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3′ Untranslated Region-Dependent Degradation of the *aceA* mRNA, Encoding the Glyoxylate Cycle Enzyme Isocitrate Lyase, by RNase E/G in *Corynebacterium glutamicum*

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We previously reported that the *Corynebacterium glutamicum* RNase E/G encoded by the *rneG* gene (NCgl2281) is required for the 5′ maturation of 5S rRNA. In the search for the intracellular target RNAs of RNase E/G other than the 5S rRNA precursor, we detected that the amount of isocitrate lyase, an enzyme of the glyoxylate cycle, increased in *rneG* knockout mutant cells grown on sodium acetate as the sole carbon source. Rifampin chase experiments showed that the half-life of the *aceA* mRNA was about 4 times longer in the *rneG* knockout mutant than in the wild type. Quantitative real-time PCR analysis also confirmed that the level of *aceA* mRNA was approximately 3-fold higher in the *rneG* knockout mutant strain than in the wild type. Such differences were not observed in other mRNAs encoding enzymes involved in acetate metabolism. Analysis by 3′ rapid amplification of cDNA ends suggested that RNase E/G cleaves the *aceA* mRNA at a single-stranded AU-rich region in the 3′ untranslated region (3′-UTR). The *lacZ* fusion assay showed that the 3′-UTR rendered *lacZ* mRNA RNase E/G dependent. These findings indicate that RNase E/G is a novel regulator of the glyoxylate cycle in *C. glutamicum*.

In most bacteria, the rate of mRNA decay depends on the initial cleavage, performed mainly by endoribonucleases of the RNase E/G family (2, 13, 31). *Escherichia coli* has two RNase E/G homologs, RNase E and RNase G. RNase E is an essential multifunctional RNase, which was initially discovered as a 5S rRNA-processing enzyme (18). RNase E also has a global role in the degradation of mRNA (2). The *E. coli* RNase G was originally identified as an endoribonuclease involved in the maturation of 16S rRNA (33, 48). RNase G is dispensable for viability (33, 48); RNase G was suggested to be involved in the regulation of central metabolism (26, 32, 42). In RNase G mutant cells, glycolysis is accelerated and pyruvic acid is consequently overproduced (42).

Both RNase E and RNase G cleave their target RNAs within single-stranded AU-rich regions (34, 38, 46). RNase E and G are known to prefer substrates with 5′-monophosphate ends to those with triphosphate ends (24, 35, 46). However, RNase E also cleaves certain mRNA substrates in a 5′-end-independent pathway referred to as "direct entry" (3, 22, 29). A recent study strongly suggested that the noncatalytic C-terminal half of RNase E has an important role in direct entry (1).

Corynebacterium glutamicum is a Gram-positive, nonpathogenic soil bacterium that has been widely applied in the industrial production of numerous metabolites, including amino acids and organic acids (5, 23, 30). *C. glutamicum* is particularly useful for large-scale production of glutamic acid and lysine. More than 2 million tons of glutamate as well as 1.5 million tons of lysine per year are produced using this bacterium (5). Since these two major metabolites produced from *C. glutamicum* are derived from tricarboxylic acid (TCA) cycle intermediates, the regulation of the TCA cycle and related pathways such as the glyoxylate cycle has been the subject of intensive study (6).

When acetate or a carbon source entering the central metabolism via acetyl coenzyme A (acetyl-CoA) is the only carbon source for an organism, the operation of the glyoxylate cycle is required to provide oxaloacetate. The glyoxylate cycle consists of 5 of the 8 reactions of the TCA cycle, and it bypasses the two decarboxylation steps via additional reactions involving isocitrate lyase (ICL) and malate synthase (MS). ICL catalyzes the cleavage of isocitrate to succinate and glyoxylate, and MS condenses glyoxylate with acetyl-CoA to form malate (17). It is known that *E. coli* and *Bacillus subtilis* do not utilize glucose and acetate simultaneously but preferentially use glucose (9, 19). On the other hand, *C. glutamicum* can coutilize glucose and acetate (17).

The expression of the *aceA* gene of *C. glutamicum* encoding ICL and that of the *aceB* gene encoding MS are positively regulated by the Lux R-type transcriptional regulator RamA (45). The *aceA* and *aceB* genes are also negatively regulated by the transcriptional regulator RamB in the presence of glucose (45). In addition, GlxR, a homolog of cyclic AMP (cAMP) receptor protein (CRP), negatively regulates the expression of *aceA* and *aceB* in a cAMP-dependent manner (45). In contrast to the situation in *E. coli* and other bacteria, the intracellular cAMP level in *C. glutamicum* is elevated during growth on glucose and lowered during growth on acetate (28). Thus, GlxR acts as a repressor of *aceA* and *aceB* during growth on glucose, when the cAMP level is high, but not during growth on acetate, when the cAMP level is low (28).

In contrast to *E. coli*, *B. subtilis*, a low-G+C Gram-positive bacterium, does not contain an RNase E/G ortholog. Instead, RNase J1/J2 and RNase Y play a central role in mRNA metabolism (4). *C. glutamicum* has one RNase E/G ortholog (NCgl2281) and one RNase J ortholog (NCgl1895), but no RNase Y. A previous study suggested that *C. glutamicum* RNase E/G was more closely related to *E. coli* RNase G than to RNase E (36). We then showed that RNase E/G is involved in the processing of 5S rRNA, although

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

Strain or plasmid	Relevant characteristic(s)	Source or reference
C. glutamicum strains		
ATCC 31831	Wild type	Laboratory stock
D2281	Δ*rneG*	37
Plasmids		
pECt	*E. coli-C. glutamicum* shuttle vector; Km^r; multiple cloning site; ColE1 *ori*; *trc* promoter; $lacI^q$	43
pECtS	Same as pECt but with Δ$lacI^q$-*trc* promoter	This study
pCrneFL	pECt with *rneG*	36
pNaceA	pECt with *aceA*	This study
pA5UZ	pECtS with *aceA* 5′-UTR-*lacZ* fusion gene	This study
pZA3U	pECt with *lacZ-aceA* 3′-UTR fusion gene	This study

it is not essential for cell viability (37). Here we show that RNase E/G is a novel posttranscriptional regulator of the glyoxylate cycle in *C. glutamicum*.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F′ (*traD36 proAB*$^+$ $lacI^q$ *lacZ*ΔM15)] and JM110 [*dam dcm supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr tsx* Δ(*lac-proAB*)/F′(*traD36 proAB*$^+$ *lac* I^q *lacZ*ΔM15)] were used for plasmid construction. The *C. glutamicum* strains and plasmids used in this study are listed in Table 1. 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 (14) containing an appropriate carbon source, 5.0 g/liter $(NH_4)_2SO_4$, 5.0 g/liter urea, 21 g/liter morpholinepropanesulfonic acid (MOPS), 1.0 g/liter K_2HPO_4, 1.0 g/liter KH_2PO_4, 0.25 g/liter $MgSO_4 \cdot 7H_2O$, 0.01 g/liter $CaCl_2$, 16.4 mg/liter $FeSO_4 \cdot 7H_2O$, 10 mg/liter $MnSO_4 \cdot H_2O$, 0.2 mg/liter $CuSO_4 \cdot 5H_2O$, 1.0 mg/liter $ZnSO_4 \cdot 7H_2O$, 0.2 mg/liter $NiCl_2 \cdot 6H_2O$, 0.2 mg/liter biotin, and 1.0 mg/liter thiamine (pH 6.8) was used as the minimal medium. Kanamycin was added when culturing cells carrying plasmid. D-Glucose, D-fructose, D-sucrose, D-ribose, L-arabinose, sodium acetate, or sodium gluconate (1% each) was added as a carbon source. The cells were grown at 30°C, and cell growth in the liquid medium was monitored by measuring the optical density at 660 nm (OD_{660}). Overnight cultures grown on CGC minimal medium containing 1% glucose were washed and then inoculated into fresh CGC minimal medium containing either 1% glucose or 1% sodium acetate.

Plasmid construction. A 2.25-kb DNA fragment containing the *aceA* gene fragment and covering 640 bp upstream of the translational start site and 282 bp downstream of the end of the coding sequence was amplified by performing PCR using the primers 5′-GGTGCTGTCAGTTCCATGG TTGTC-3′ and 5′-GAGGATCCGAACACGCAACGAGAG-3′ (the artificially generated NcoI and BamHI sites are underlined). The amplified fragment was digested with NcoI and BamHI. The digested DNA fragment was cloned into *E. coli-C. glutamicum* shuttle vector plasmid pECt (43) digested with the same enzymes. The newly constructed plasmid was named pNaceA.

A PCR protocol was used to delete the DNA fragment containing $lacI^q$ and the *trc* promoter from the vector plasmid pECt. A 5.7-kb DNA fragment was amplified by PCR using the primers 5′-CGGATAACCGCGGC ACACAGGAAAC-3′ and 5′-GGCATACTCCGCGGCATCGTATAA C-3′ (the artificially generated SacII sites are underlined). The amplified fragment was digested with SacII and subjected to self-ligation. The newly constructed vector plasmid was named pECtS.

A 0.67-kb upstream region of the *aceA* gene containing the promoter sequence, the 5′ untranslated region (5′-UTR), and the first 14 bp of the coding region was amplified by performing PCR using the primers 5′-G GTGCTGTCAGTTCCATGGTTGTC-3′ and 5′-GCGGTACGTGGATC CCCAACGTTT-3′ (the artificially generated NcoI and BamHI sites are underlined). A 3.09-kb DNA fragment containing the *E. coli lacZ* gene fragment and covering 4 bp upstream of the translational start site and 3 bp downstream of the end of the coding sequence was amplified by performing PCR using the primers 5′-CACAGGATCCAGCTATGACCATG ATT-3′ and 5′-CATGGCCTGCCCGGTACCTATTATT-3′ (the artificially generated BamHI and KpnI sites are underlined). The PCR products were digested with BamHI and ligated. The ligated DNA fragment was digested with NcoI and KpnI. The digested DNA fragment was cloned into plasmid pECtS digested with the same enzymes. The newly constructed plasmid was named pA5UZ.

A two-step PCR protocol was used to generate the gene fusion product consisting of *lacZ* and the *aceA* 3′-UTR. A 3.1-kb DNA fragment was amplified by PCR using the primers 5′-CGCCATGGAAAGGAATAATT ACTCTAATGACCATGATTACGGATTCAC-3′ and 5′-CTGTAGGTCC TAGTTTTTTTGACACCAGACCAACT-3′ (the artificially generated NcoI site is underlined). The 5′ primer contains the Shine-Dalgarno (SD) sequence of the *C. glutamicum pyc* gene and the first 22 bp of the coding region of the *E. coli lacZ* gene. The 3′ primer contains the *E. coli lacZ* gene fragment lacking the last 3 bp of the coding region and a 15-bp downstream region of the *aceA* gene containing the last 6 bp of the coding region in frame. A 153-bp DNA fragment was amplified by PCR using the primers 5′-GTCTGGTGTCAAAAAAACTAGGACCTACAGGTTCT-3′ and 5′-TCTTTCGGAAGCTTTGCAGTCAACA-3′ (the artificially generated HindIII site is underlined). The 5′ primer contains the *lacZ* gene fragment lacking the last 3 bp of the coding region and a 20-bp downstream region of the *aceA* gene containing the last 6 bp of the coding region in frame. The two amplicons were annealed to each other and used in a second round of PCR to generate the *lacZ-aceA* 3′-UTR fusion gene using the primers 5′-CGCCATGGAAAGGAATAATTACTCTAATGAC CATGATTACGGATTCAC-3′ and 5′-TCTTTCGGAAGCTTTGCAGTC AACA-3′ (the artificially generated NcoI and HindIII sites are underlined). The amplified fragment was digested with NcoI and HindIII. The digested DNA fragment was cloned into the pECt plasmid (43) digested with the same enzymes. The newly constructed plasmid was named pZA3U.

Analysis of cellular proteins. Cells suspended in sodium phosphate buffer (50 mM, pH 7.0) were disrupted by sonication. After the removal of unbroken cells, cell lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the gel was stained with Coomassie brilliant blue. The 45-kDa protein band was cut out and analyzed by mass spectrometry (MALDI-TOF/TOF ultrafleXtreme; Bruker Daltonics, Inc., Billerica, MA).

Total RNA extraction. Total cellular RNA from *C. glutamicum* ATCC 31831 and D2281 was isolated as described previously (37). Briefly, overnight cultures grown on CGC minimal medium containing 1% glucose at 30°C were washed and then inoculated into fresh CGC minimal medium containing 1% sodium acetate. Two volumes of RNA Protect bacterial reagent (Qiagen, Valencia, CA) were added directly to one volume of exponentially growing cultures at an OD_{660} of ~1 (in logarithmic phase) to stabilize cellular RNAs. The cells were harvested by centrifugation at 5,000 × *g* for 10 min at 25°C, and total cellular RNAs were isolated using an RNeasy minikit (Qiagen). Total RNA was treated with DNase I at 37°C for 1 h.

Northern hybridization. For Northern analysis of *aceA* mRNA and 16S rRNA, total RNAs were separated using the formaldehyde-free RNA gel kit (1% agarose) (Amresco, Solon, OH). A hybridization probe labeled with digoxigenin-dUTP (DIG-dUTP) was generated using the PCR DIG probe synthesis kit (Roche Diagnostics, Basel, Switzerland). For the *aceA* mRNA, the hybridization probe was amplified by PCR using the primers 5′-CTACACCGCAGACCAGGTAG-3′ and 5′-CTGGCCTTCTTCAGTG GAAC-3′. For the 16S rRNA, the hybridization probe was amplified by PCR using the primers 5′-GTGGAGAGTTTGATCCTGGCTCAGGA C-3′ and 5′-GAAAGGAGGTGATCCAGCCGCACCTTC-3′. Northern hybridizations were performed according to the procedures described in the DIG Northern starter kit (Roche Diagnostics). The hybridization tem-

TABLE 2 Primers used in qRT-PCR analysis

Target gene	5′ primer sequence	3′ primer sequence	Reference or source
16S rRNA-1	AGAGTTTGATCCTGGCTCA	ACGTGTTACTCACCCGTTCG	21
16S rRNA-2	ACGTTCCCGGGCCTTGTACA	CGGCTACCTTGTTACGAC	21
aceA	ATGTCAAACGTTGGAAAGCC	TGTGCTCCTCGATGACGGAA	21
aceB	TGACTGAACAGGAACTGTTGTC	AGGGAGTACCGCTTCGGTTA	21
ack	CTCCGGTTCATCTTCCATCA	GAGATCGAACGCCAGGTTTA	This study
pta	CTCTGATCACCACGGTCAAC	TCCTACACCGATGATGAGAG	This study
ramA	GGATACCCAGCGGATTAAAG	CGATTGTCCTGCAACACAGT	This study
gltA	TGTTTGAAAGGGATATCGTG	AGTCTCAGACAGCATCTTGC	21
acn	GCACCCTTGAAGTTGGCGAC	CGGTACGAAGAAGGTTCTCT	21
icd	AAGCACCGCTGCTCGCGACCTA	CGTCCAGCGAGTGAAATGTC	21

perature was 50°C. Positive hybridization bands were detected using the CDP-Star reagent (Roche Diagnostics) with exposure times between 30 s and 15 min. The amount of RNA was measured on the basis of the band intensity by using the Just TLC software (Sweday, Lund, Sweden). For the rifampin chase experiment, exponentially growing cells were treated with 150 μg/ml rifampin to inhibit the *de novo* synthesis of RNA, and after the specified times, total RNA was extracted and analyzed as described above.

qRT-PCR. The mRNA was quantified using the Eco Real-Time PCR System (Illumine, Inc., San Diego, CA). Primers used in this quantitative real-time PCR (qRT-PCR) analysis are listed in Table 2. A 50-ng total RNA sample was used for each RT-PCR with each primer pair using the QuantiFast SYBR green RT-PCR kit (Qiagen) according to the manufacturer's instructions. Negative controls with no reverse transcriptase were included with each RNA sample to rule out DNA contamination. Amplicons were run on a 2% (wt/vol) agarose gel to verify that only a single band was produced. The target gene transcripts were normalized to the reference gene transcript (16S rRNA) from the same RNA sample. Each gene was analyzed using RNA isolated from three independent samples. The cycle threshold (C_T) for each sample was generated according to the procedures described in the Eco real-time PCR system user guide.

Primer extension analysis. Primer extension analysis was performed as described previously (37). Briefly, the 5′ end of *aceA* mRNA was determined by nonradioactive primer extension analysis. Aliquots of 16 μg of total RNA extracted from ATCC 31831 and D2281 cells were used for primer extension with Superscript II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA) and the biotinylated oligonucleotide 5′-GTGAT GCCGTTCCAACGAG-3′, which was complementary to a region within the *aceA* mRNA coding region. The primer extension products were separated on a denaturing polyacrylamide gel (8 M urea, 6% gel) together with sequencing ladders obtained using the same primer. The template for the sequencing ladders was PCR amplified using the primers 5′-GGTGC TGTCAGTTCCATGGTTGTC-3′ and 5′-GAGGATCCGAACACGCAA CGAGAG-3′. The separated products were detected by chemiluminescence using a Phototope-Star detection kit (New England BioLabs Inc., Beverly, MA).

3′ RACE. 3′ rapid amplification of cDNA ends (3′-RACE) analysis was performed as described previously (37). Total RNA was treated with DNase I and dephosphorylated with calf intestinal phosphatase. Dephosphorylated RNA was ligated with the 3′ RNA adapter 5′-P-UUCACUGU UCUUACAGGUUCGCCGGCG-idT-3′ (Gene Design Inc., Ibaraki, Japan) containing 3′-inverted deoxythymidine (3′idT) at 10°C overnight. Further treatment of the RNA with the ligated 3′ RNA adapter and reverse transcription using the primer 5′-CGAACCTGTAAGAACAGTGAA-3′ complementary to the 3′ adapter and Ready-To-Go You-Prime first-strand beads (GE Healthcare, Buckinghamshire, United Kingdom) were performed. The products of the reverse transcriptions were amplified by two consecutive PCRs, using the adapter-specific primer used for RT-PCR and the *aceA* mRNA-specific primer 5′-GCTTCACCGCTGTTAAG CAC-3′. The 3′ RACE products were separated on a 2% agarose gel. The band of the stable 3′ end was cut out and sequenced using the Applied Biosystems 3730xl DNA analyzer (Applied Biosystems, Foster City, CA).

β-Galactosidase activity. β-Galactosidase activity was determined by the method of Miller (40). Overnight cultures grown on CGC minimal medium containing 1% glucose at 30°C were washed and then inoculated into fresh CGC minimal medium containing either 1% glucose or 1% sodium acetate. To synthesize the LacZ protein from the *lacZ-aceA* 3′ UTR fusion gene, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM when cell growth reached the early exponential phase (OD_{660} of ~0.3). After 5 h of cultivation, the culture was subjected to the β-galactosidase activity assay.

Determination of glucose and sodium acetate concentrations. Glucose concentrations in the culture supernatant were measured using the Biotech-analyzer AS-210 (SakuraSeiki, Tokyo, Japan) with a glucose oxidase sensor. Sodium acetate concentrations in the culture supernatant were measured using the Agilent 7100 capillary electrophoresis (CE) system with the Agilent organic acid solution kit (Agilent Technologies, Santa Clara, CA).

RESULTS

Overproduction of the ICL protein in the Δ*rneG* mutant. In order to examine whether RNase E/G participates in regulation of carbon metabolism at the mRNA level, we first compared protein expression patterns between the wild type and the Δ*rneG* mutant when cells were grown on various kinds of sugars and organic acids as the sole carbon source. The total cellular proteins obtained from the cultures were subjected to SDS-PAGE. The carbon sources examined were D-glucose, D-fructose, D-sucrose, D-ribose, L-arabinose, sodium acetate, and sodium gluconate. Although most *C. glutamicum* strains do not have the ability to utilize L-arabinose, *C. glutamicum* ATCC 31831, which we used as the wild-type strain in this study, is able to utilize L-arabinose as the sole carbon source (27). As shown in Fig. 1, a Δ*rneG* mutant strain, D2281, grown on sodium acetate as the sole carbon source synthesized a large amount of protein with a molecular mass of ~45 kDa, compared with the wild type; the Δ*rneG* mutant produced 2.3-fold ± 0.4-fold ($n = 3$) more 45-kDa protein than the wild type. The overproduction of the 45-kDa protein reverted to wild-type levels when the plasmid pCrneFL, carrying the *rneG* gene (36), was introduced into the D2281 strain (data not shown). The 45-kDa protein band was identified as ICL with a molecular mass of 47.3 kDa by mass spectrometry. Overproduction of ICL in the D2281 strain was also observed when cells were grown on sodium acetate plus glucose (data not shown).

Increased stability of *aceA* mRNA in the Δ*rneG* mutant. It was likely that overproduction of ICL by the Δ*rneG* mutant was due to the increased stability of the *aceA* mRNA encoding ICL. To examine the effect of the Δ*rneG* mutation on the stability of the *aceA* mRNA, we first carried out Northern hybridization. The amount of *aceA* mRNA in the Δ*rneG* mutant grown on sodium

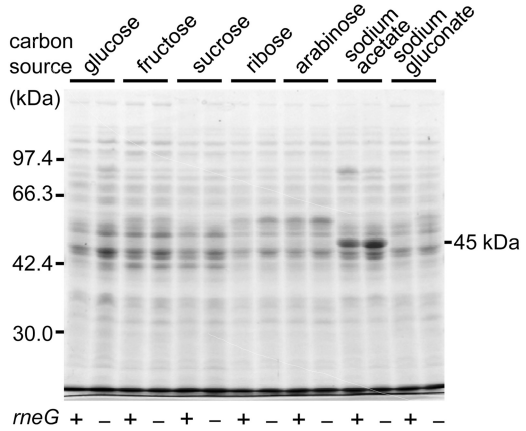

FIG 1 Overproduction of the ICL protein in the Δ*rneG* mutant. ATCC 31831 (wild type [+]) and D2281 (Δ*rneG* [−]) cells were grown on various kinds of sugars and organic acids as the sole carbon source. The total cellular proteins obtained from the cultures were analyzed by 10% SDS-PAGE. A Coomassie brilliant blue-stained gel is shown. The carbon sources are shown at the top of the gel. The position of the 45-kDa protein is shown to the right of the gel.

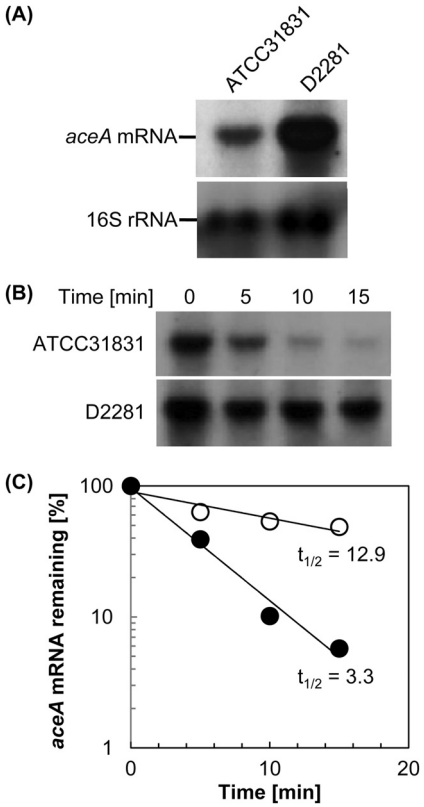

FIG 2 Increased stability of *aceA* mRNA in the Δ*rneG* mutant. (A) Intracellular levels of *aceA* mRNA and 16S rRNA in ATCC 31831 (wild type) and D2281 (Δ*rneG*) strains. Total RNAs from ATCC 31831 and D2281 were analyzed by Northern hybridization, as described in Materials and Methods, using specific probes for *aceA* mRNA and 16S rRNA. (B) Measurement of the half-lives of *aceA* mRNA in the ATCC 31831 and D2281 strains. Rifampin was added to exponentially growing cultures of ATCC 31831 and D2281 at time zero, and at the indicated times, total RNAs were isolated and analyzed by Northern hybridization using a specific probe for *aceA* mRNA. (C) The intensities of the hybridized bands in Fig. 2B were quantified with Just TLC software and the half-life ($t_{1/2}$) (min) of *aceA* mRNA was calculated. Filled circles, ATCC 31831; open circles, D2281. The half-lives shown are representative of those from three independent rifampin chase experiments with comparable results.

acetate as the sole carbon source was significantly higher than that in the wild type (Fig. 2A). We expected that RNase E/G is involved in degradation of not only *aceA* mRNA but also other mRNAs encoding proteins involved in acetate metabolism. Therefore, we performed qRT-PCR analysis. Total RNA was prepared from the cultures growing exponentially on sodium acetate as the sole carbon source. The RNA was subjected to qRT-PCR analyses using specific primer sets for genes involved in acetate metabolism. The selected genes were *aceA* (encoding ICL), *aceB* (encoding MS), *ack* (encoding acetate kinase), *pta* (encoding phosphotransacetylase), *ramA* (encoding RamA, which positively regulates *aceA*), *gltA* (encoding citrate synthase), *acn* (encoding aconitase), and *icd* (encoding isocitrate dehydrogenase [ICD]). As shown in Fig. 3, the *rneG* deletion resulted in an increase in *aceA* mRNA level of approximately 3-fold. However, the *rneG* deletion did not affect the amounts of the other mRNAs. These results suggest that RNase E/G specifically degrades the *aceA* mRNA among these mRNAs, although this does not exclude the possibility that the RNase E/G degrades mRNAs other than the *aceA* mRNA.

We then performed a rifampin chase experiment. Exponentially growing cells on sodium acetate as the sole carbon source were treated with 150 μg/ml rifampin to prevent further initiation of transcription. Total RNA was isolated at various times after the addition of rifampin, and the rates of decay of the *aceA* mRNA in the wild type and the Δ*rneG* mutant were determined. The *aceA* mRNA degraded with a half-life of 3.3 min in the wild type. In contrast, it degraded with a half-life of 12.9 min in the Δ*rneG* mutant (Fig. 2B and C). The prolonged half-life of the *aceA* mRNA in the Δ*rneG* mutant was also confirmed by qRT-PCR (data not shown). These results indicate that the overproduction of ICL in the Δ*rneG* mutant is due to the increased stability of the *aceA* mRNA.

Involvement of the 3′-UTR of *aceA* mRNA in RNase E/G-dependent degradation. To determine the cleavage site of the *aceA* mRNA by RNase E/G, we first carried out primer extension analysis using total RNA isolated from wild-type and Δ*rneG* mutant cells grown on sodium acetate as the sole carbon source. In the case of *E. coli*, the degradation of many mRNAs is often initiated by RNase E cleavage in the 5′ untranslated region (5′-UTR) of the mRNA (25). However, as shown in Fig. 4A, only one 5′ end, which corresponded to the previously identified transcriptional start site of the *aceA* gene (17), was detected both in the wild type and in the Δ*rneG* mutant (Fig. 4A). This primer extension analysis suggested that RNase E/G-mediated degradation of *aceA* mRNA is 5′-UTR independent. It is also reported that several *E. coli* mRNAs, such as those of the *rpsO* and *cspA* genes, are subjected to RNase E cleavage in their 3′-UTR (20, 22). Therefore, we next performed 3′ RACE analysis to estimate the sites of RNase E/G cleavage in the *aceA* mRNA using total RNAs isolated from the wild type and the Δ*rneG* mutant harboring the pNaceA plasmid. The pNaceA plasmid expresses the *aceA* mRNA from the native promoter. As shown in Fig. 4B, a stable 3′ end of the *aceA* mRNA was detected in the Δ*rneG* mutant harboring pNaceA, while such a stable 3′ end was not seen in the wild type harboring pNaceA. This might be because the RNase E/G cleavage product is degraded rapidly by 3′-to-5′ exoribonucleases. The major PCR prod-

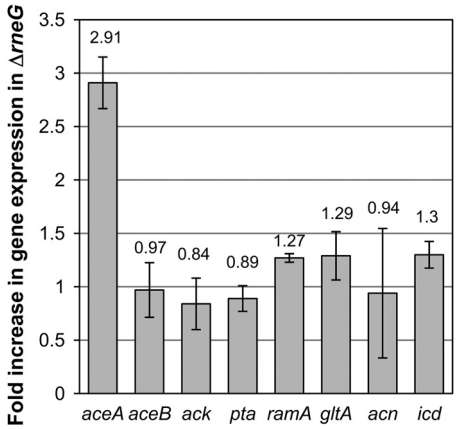

FIG 3 Relative expression levels of genes involved in acetate metabolism. qRT-PCR was used for analysis of total RNA isolated from ATCC 31831 (wild type) and D2281 (Δ*rneG*) cells grown on CGC minimal medium containing 1% sodium acetate. Each column represents the gene of interest shown on the *x* axis. The fold increase in gene expression in the Δ*rneG* mutant corresponds to the ratio of the transcript level of each gene in the wild-type strain to that of the corresponding gene in the Δ*rneG* mutant. The levels of gene transcripts were measured in triplicate and normalized using 16S rRNA transcript levels.

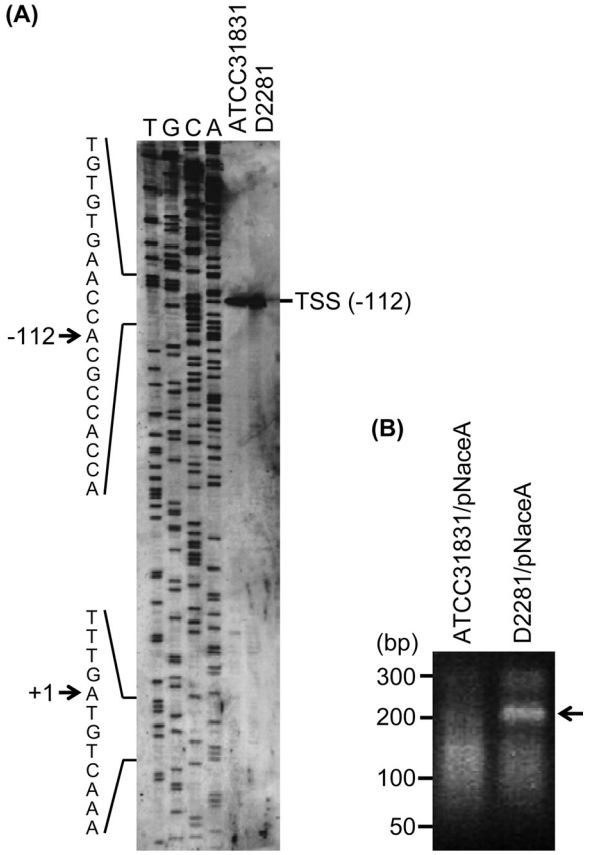

FIG 4 Estimation of the sites of RNase E/G cleavage in *aceA* mRNA. (A) Primer extension analysis of *aceA* transcripts from ATCC 31831 and D2281 cells. Primer extension products derived from the total RNAs of ATCC 31831 (wild type) and D2281 (Δ*rneG*) grown on CGC minimal medium containing 1% sodium acetate were separated on a denaturing polyacrylamide gel along with sequencing ladders (T, G, C, and A) obtained using the same primer. Parts of the *aceA* transcript sequence are indicated on the left side. The positions of the transcriptional start site (TSS) and the translational start site are indicated by arrows. The translational start site of the *aceA* transcript is defined as +1. The detected 5′ end of the *aceA* transcript corresponded to the TSS (−112). (B) 3′ RACE products derived from total RNA isolated from ATCC 31831 and D2281 cells harboring pNaceA. 3′ RACE products were obtained as described in Materials and Methods. The pNaceA plasmid expresses the *aceA* mRNA from the native promoter. A 50-bp ladder marker is shown on the left side. The position of the stable 3′ end detected in the Δ*rneG* mutant is shown on the right side. The band of the stable 3′ end was cut out and sequenced.

uct obtained from the Δ*rneG* mutant harboring pNaceA was isolated from the 2% agarose gel and subjected to sequencing analysis. The 3′ end was located at 63 nucleotides (nt) downstream of the end of the *aceA* coding sequence. Using the RNA fold software (mfold [http://mfold.rna.albany.edu/?q=mfold]), the secondary structure of the *aceA* 3′-UTR was predicted. As shown in Fig. 5, a GC-rich hairpin loop followed by a run of U residues was found at the 3′ end of the transcript, which resembles typical bacterial rho-independent transcription terminators. It was also predicted that the UAG stop codon can be occluded by base pairing with sequences further downstream in the 3′-UTR (Fig. 5). In addition, a single-stranded AU-rich region was found between the two stem-loops (Fig. 5). Considering the substrate preference of *E. coli* RNase E and G, RNase E/G cleavage might occur within this AU-rich region, which is somewhat reminiscent of the *E. coli rpsO* and *cspA* mRNA (20, 22).

3′-UTR-dependent overproduction of the LacZ protein from a *lacZ-aceA* 3′-UTR fusion gene in the Δ*rneG* mutant. In order to verify the possibility that the 3′-UTR is involved in stability control by RNase E/G, a *lacZ-aceA* 3′-UTR fusion gene was constructed as described in Materials and Methods (Fig. 6B). An *aceA*-5′-UTR-*lacZ* gene was also constructed as a control (Fig. 6A). The plasmid pA5UZ, carrying a 0.67-kb upstream region of the *aceA* gene containing the promoter sequence, the 5′-UTR, and the first 14 bp of the coding region, joined in frame with the *E. coli lacZ* gene, was introduced into the wild type and the Δ*rneG* mutant. As shown in Fig. 6C, in both the wild-type and Δ*rneG* mutant cells, the expression of the ICL-LacZ fusion protein was repressed in glucose minimal medium. On the other hand, in both the wild-type and Δ*rneG* mutant cells, the expression of the ICL-LacZ fusion protein significantly increased in sodium acetate minimal medium (Fig. 6C). As expected, there was no significant difference in the expression of the ICL-LacZ fusion protein between the wild type and the Δ*rneG* mutant (Fig. 6C). β-Galactosidase activities were 8.2- and 9.9-fold higher in sodium acetate minimal medium than in glucose minimal medium in the wild type and in the Δ*rneG* mutant, respectively (Fig. 6A). In agreement with the results of SDS-PAGE, there was no significant difference in β-galactosidase activity between the wild type and the Δ*rneG* mutant (Fig. 6A). These results confirmed that the RNase E/G-mediated degradation of the *aceA* mRNA is 5′-UTR independent.

Plasmid pZA3U, carrying the *E. coli lacZ* gene lacking the last 3 bp of the coding region, joined with a 149-bp downstream region of the *aceA* gene containing the last 9 bp of the coding region and the 3′-UTR in frame, was introduced into the wild type and the Δ*rneG* mutant. In the absence of IPTG, the expression of the LacZ protein from the *lacZ-aceA* 3′-UTR fusion gene was not detected on a Coomassie brilliant blue-stained gel (data not shown). In the presence of 0.1 mM IPTG, the LacZ protein was overproduced in the Δ*rneG* mutant both in glucose and in sodium acetate minimal medium (Fig. 6C). β-Galactosidase activities were 1.6- and 2.3-fold higher in the Δ*rneG* mutant than in the wild type in glucose

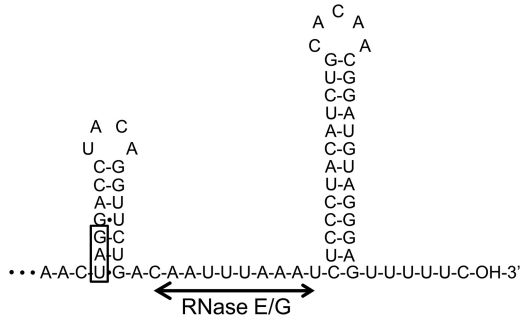

FIG 5 Predicted secondary structure of the *aceA* 3′-UTR. RNA fold software (mfold [http://mfold.rna.albany.edu/?q=mfold]) was used to predict the secondary structure of the *aceA* 3′-UTR. The UAG stop codon is boxed. The horizontal line represents the possible cleavage site of RNase E/G (see the text for details).

and in sodium acetate minimal medium, respectively (Fig. 6B). These results indicate that RNase E/G-mediated degradation of the *aceA* mRNA is 3′-UTR dependent.

Growth of the Δ*rneG* mutant on sodium acetate as the sole carbon source. We examined the growth of the wild type and the Δ*rneG* mutant on sodium acetate as the sole carbon and energy source. Overnight cultures of the wild type and the Δ*rneG* mutant grown on CGC minimal medium containing 1% glucose were washed and then inoculated into fresh CGC minimal medium containing either 1% glucose or 1% sodium acetate. Cell growth was monitored by measuring the OD_{660}. The growth pattern and glucose consumption rate of the Δ*rneG* mutant on glucose minimal medium were very similar to those of the wild type (Fig. 7A). On the other hand, the growth of the wild type on CGC minimal medium containing 1% sodium acetate started after a lag phase of 2 to 3 h, while such a lag phase was not observed for the Δ*rneG* mutant (Fig. 7B). However, the growth rates were almost identical between the wild-type and Δ*rneG* mutant strains. The doubling times in sodium acetate medium were 147 ± 5.0 min in the wild type and 153 ± 4.0 min in the Δ*rneG* mutant. The Δ*rneG* mutant consumed sodium acetate faster than did the wild type, while the sodium acetate consumption rate of the Δ*rneG* mutant was almost the same as in the wild type (Fig. 7B). These results suggest that RNase E/G is involved in the adaptation to the change of carbon source from glucose to acetate.

DISCUSSION

In this study, we searched for *in vivo* target RNAs of RNase E/G other than the 5S rRNA precursor. We found that ICL was overproduced in the Δ*rneG* mutant strain compared with the wild-type strain when cells were grown on sodium acetate minimal medium. The *aceA* mRNA accumulated in the Δ*rneG* mutant cells. The rifampin chase experiment showed that accumulation of *aceA* mRNA was due to its increased stability. These results indicate that RNase E/G is involved, at least in part, in the degradation of *aceA* mRNA. qRT-PCR analysis also suggests that RNase E/G specifically degrades *aceA* mRNA, although this does not exclude the possibility that RNase E/G degrades mRNAs other than the *aceA* mRNA. Since ICL is a key enzyme in the glyoxylate cycle, we conclude that RNase E/G is a novel regulator of the glyoxylate cycle in *C. glutamicum*. In order to identify other target RNAs, it is necessary to carry out comprehensive transcriptome and/or proteome analyses in the Δ*rneG* mutant cells.

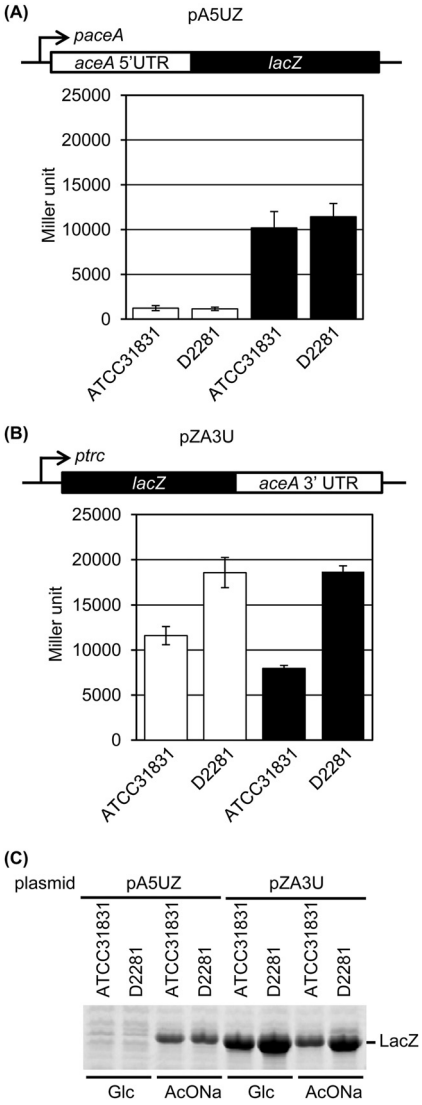

FIG 6 Effect of the Δ*rneG* mutation on the expression of the *aceA-lacZ* fusion genes. (A) β-Galactosidase activity in ATCC 31831 (wild type) and D2281 (Δ*rneG*) cells harboring the pA5UZ plasmid. Plasmid pA5UZ carries the *aceA* 5′-UTR-*lacZ* fusion gene. A schematic diagram of the *aceA* 5′-UTR-*lacZ* fusion gene and the *aceA* promoter *paceA* is shown at the top. The wild type and the Δ*rneG* mutant were grown on CGC minimal medium containing either 1% sodium acetate (filled bars) or 1% glucose (blank bars). (B) β-Galactosidase activity in ATCC 31831 and D2281 cells harboring the pZA3U plasmid. Plasmid pZA3U carries the *lacZ-aceA* 3′-UTR fusion gene. A schematic diagram of the *lacZ-aceA* 3′-UTR fusion gene and the *trc* promoter *ptrc* is shown at the top. The wild type and the Δ*rneG* mutant were grown on CGC minimal medium containing either 1% sodium acetate (filled bars) or 1% glucose (blank bars). To synthesize the LacZ protein from the *lacZ-aceA* 3′-UTR fusion gene, IPTG was added to a final concentration of 0.1 mM. Enzyme activity is expressed in Miller units. All the values are derived from at least three independent cultivations, and the error bars represent the standard deviations. (C) Expression of the LacZ protein expressed from the pA5UZ and pZA3U plasmids was examined by 7.5% SDS-PAGE. The position of the LacZ protein is shown to the right of the gel.

What is the physiological meaning of the regulation of *aceA* expression by RNase E/G? In the case of *E. coli*, the flux between the TCA cycle and the glyoxylate cycle is well balanced by several factors. First, the genes of the glyoxylate cycle are induced only

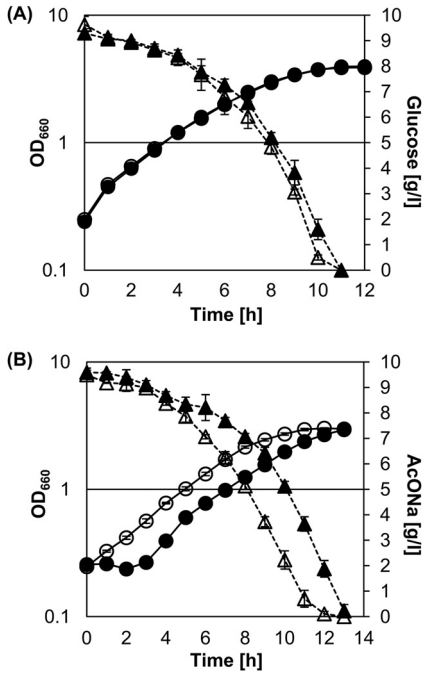

FIG 7 Growth and carbon consumption of the ATCC 31831 (wild type) and D2281 (Δ*rneG*) strains. For the growth curve, overnight cultures of the wild type (filled circles) and the Δ*rneG* mutant (open circles) grown on CGC minimal medium containing 1% glucose were washed and then inoculated into fresh CGC minimal medium containing either 1% glucose (A) or 1% sodium acetate (B). Cell growth was monitored by measuring the OD_{660}. The consumption (filled triangles, ATCC 31831; open triangles, D2281) of glucose (A) and sodium acetate (B) was determined via an enzyme electrode glucose sensor and CE, respectively. All values are from at least three independent cultivations, and the error bars represent the standard deviations.

when acetate or fatty acids are the sole carbon source (17). In *E. coli*, the ICL and MS genes are organized as an operon together with the *aceK* gene encoding ICD kinase/phosphatase (17). AceK phosphorylates ICD; this lowers ICD turnover and prevents a high flux of isocitrate through the TCA cycle (10, 16). Since dephosphorylation of ICL leads to loss of catalytic activity (41), phosphorylation is assumed to increase the flux of isocitrate through the glyoxylate cycle in *E. coli* (11).

In contrast to the case with *E. coli*, the induction of the *C. glutamicum aceA* and *aceB* genes in the presence of acetate occurs independently of the presence or absence of an additional carbon and energy source (17). In addition, the *aceA* and *aceB* genes are not organized as an operon. Instead, the two genes are clustered on the chromosome and transcribed in divergent directions (17). Furthermore, there is no evidence for phosphorylation control of ICD in *C. glutamicum* (15). Overall, the flux between the TCA cycle and the glyoxylate cycle has to be well balanced, and the regulation of the *aceA* and *aceB* genes in *C. glutamicum* is assumed to be quite different from that seen in other bacteria. In the case of *C. glutamicum*, we showed that the expression of ICL is negatively regulated by RNase E/G. This is the first report showing that the glyoxylate cycle is regulated at the mRNA level. A biochemical study suggested that the inhibition or activation of MS plays only a minor role in controlling the carbon flow in the glyoxylate cycle (17); therefore, it is reasonable that RNase E/G specifically degrades *aceA* mRNA. We assume that the glyoxylate cycle is switched off instantly when acetate is all consumed. Therefore, RNase E/G-mediated degradation of *aceA* mRNA may permit quick changes in cell metabolism. As shown in Fig. 7B, the growth of the wild type and the Δ*rneG* mutant on sodium acetate minimal medium suggests that RNase E/G is involved in the adaptation to the change of carbon source from glucose to acetate, although further studies are necessary to clarify this hypothesis.

We were unable to detect new 5′ termini corresponding to the sites of RNase E/G cleavage in the 5′-UTR (Fig. 4A). In addition, there was no significant difference in the expression of LacZ protein from the *aceA* 5′-UTR-*lacZ* fusion gene between the wild type and the Δ*rneG* mutant (Fig. 6A and C). These results indicate that the RNase E/G-mediated degradation of *aceA* mRNA is 5′-UTR independent. Rather, the results of 3′ RACE analysis and the β-galactosidase activity assay indicate that degradation of *aceA* mRNA by RNase E/G is 3′-UTR dependent. The secondary structure of the 3′-UTR of *aceA* mRNA suggests a possible mechanism for the degradation of *aceA* mRNA in *C. glutamicum* (Fig. 5). RNase E/G cleavage truncates the *aceA* mRNA at its 3′ end, leaving the 5′ terminus intact. The preferred RNase E/G cleavage site is located in the single-stranded AU-rich region between two stem-loops (Fig. 5). This situation resembles the mechanisms involved in the cleavage of the *E. coli cspA* and *rpsO* mRNAs. In *E. coli*, RNase E cleaves *cspA* mRNA at a single site between two stem-loops (22). RNase E also controls the stability of the *rpsO* mRNA by the removal of a stable 3′ terminator stem-loop (8, 12). Since the RNase E cleavage site of *cspA* mRNA was determined by an *in vitro* RNase E assay (22, 29), it is necessary to perform an *in vitro* RNase E/G cleavage assay for *aceA*.

The strong stem-loop structure found at the 3′ end of many bacterial mRNAs, which functions as the transcription terminator structure, is resistant to 3′-to-5′ exonucleolytic degradation (39, 44). As shown in Fig. 4B, a stable 3′ end of the *aceA* mRNA was not detected in the wild type even if the amount of *aceA* transcript was increased by the introduction of the plasmid pNaceA. This suggests that RNase E/G cleavage generates a fragment with an unprotected 3′ end which is degraded rapidly by 3′-to-5′ exoribonucleases. In the case of *E. coli*, after the initial endoribonucleolytic cleavage, the degradation products are rapidly degraded by 3′-to-5′ exoribonucleases such as polynucleotide phosphorylase (PNPase) (2). After RNase E cleavage, the *rpsO* mRNA lacking the 3′ terminator stem-loop becomes a substrate for PNPase (7). Since *C. glutamicum* has a PNPase homolog (NCgl1900), this PNPase homolog may degrade *aceA* mRNA promptly after RNase E/G cleavage. Future studies will be necessary to understand how different sequences and structures located at the 3′ end affect the access of RNase E/G to its internal target site.

In the wild type, β-galactosidase activity expressed from the *lacZ-aceA* 3′-UTR fusion gene was about 1.5-fold lower in sodium acetate medium than in glucose medium, while such repression was not seen in the Δ*rneG* mutant (Fig. 6B). When *C. glutamicum* cells are grown on acetate, a large amount of *aceA* mRNA is synthesized. Therefore, it is possible that expression of the *rneG* gene increases to degrade the *aceA* mRNA efficiently when grown on acetate. It is also possible that some unknown factor(s) also participates in the 3′-UTR-dependent degradation of *aceA* mRNA. It is known that an RNA chaperone, Hfq, helps RNase E-dependent degradation of mRNAs in *E. coli*. Hfq facilitates the pairing of small RNAs with their target mRNAs (47). Although *C. glutamicum* does not have an Hfq homolog, it is possible that an uniden-

tified functional counterpart of Hfq and/or a small RNA, which may be induced when grown on acetate, is involved in the 3′-UTR-dependent degradation of *aceA* mRNA by the *C. glutamicum* RNase E/G.

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