

Full Paper

RNase E/G-dependent degradation of *metE* mRNA, encoding methionine synthase, in *Corynebacterium glutamicum*

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Corynebacterium glutamicum is used for the industrial production of various metabolites, including L-glutamic acid and L-lysine. With the aim of understanding the post-transcriptional regulation of amino acid biosynthesis in this bacterium, we investigated the role of RNase E/G in the degradation of mRNAs encoding metabolic enzymes. In this study, we found that the cobalamin-independent methionine synthase MetE was overexpressed in $\Delta rneG$ mutant cells grown on various carbon sources. The level of *metE* mRNA was also approximately 6- to 10-fold higher in the $\Delta rneG$ mutant strain than in the wild-type strain. A rifampicin chase experiment showed that the half-life of *metE* mRNA was approximately 4.2 times longer in the $\Delta rneG$ mutant than in the wild-type strain. These results showed that RNase E/G is involved in the degradation of *metE* mRNA in *C. glutamicum*.

Key Words: *Corynebacterium glutamicum*; *metE*; mRNA degradation; RNase E/G

Introduction

Corynebacterium glutamicum is a Gram-positive, non-pathogenic soil bacterium that is a workhorse for amino acid production. More than 2.5 million tons of the flavor enhancer L-glutamic acid and one million tons of the feed additive L-lysine per year are produced using *C. glutamicum* (Hirasawa and Shimizu, 2016). This bacterium has also been widely used for the production of various other metabolites, including D-amino acids, organic acids, diamines, fuels, proteins, and aromatic compounds (Becker and Wittmann, 2012; Kogure et al., 2016; Kubota

et al., 2016; Matsuda et al., 2014). *C. glutamicum* belongs to the mycolic acid-containing actinomycetes, which also includes the genera *Mycobacterium*, *Nocardia*, and *Rhodococcus*.

Ribonucleases (RNases) play an important role in the post-transcriptional regulation of gene expression (Mackie, 2013). In most bacteria, such as *E. coli*, mRNA decay depends on the initial cleavage, which is mainly catalyzed by RNase E/G family enzymes (Arraiano et al., 2010). RNase E/G endoribonucleolytically cleaves single-stranded AU-rich regions (Mackie, 2013). In *E. coli*, mRNA decay is often initiated by RNase E via one of two pathways. In one pathway, RNase E directly accesses internal sites in target mRNAs with 5'-terminal triphosphate (Clarke et al., 2014). The other pathway is 5' end-dependent, and it is stimulated by the removal of two of the three phosphates from the 5' terminus of primary transcripts (Celesnik et al., 2007; Deana et al., 2008; Luciano et al., 2017). *E. coli* has two RNase E/G family enzymes, RNase E and RNase G, and RNase E is essential for cell viability in *E. coli* (Apprion and Lassar, 1978; Arraiano et al., 2010). In contrast, *C. glutamicum* has only one RNase E/G encoded by *rneG*, and it is not essential (Maeda and Wachi, 2012a). The *C. glutamicum* RNase E/G is involved in 5'-end processing of the 5S rRNA and 3'-end processing of the 4.5S rRNA (Maeda and Wachi, 2012a; Maeda et al., 2017). In addition, the RNase E/G degrades *aceA* mRNA, which encodes the glyoxylate cycle enzyme isocitrate lyase. Degradation of *aceA* mRNA by RNase E/G depends on the 3'-untranslated region (3'-UTR) (Maeda and Wachi, 2012b). It was also reported that the transcriptional termination factor Rho and RNase E/G play a central role in FMN riboswitch regulation in *C. glutamicum* (Takemoto et al., 2015). In *C. glutamicum*, both Rho and RNase E/G are individually dispensable, whereas simultaneous disruption is synthetic lethal (Takemoto et al., 2015).

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Table 1. *C. glutamicum* strains and plasmids used in this study.

Strain or Plasmid	Relevant genotype or description	Source or reference
Strains		
<i>C. glutamicum</i>		
ATCC31831	Wild type	Laboratory stock
D2281	The same as ATCC31831 but Δ rneG	Maeda and Wachi (2012a)
Plasmids		
pECtS	The same as pECt but Δ lacI ^q -trc promoter	Maeda and Wachi (2012b)
pCmE-FL	pECtS with <i>C. glutamicum</i> metE	This study

Table 2. Primers used in qRT-PCR analysis.

Target gene	5' primer sequence	3' primer sequence	Reference or source
16S rRNA-1	CTGGCGGCGTGCTTAACACA	CCCAGTTTCCCAGGCTTATC	This study
16S rRNA-2	CGCTGCGGTGAATACGTTCC	CTTCCGGTACGGCTACCTTG	This study
metE	GAGCGAAGCGTGAAGTGAAG	ACGTCCTGCAAACGGAACGG	This study

In this study, we showed that RNase E/G is involved in the degradation of *metE* mRNA encoding cobalamin-independent methionine synthase in *C. glutamicum*.

Materials and Methods

Media and growth conditions. L broth containing 1% polypeptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose (pH 7.0) was used as the complex medium. CGC medium (Eikmanns et al., 1991) containing an appropriate carbon source, 5.0 g/L (NH₄)₂SO₄, 5.0 g/L urea, 21 g/L morpholinepropanesulfonic acid (MOPS), 1.0 g/L K₂HPO₄, 1.0 g/L KH₂PO₄, 0.25 g/L MgSO₄·7H₂O, 0.01 g/L CaCl₂, 16.4 mg/L FeSO₄·7H₂O, 10 mg/L MnSO₄·H₂O, 0.2 mg/L CuSO₄·5H₂O, 1.0 mg/L ZnSO₄·7H₂O, 0.2 mg/L NiCl₂·6H₂O, 0.2 mg/L biotin, and 1.0 mg/L thiamine (pH 6.8) was used as the minimal medium. When necessary, media were supplemented with 20 µg/mL kanamycin. Glucose, fructose, sucrose, ribose, L-arabinose, sodium acetate, sodium lactate, or sodium gluconate (1% w/v) was added as a carbon source. *C. glutamicum* was grown aerobically at 30°C, and cell growth in liquid medium was monitored by measuring the optical density at 660 nm (OD₆₆₀). *E. coli* JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/F'(traD36 proAB⁺ lacI^q lacZΔM15)*] and JM110 [*dam dcm supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr tsx Δ(lac-proAB)/F'(traD36 proAB⁺ lacI^q lacZΔM15)*] cells were used for all genetic manipulations. *E. coli* strains were cultivated at 37°C in L medium supplemented with 50 µg/mL kanamycin when necessary.

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. A 3.14-kb DNA fragment containing the *metE* gene with 445 bp upstream region, which contains its own promoter sequence, and 455 bp downstream region, which contains a Rho-dependent terminator-like sequence, was amplified by PCR using the primers 5'-CGCGTTGAATTCTGCAAAACCCC-3' and 5'-GGGAATGTGACGACGAGTTGGCCG-3' (the artificially generated *Eco*RI and *Sal*I sites are underlined).

The amplified fragment was digested with *Eco*RI and *Sal*I, and then the digested DNA fragment was cloned into the *E. coli*-*C. glutamicum* shuttle vector plasmid pECtS (Maeda and Wachi, 2012b) digested with the same enzymes to construct plasmid pCmE-FL.

Analysis of cellular proteins. The total cellular proteins of *C. glutamicum* were prepared as described previously (Maeda and Wachi, 2012b). Briefly, cells were suspended in sodium phosphate buffer (50 mM, pH 7.0) and disrupted by sonication. After removal of the unbroken cells, the proteins in the lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the gel was stained with Coomassie brilliant blue. The 80-kDa protein band was cut out and analyzed by mass spectrometry (MALDI-TOF/TOF ultrafleXtreme; Bruker Daltonics, Inc., Billerica, MA) after in-gel trypsin digestion.

Total RNA purification. Total cellular RNA from *C. glutamicum* cells was isolated as described previously (Maeda and Wachi, 2012b). Briefly, overnight cultures grown in CGC medium containing a carbon source (1%) at 30°C were washed and then inoculated into fresh CGC minimal medium containing a carbon source (1%). Then, two volumes of RNA Protect bacterial reagent (Qiagen, Valencia, CA) were added directly to one volume of exponentially growing cultures (OD₆₆₀ of ~1; mid-exponential growth phase) to stabilize cellular RNA. The cells were harvested by centrifugation at 5,000 × g for 10 min at 25°C, and total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated with DNase I at 37°C for 1 h.

qRT-PCR. The mRNA was quantified using the Eco Real-Time PCR System (Illumina, Inc., San Diego, CA) as described previously (Maeda and Wachi, 2012b). Primers used in this quantitative real-time PCR (qRT-PCR) analysis are listed in Table 2. Briefly, 50 ng of total RNA was used in each RT-PCR using the QuantiFast SYBR green RT-PCR kit (Qiagen) according to the manufacturer's in-

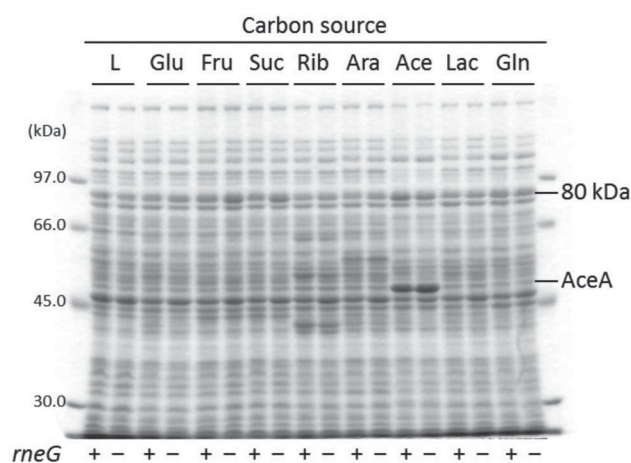


Fig. 1. Overproduction of MetE protein in the $\Delta rneG$ mutant.

ATCC31831 (wild-type [+]) and D2281 ($\Delta rneG$ [-]) cells were grown to the mid-exponential growth phase in media with various sugars and organic acids as the sole carbon source. Then, the total cellular proteins were analyzed by 10% SDS-PAGE, and stained with Coomassie brilliant blue. The positions of the 80-kDa protein and AceA are shown on the right of the gel. The carbon sources are shown at the top of the gel. L: L broth, Glu: glucose, Fru: fructose, Suc: sucrose, Rib: ribose, Ara: L-arabinose, Ace: acetate, Lac: lactate, and Gln: gluconate.

structions. Negative controls, with no reverse transcriptase, were included for each RNA sample to rule out genomic DNA contamination. Target gene transcript levels were normalized to the reference gene transcript (16S rRNA) in the same RNA sample. Each gene was analyzed using RNA isolated from at least three independent samples.

Results

Overproduction of MetE protein in the $\Delta rneG$ strain

We previously reported that isocitrate lyase encoded by *aceA* was overproduced in $\Delta rneG$ mutant cells grown on acetate as a single carbon source (Maeda and Wachi, 2012b). We performed a proteome analysis using cells grown to a stationary phase on various sugars and organic acids as a sole carbon source (Maeda and Wachi, 2012b). In the present study, we compared the protein expression patterns between wild-type and $\Delta rneG$ cells in the exponential growth phase to identify additional target mRNAs of RNase E/G. Total cellular proteins obtained from cells grown to the mid-exponential growth phase in complex or minimal medium with glucose, fructose, sucrose, ribose, L-arabinose, acetate, lactate, or gluconate as the sole carbon source were separated by SDS-PAGE. As shown in Fig. 1, a protein of ~80-kDa was overproduced in $\Delta rneG$ cells grown on fructose or sucrose as the sole carbon source, and as previously reported, AceA was overproduced in $\Delta rneG$ cells grown on acetate (Maeda and Wachi, 2012b). Overproduction of the 80-kDa protein was not observed apparently when the cells were grown on other carbon sources. This 80-kDa protein band was identified as MetE by mass spectrometry, with a deduced molecular mass of 81.3 kDa. The number of peptides matched and sequence coverage were 29 and 64.3% respectively for MetE in MASCOT analysis (Matrix Science Ltd., London, UK). To observe the effect of the $\Delta rneG$ mutation on

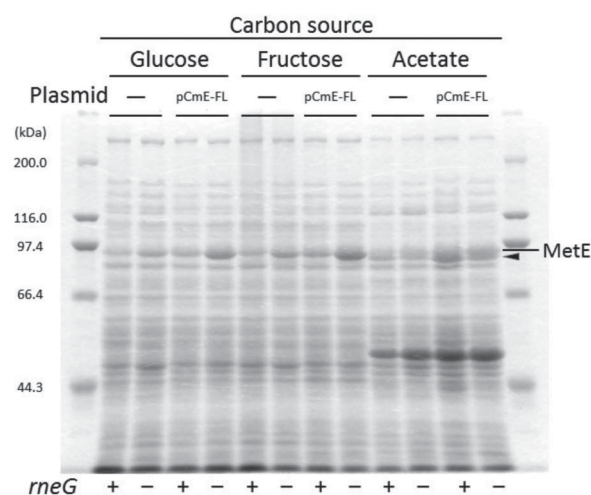


Fig. 2. The effect of the carbon source on MetE overproduction in $\Delta rneG$ mutant cells.

Wild-type (+) and $\Delta rneG$ (-) mutant cells harboring a *metE* overexpression plasmid (pCmE-FL) were grown to the mid-exponential growth phase on glucose, fructose, or acetate as the sole carbon source. The total cellular proteins were analyzed by 10% SDS-PAGE, and stained with Coomassie brilliant blue. The position of MetE is shown on the right of the gel. The carbon sources are shown at the top of the gel. A protein band (indicated by an arrowhead) migrated just below MetE, which is overproduced in the wild-type cells harboring pCmE-FL grown on acetate, is malate synthase encoded by *aceB*. The expression of *aceB* is induced when grown on acetate.

the expression of *metE* more precisely, we constructed a *metE*-overexpressing plasmid named pCmE-FL, which harbors the *metE* gene with its own promoter and terminator. Then, wild-type and $\Delta rneG$ mutant strains harboring pCmE-FL were grown on glucose, fructose, or acetate as the sole carbon source and the cells were harvested at the mid-exponential growth phase. As shown in Fig. 2, MetE overexpression in $\Delta rneG$ mutant cells harboring pCmE-FL was not clearly affected by the carbon source. This result indicates that overproduction of MetE in exponentially growing $\Delta rneG$ cells is independent of the carbon source. It is possible that the basal expression levels of *metE* were higher when cells were grown on fructose or sucrose than those on other carbon sources, which made the overproduction of MetE protein more prominent in the $\Delta rneG$ mutant grown on fructose or sucrose in Fig. 1.

We also examined whether $\Delta rneG$ cells overproduced MetE protein at the stationary phase. Wild-type and $\Delta rneG$ mutant strains harboring pCmE-FL were grown on fructose as a sole carbon source, and cells were harvested at both the mid-exponential growth phase and stationary phase. As shown in Fig. 3, the $\Delta rneG$ mutant harboring pCmE-FL overproduced MetE at the exponential growth phase, but this was not so obvious at the stationary phase. Even in the wild-type cells, the expression level of MetE protein was decreased at the stationary phase compared with that at the exponentially growing phase.

Increased stability of *metE* mRNA in $\Delta rneG$ cells

The proteome analysis shown in Figs. 1–3 suggested that overproduction of MetE in exponentially growing $\Delta rneG$

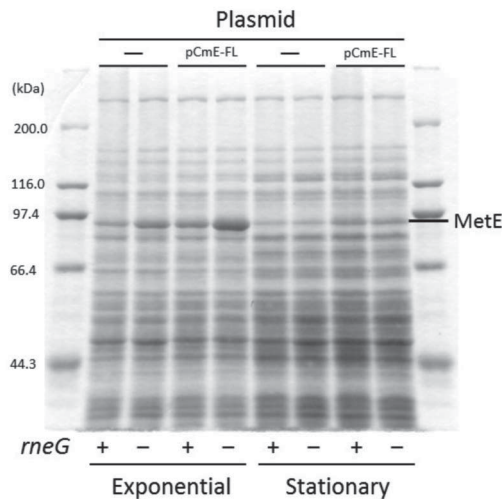


Fig. 3. The effect of the growth phase on MetE overproduction in $\Delta rneG$ mutant cells.

Wild-type (+) and $\Delta rneG$ (–) mutant cells harboring the pCmE-FL plasmid were grown to the mid-exponential growth phase or stationary phase on fructose as the sole carbon source. Then, the total cellular proteins obtained were analyzed by 10% SDS-PAGE, and stained with Coomassie brilliant blue. The position of MetE is shown on the right of the gel.

cells resulted from a defect in *metE* mRNA degradation by RNase E/G. To examine the effect of the $\Delta rneG$ mutation on the stability of *metE* mRNA, we first performed a qRT-PCR analysis. Total RNA was extracted from wild-type and $\Delta rneG$ cells growing exponentially on glucose, fructose, or acetate as a sole carbon source. As shown in Fig. 4, the level of *metE* mRNA in $\Delta rneG$ cells was approximately 6.1-fold higher than that in wild-type cells when grown on fructose as the sole carbon source. In addition, increased levels of *metE* mRNA were also observed in the $\Delta rneG$ mutant when the cells were grown on glucose or acetate as the sole carbon source. The levels of *metE* mRNA in the $\Delta rneG$ mutant were approximately 10.8-fold and 9.7-fold higher than that in the wild-type cells when grown on either glucose or acetate as the sole carbon source, respectively (Fig. 4). These results suggest that RNase E/G degrades *metE* mRNA regardless of the carbon source.

We also examined mRNA levels at the stationary phase. The expression levels of *metE* mRNA were decreased to about 1/20 both in wild-type and $\Delta rneG$ mutant cells at the stationary phase compared with those at the exponential phase; that is, the increased levels of *metE* mRNA in $\Delta rneG$ mutant were still observed in the stationary phase (data not shown). This suggests that RNase E/G degrades *metE* mRNA both at the exponential and stationary phase. It is possible that the effect of the $\Delta rneG$ mutation is more prominent for highly expressed genes when observed at protein levels. Previously identified RNase E/G target mRNAs, *adhE* of *E. coli* and *aceA* of *C. glutamicum*, are also highly expressed genes (Maeda and Wachi, 2012b; Umitsuki et al., 2001).

Next, we measured the half-lives of *metE* mRNA in wild-type and $\Delta rneG$ cells grown on fructose as a sole carbon source. Exponentially growing cells were treated with 150 $\mu\text{g/mL}$ rifampicin to prevent further initiation of transcrip-

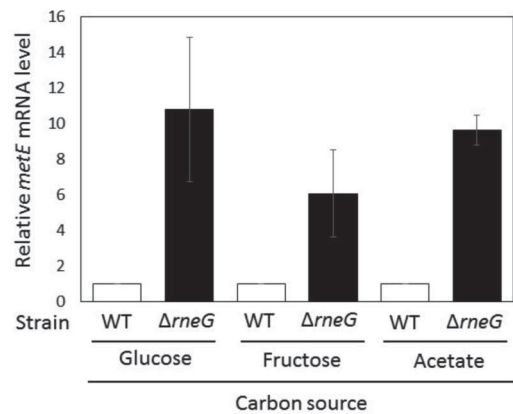


Fig. 4. Relative *metE* mRNA levels in wild-type and $\Delta rneG$ cells grown on glucose, fructose, or acetate as the sole carbon source.

The total cellular RNA was extracted from exponentially growing cells. Relative *metE* mRNA levels were measured by qRT-PCR and normalized to 16S rRNA transcript levels. The levels are presented relative to that in wild-type cells, which was set to 1. The values are the means (with standard deviation) from at least three independent experiments.

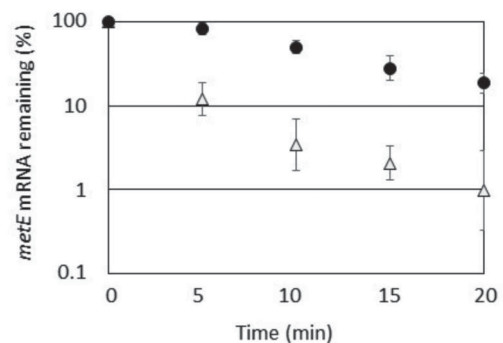


Fig. 5. Increased stability of *metE* mRNA in $\Delta rneG$ mutant cells.

The half-lives of *metE* mRNA in wild-type (open triangles) and $\Delta rneG$ mutant (filled circles) cells were determined. Rifampicin was added to exponentially growing cultures at time zero, and at the indicated time points, the total RNA was isolated and analyzed by qRT-PCR using specific primers for *metE* mRNA and normalized to 16S rRNA transcript levels. The half-lives were calculated from three independent experiments with standard deviations.

tion. Then, total RNA was isolated at various time points after the addition of rifampicin, and the rates of decay of *metE* mRNA were determined in wild-type and $\Delta rneG$ cells (Fig. 5). *metE* mRNA showed a half-life of 2.1 min in wild-type cells, and a prolonged half-life of 8.9 min in $\Delta rneG$ cells. These results indicated that the overexpression of MetE in the $\Delta rneG$ mutant results from the increased stability of *metE* mRNA.

Discussion

In this study, we have, for the first time, identified *metE* mRNA as a substrate of RNase E/G, in addition to *aceA* mRNA. We showed that MetE was overproduced in $\Delta rneG$ mutant cells when compared to the levels in wild-type cells during the exponential growth phase. The levels of *metE* mRNA were also increased in $\Delta rneG$ mutant cells. A rifampicin chase experiment showed that the half-life of

metE mRNA was prolonged in $\Delta rneG$ mutant cells. These results indicate that RNase E/G is involved in the degradation of *metE* mRNA in *C. glutamicum*.

Methionine is synthesized from homoserine in *C. glutamicum*. Homoserine is converted to *O*-acetylhomoserine by MetX (homoserine *O*-acetyltransferase) (Rückert et al., 2003). Then, homocysteine is synthesized by a trans-sulfuration pathway with MetB (cystathionine γ -synthase) and MetC (cystathionine β -lyase), or direct sulfhydrylation with MetY (*O*-acetylhomoserine sulfhydrylase) (Lee and Hwang, 2003; Rückert et al., 2003). The final step in L-methionine biosynthesis is catalyzed by methionine synthase. *C. glutamicum* possesses two methionine synthases, one encoded by *metE* and the other encoded by *metH* (Rückert et al., 2003). MetE is cobalamin (vitamin B₁₂) independent, whereas MetH is cobalamin dependent. Our preliminary experiment showed that a $\Delta metE$ mutant was auxotrophic for methionine, suggesting that MetE but not MetH plays a major role in methionine synthesis, at least, under our experimental conditions (data not shown). The transcriptional repressor McbR regulates the expression of almost all enzymes involved in methionine biosynthesis (Rey et al., 2003, 2005; Suda et al., 2008). Methionine production by fermentation has been explored by overproducing biosynthetic enzymes and/or deleting McbR in *C. glutamicum*, but productivity was still low for commercial purposes (Li et al., 2016; Park et al., 2007; Qin et al., 2015). Overproduction of MetE by the *rneG* mutation might be applied for improving the productivity of methionine.

It was previously shown that acidic stress induced oxidative stress in *C. glutamicum*. Oxidative stress caused MetE inactivation by *S*-mycothiolation, which resulted in methionine synthesis impairment. Under these conditions, MetE protein levels were increased (Follmann et al., 2009; Liu et al., 2016). In *E. coli*, it is also reported that oxidative stress causes MetE inactivation by *S*-glutathionylation, which results in methionine limitation (Hondorp and Matthews, 2004). Since rapid mRNA degradation permits fast change in gene expression, the degradation of *metE* mRNA by RNase E/G might be involved in the stress response mechanism.

Several studies in *E. coli* and other bacteria have shown that mRNA decay is frequently initiated by RNase E cleavage in the 5'-UTR (Hankins et al., 2007; Kaberdin and Bläsi, 2006). The 5'-UTRs can contain regulatory elements involved in up- or down-regulation of translation, including ribosome binding signals, translation repressors, low molecular-weight effectors, and small RNA with corresponding effects on mRNA stability (Kaberdin and Bläsi, 2006). In contrast, strong stem-loop structures such as the transcription terminator at the 3' end of many bacterial mRNAs are resistant to 3' to 5' exoribonucleolytic degradation (McLaren et al., 1991; Spickler and Mackie, 2000). Our previous study showed that the degradation of *adhE* mRNA was 5'-UTR dependent in *E. coli* (Ito et al., 2013; Umitsuki et al., 2001). We also showed that RNase E/G cleavage of *aceA* mRNA is dependent on the 3'-UTR in *C. glutamicum* (Maeda and Wachi, 2012b). The 3'-UTR of *aceA* mRNA contains a typical Rho-independent termina-

tor. RNase E/G cleavage removes this Rho-independent terminator and generates an unprotected 3' end, which is rapidly degraded by 3' to 5' exoribonucleases (Maeda and Wachi, 2012b). In these studies, *lacZ* assay fused with 5'- or 3'-UTR was valid for estimating cleavage sites. Therefore, we constructed plasmids harboring *metE* 5'-UTR-*lacZ* and *lacZ*-*metE* 3'-UTR fusion genes. However, neither *metE* 5'-UTR-*lacZ* nor *lacZ*-*metE* 3'-UTR showed significant difference in the expression between wild-type and $\Delta rneG$ strains (data not shown). This suggests that the mechanism for *metE* mRNA degradation by RNase E/G is different from 5'- or 3'-UTR dependent mechanism. Unlike in *aceA* mRNA, a Rho-dependent terminator-like sequence (i.e., C-rich and G-poor sequences that lack obvious secondary structure followed by a *boxA* signal) was found at the 3' end of the *metE* transcript (Friedman and Olson, 1983; Richardson and Richardson, 1996). It was previously shown that RNase III-mediated degradation of *mraZ* mRNA in *C. glutamicum* is coding region dependent (Maeda et al., 2016). Secondary structures within the coding region would slow down translation by ribosomes, which consequently increases the probability of degradation by endoribonucleases such as RNase III and RNase E/G (Braun et al., 1998; Maeda et al., 2016). Determination of RNase E/G cleavage site in *metE* mRNA remains to be done.

Acknowledgments

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