRNA, we also identified that the base-pairing between the FnrS and the 5′ TIR region of *metE*$^+$ mRNA is necessary for the down-regulation of *metE*$^+$ gene expression. As examples, *metE-2*(A-2 T)(A-3C)*::lacZ* (op) or *metE54*(G-8C)*::lacZ* (op) would result in a new base-paring with FnrS II and FnrS 54, separately. In both bacteria carrying each one of the above *metE::lacZ* mutant fusion gene, showed lower β-galactosidase activity in the presence of FnrS II and FnrS 54, separately (Fig. 6). In such way, both FnrS mutants can restore their repressing activity.

From our data, FnrS can hybridize with *metE*$^+$ 5′ TIR RNA (Fig. 5 and 6), and that could hinder the ribosome binding-site (SD) and translational initiation site (ATG) of *metE*$^+$ mRNA from the recognition by the ribosomal small subunit. However, it remains unknown for whether the ribonucleases, RNase III and RNase E, participate in the destabilization of the *metE*$^+$ mRNA. Although, the recent studies showed that new RNase III cleavage sites of mRNAs were verified in *E. coli* and those revealed an increased regulation of mRNA(s) (Gordon et al., 2017). FnrS is induced under an anaerobic state, and no RNase III cutting analytic data was performed under such conditions. Also, the sequences of the RNase E cutting site were also recapitulated by the *in vivo* analyses (Chao et al., 2017). A core motif, from -2 to +3, was delineated. The 5 nucleotides, $R^{-2}N^{-1}|W^{+1}U^{+2}U^{+3}$ (R as G/A, W as A/U and N as any nucleotide), were presented (Chao et al., 2017). The +2 residue U is quite conserved and is essential for the RNase E cleavage. Through scanning, we have found the similar RNase E cutting sequences in the *metE*$^+$ 5′ TIR region (our unpublished data). However, the *metE*$^+$ gene was not found in the RNase E cutting profile of *Salmonella enterica* (Chao et al., 2017). Again, the profile for RNase E cutting was not determined under anaerobic conditions.

Through our study, FnrS forms the complementary base-pair to the 5′ mRNA of *metE*$^+$ TIR region and that might lead to the hidden SD region and translational initiation site of *metE*$^+$, resulting in an inefficient translation and decreased mRNA levels. Alternatively, the likely RNase III or RNase E cutting site in the *metE*$^+$ mRNA can be exposed after FnrS binding, and it can be cut by RNase III or RNase E and immediately degraded by other RNase enzymes. Further study would be focusing on distinguishing between these two possibilities.

Also, our data, presented here, indicated that the FnrS could repress the *metE*$^+$ gene expression mediated by the base-pairing mechanism under an anaerobic environment. This regulation is important for the bacteria to adapt in oxygen-limited condition, in which the bacteria could save the energy for the biosynthesis of methionine, and thus, FnrS plays an important role for this anaerobic adjustment.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.micres.2019.126319.

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