RNase E Is Required for Induction of the Glutamate-Dependent Acid Resistance System in *Escherichia coli*

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The *Escherichia coli* RNase E is an essential endoribonuclease involved in processing and/or degradation of rRNAs, tRNAs, and non-coding small RNAs as well as many mRNAs. It is known that RNase E activity is somehow regulated by an RNA-binding protein Hfq, at least in some cases. We searched for proteins that showed changes in expression in both *hfq::cat* and *rne-1* mutant cells as compared with the wild type, and found that a protein band of 49-kDa decreased in these mutant cells at 42 °C, the restrictive temperature for *rne-1*. N-terminal amino acid sequencing identified it as a mixture of GadA and GadB, two isozymes of glutamate decarboxylase involved in glutamate-dependent acid resistance. The *rne-1* mutant as well as the *hfq* mutant showed decreased survival under acidic conditions (pH 2.5). Hfq is known to regulate the expression of GadA/B in RpoS- and GadY small RNA-dependent ways. We examined the expression of these two regulators in *rne-1* mutant cells. In the mutant cells, the induction of GadY was defective at 42 °C, but the expression of RpoS was normal. These results indicate that RNase E is required for induction of the glutamate-dependent acid resistance system in a RpoS-independent manner.

Key words: acid resistance; GAD; glutamate decarboxylase; Hfq; RNase E

RNase E is an essential endoribonuclease in *Escherichia coli*. The phenotype of its temperature-sensitive mutation *rne-1* (originally called *ams-1*) was initially described as increasing the chemical half-life of total cellular RNA.1,2 Another independently isolated mutation, *rne-3071*, was implicated in the maturation process of 5S rRNA from its precursor 9S rRNA.3 RNase E plays an important role in all aspects of RNA metabolism in *E. coli*, including processing and/or decay of rRNAs, tRNAs, non-coding small RNAs, and mRNAs. It forms a multiprotein complex called a degradosome with an exoribonuclease PNase, an RNA helicase RhlB, enolase, and several other minor components.4–6 The degradosome is believed to function as a major RNA degradation machine in *E. coli*. Endoribonucleolytic cleavage by RNase E depends on 5'-end structures7,8 and occurs within single-stranded A and/or U-rich segments.9–11

The Hfq protein, which is encoded by the *hfq* gene,12 was initially identified as a bacterial host factor required for replication of bacteriophage Qβ RNA.13,14 Inactivation of the *hfq* gene in *E. coli* causes a wide variety of phenotypes and alters the expression of many proteins.15,16 An increasing amount of evidence indicates that Hfq pleiotropically regulates the expression of many genes. Hfq often regulates gene expression by modulating mRNA stability and/or translation efficiency via binding to small non-coding RNAs. Hfq binds to several small non-coding RNAs, such as DsrA, RprA, RyhB, Spot42 RNA, and OxyS, as well as to other small RNAs of unknown function. Hfq facilitates their interaction with target mRNAs.17–23

The regulatory function of Hfq in gene expression is somehow mediated by RNase E activity, at least in some cases. It has been reported that Hfq stimulates the decay of *ompA* mRNA by RNase E by interfering with ribosome binding.24–26 We have also reported that Hfq and RNase E are involved in expression of the cell division protein FtsZ.27,28 RNase E and Hfq are also implicated in the regulation of *ptsG* mRNA. In this case, it was found that RNase E and Hfq form a ribonucleoprotein complex that includes a non-coding small RNA. The Hfq-small RNA complex might guide RNase E to the target RNA.29–31

Here we report that the expression of GadA/B decreased both in *hfq::cat* and *rne-1* mutant cells. The *rne-1* mutant as well as *hfq::cat* showed decreased
survival under acidic conditions (pH 2.5) as compared with the wild type. These results indicate that RNase E plays an important role in induction of the glutamate-dependent acid resistance system in E. coli.

Materials and Methods

Bacterial strains and media. E. coli K-12 strain CSH26 (ΔftrA Δ(ftrA-pro) thi) and its derivatives HAT10 (the same as CSH26 but hfg10::cat) and HAT103 (the same as CSH26 but zpc-726::Tn10 mecJ) were used in this study. Cells were grown in modified Lennox (LB) broth containing 1% Polypepton (Wako Pure Chemical Industries, Osaka, Japan), 0.5% yeast extract, 0.5% NaCl, and 1% glucose (pH 7.0) or modified Luria-Bertani (LB) broth containing 1% Polypepton, 0.5% yeast extract, and 1% NaCl, which was buffered with either 100 mM morpholinepropanesulfonic acid (MOPS; pH 8.0) or 100 mM morpholinethanesulfonic acid (MES; pH 5.5).

Analysis of cellular proteins. Cells grown in LB broth at 30 or 42 °C for 3 h were harvested, suspended in sodium phosphate buffer (50 mM, pH 7.0), and disrupted by sonication. After unbroken cells were removed, cell lysates were analyzed by SDS–polyacrylamide gel electrophoresis. The 49-kDa protein band was blotted onto PVDF membrane, and its N-terminal amino acid sequence was determined with a protein sequencer (ABI model 492 Procise Protein Sequencer, Applied Biosystems, Foster City, CA).

GAD assay. GAD assay was carried out using GAD reagent with minor modifications. The GAD reagent consisted of 1 g of L-glutamic acid, 0.05 g of bromocresol green, 90 g of NaCl, and 3 ml of Triton X-100 per liter. When decarboxylation of L-glutamic acid occurs, the pH of GAD reagent progressively increases and the pH indicator, bromocresol green, changes from yellow to green. Cells were harvested, washed with saline (0.85% NaCl), and suspended in the same solution. An aliquot of cell suspension was transferred to a new tube, and 1 ml of GAD reagent was added. The reaction mixtures were incubated for 1 h at 35 °C.

Acid resistance assay. Cells were grown in LB broth at 42 °C for 2 h to allow GAD expression. An aliquot of culture (100 μl) was added directly into 300 μl of LB adjusted to pH 2.5 with HCl. After 5 min of incubation at 42 °C, cell suspensions were spotted on L agar plates after serial dilutions with phosphate buffered saline (10 mM sodium phosphate buffer pH 7.0, 130 mM NaCl). Colonies were observed after 24 h of incubation at 30 °C.

Northern hybridization. Total cellular RNA was isolated and analyzed by Northern hybridization, as described previously. Briefly, cells grown at 30 or 42 °C for 3 h were harvested and washed with ice-cold TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). The cells were suspended in 100 μl of TE and extracted directly by vigorously mixing them with an equal volume of equilibrated phenol/chloroform. After centrifugation (10,000 × g, 10 min), part of the upper layer was used for analysis. RNA samples were fractionated by formamide-agarose gel electrophoresis (6.5% formaldehyde, 20 mM MOPS pH 7.0, 5 mM sodium acetate, 1 mM EDTA, 1% agarose). RNA samples were transferred onto positively charged nylon membrane (Hybond-N+, Amersham Bioscience, Piscataway, NJ) by the capillary method. DNA probes were prepared by PCR amplification using sets of primers: 5’-CCTACTTCTTTATGAGATCCTAAATC-3’ and 5’-CAATACGCACAGCAAGCT-TGAAAAAGG-3’ for gadvB; 5’-ATGATGACTATGATCAGCATGACGCTGAAAGTTC-3’ and 5’-CTTCGATATCTCCGCCGTGGACCG-GGGACC-3’ for gadY and 5’-ACTCATGGAAT-GTGCTTAATTGCG-3’ and 5’-CCCTCCGCAGAACGGTCAGTGCC-3’ for gadX. The gadBC probe also hybridizes with gadvA mRNA. Amplified DNA fragments were labeled with a Gene Images Kit (Amersham Bioscience) and used for Northern hybridization.

Results

Decreased expression of GadA and GadB proteins in hfg::cat and me-1 mutant cells

RNase E is involved in the regulatory mechanism of gene expression via degradation and/or processing of mRNAs. The action of RNase E on mRNAs is somehow regulated by the RNA-binding protein Hfq, at least in some cases. The involvement of RNase E and Hfq in gene expression is implicated in ompA mRNA, ftsZ mRNA, ftsG mRNA, and ptsG mRNA. In order to investigate the Hfq- and RNase E-dependent regulatory mechanism of gene expression, we searched for proteins which showed changes in expression in both hfg::cat and me-1 mutant cells. The hfg::cat and me-1 mutant strains were grown in LB broth at 30 or 42 °C for 3 h. Cells were harvested and cell lysates were subjected to SDS–polyacrylamide gel electrophoresis. We found a 49-kDa protein band in the wild-type cells grown at 42 °C that was greatly decreased both in the hfg::cat cells and me-1 mutant cells (Fig. 1). This band was not seen in cell lysates grown at 30 °C even in the wild-type cells. The N-terminal amino acid sequence, MD(Q/K)K(L/Q)(L/V)TD, was obtained for this band. This can be considered to be a mixture of MDQKL LTD and MDKKQVTD, which correspond to the N-terminal sequences of GadA and GadB respectively. GadA and GadB are two isozymes of glutamic acid decarboxylase (GAD) with very similar amino acid sequences that are involved in glutamate-dependent acid resistance. These changes were also confirmed by GAD activity assay. As shown in Fig. 2, GAD activity
was induced at 42°C in the wild type, but very weak or almost no induction of GAD activity was seen in the hfq::cat and rne-1 mutants.

**hfq::cat and rne-1 mutants were defective in induction of GAD under acidic conditions**

It is known that GAD production is induced under acidic culture conditions. Hence we investigated whether hfq::cat and rne-1 mutations affect the induction of GAD under acidic conditions. Cells were grown at 30 or 42°C for 3h in LB medium, which was buffered with MES at pH 5.5 or MOPS at pH 8.0. As shown in Fig. 3, the wild-type strain did not show GAD activity when grown at 30°C either at pH 5.5 or at 8.0. GAD activity was induced when grown at 42°C at pH 5.5, but not at pH 8.0. This indicates that GAD induction occurs when cells are grown at 42°C at pH 5.5, at least in this strain under our experimental conditions. The hfq::cat and rne-1 mutant strains were both defective in induction of GAD under the same conditions.

In the experiments detecting 49-kDa GadA/B proteins, the cells were grown in L broth containing 0.1% glucose at 42°C. It is probable that L broth became acidic during cultivation due to oxidation of glucose. Measurement of the pH of the culture broth revealed that this was the case. The pH of cultures reached 5.5–5.7 after 2h at 42°C. Taken together with the above results, it is most likely that acidification of culture broth and a culture temperature of 42°C induced the expression of GadA/B in L broth too. We used L broth and a culture temperature of 42°C to induce GAD in subsequent experiments for experimental convenience.

**hfq::cat and rne-1 mutant strains were deficient in acid resistance**

It is known that GAD is necessary for the process of internal proton consumption that maintains an intracellular pH suitable for cell survival. We assayed the ability of the hfq::cat and rne-1 mutant strains to survive under acidic conditions. Cells were grown in L broth at 42°C for 2h were challenged at pH 2.5 at 42°C. After 5 min of acid challenge, cell viability was examined by spotting cell suspensions on L agar plates after serial dilutions (1/10 each).
This indicates that the hfq::cat and rne-1 mutant strains are deficient in acid resistance. The effect of mutation on acid resistance was severer in hfq::cat mutant cells than in rne-1 (Fig. 4), while that on the expression of GAD activity appeared greater in rne-1 than in hfq::cat (Fig. 2). This is probably because the Hfq protein is also required for the expression of other RpoS-dependent acid resistance systems.44)

**Effects of the rne-1 mutation on the expression of RpoS and GadY**

It is known that Hfq participates in GAD expression through RpoS and GadY small RNA. Hfq is required for the biosynthesis of RpoS,16,43) Transcription of gadX, encoding one of the positive regulators of gadA/gadBC, is RpoS-dependent,44) and the stability of gadX mRNA is regulated by the action of GadY small RNA.45) Expression of GadY is also RpoS-dependent.45) GadY-mediated regulation of gadX mRNA requires Hfq.45) Thus the hfq mutant is defective in the induction of GAD. In order to investigate at which step(s) RNase E is required, the effect of the rne-1 mutation on the expression of these regulators was examined in comparison with hfq::cat.

As shown in Fig. 5A, expression of gadA mRNA and gadBC mRNA was induced in wild-type cells grown in L broth at 42 °C, but not in the hfq::cat or rne-1 mutant cells. Under these conditions, the amount of rpoS mRNA greatly decreased in hfq::cat mutant cells, as reported previously.16,43) On the other hand, rpoS mRNAs were detected in the rne-1 mutant cells as well as in the wild type, although an extra band of smaller size appeared in the rne-1 mutant cells at 42 °C. To examine whether the rne-1 mutation affects synthesis of the RpoS protein, Western blotting of RpoS was carried out (Fig. 5B). The rne-1 mutant strain produced almost the same amount of RpoS protein as the wild type at 42 °C, while the hfq::cat mutant strain produced a very small amount of RpoS, or almost none. This indicates that the rne-1 mutation does not affect the expression of RpoS, unlike hfq::cat. However, the amount of GadY small RNA significantly decreased in the rne-1 mutant cells as well as in hfq::cat. GadY small RNA was expressed in the wild-type cells at 30 and 42 °C, but almost no expression was observed in the hfq::cat mutant cells at either temperature as reported previously.45) A very small amount of GadY was detected in rne-1 mutant cells at 30 °C, but almost no GadY at 42 °C. Since GadY is known to regulate the stability of gadX mRNA,45) the expression of gadX mRNA was also examined. Induction of gadX mRNA was observed in wild-type cells at 42 °C, but almost no gadX mRNA was detected in the hfq::cat or rne-1 mutant cells. These results suggest that RNase E is involved in the GadY-mediated regulation of GAD expression.

**Discussion**

GadA and GadB are highly homologous glutamate decarboxylase isoforms that contribute to the glutamate-dependent acid resistance system in *E. coli*. These two enzymes convert intracellular glutamate to γ-aminobutyrate, consuming an intracellular proton. A putative amino acid antiporter, GadC, exchanges intracellular γ-aminobutyrate for external glutamate.38–42) Regulation of the genes involved in glutamate-dependent acid resistance is extremely complex. The genes gadB and gadC form an operon, while gadA maps to a different locus.38) The LuxR-like regulator GadE and the AraC-like regulators, GadX and GadW, are implicated in gadA/gadBC expression under some circumstances.46,47) In addition, stationary-phase sigma factor RpoS, the cyclic AMP receptor protein CRP, and the histone-like protein H-NS are also considered to be regulators involved in modulating gadA/gadBC expression.46) Expression of gadX is RpoS-dependent and is induced in the stationary phase of growth in complex media.44,47) On the other hand, expression of gadE is induced during the exponential phase of growth in minimal media, probably through the EvgAS two-component signal transduction system.47) Since some of the experiments were done under experimental conditions in which evgA was artificially overproduced, the physiological significance of the regulation by EvgAS remains obscure. However, since we used complex media such as L and LB in our experiments, the contribution of the EvgAS regulatory system appears negligible. In fact, we did not detect expression of evgA mRNA under our experimental conditions (data not shown).

Here we found that the rne-1 mutant strain as well as hfq::cat is deficient in acid resistance. This is at least

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![Fig. 5. Effects of hfq10::cat and rne-1 Mutations on the Expression of gadA/gadBC, rpoS, gadY, and gadX Genes.](image-url)
partly due to a defect in the induction of GAD activity under acid conditions. Recently it was reported that a novel small RNA, GadY, positively regulates the stability of gadX mRNA. It has been suggested that Hfq is required for GadY base pairing with gadX mRNA. Hfq is also required for RpoS biosynthesis via other small RNAs, DsrA and RprA.

There is no report that RNase E is involved in the gene expression of gadA/gadBC. Here we found that the amounts of GadY small RNA as well as gadX mRNA significantly decreased in rne-1 mutant cells. It is known that non-coding small RNAs such as DsrA, RyhB, and DicF are processed by RNase E. It is possible that RNase E is also involved in GadY biosynthesis or degradation. Three molecular species of different lengths have been detected for GadY. RNase E might catalyze the processing of a putative GadY precursor, which is unstable. It is also possible that RNase E is involved in the processing of gadX mRNA. Previously we reported that RNase E-cleavage is required for efficient translation of ftsZ mRNA. It is possible that unprocessed gadX mRNA is also deficient in translation and hence can be attacked by other RNases. Since GadY small RNA is indeed one of the targets of Hfq, it is likely that the Hfq protein also participates in this RNase E-mediated regulation of GadY-gadX. This point should be investigated further.

Even though it is now well known that RNase E participates in many reactions in RNA metabolism, there are very few reports showing the physiological phenotypes of rne mutants. One exception is its role in cell division: RNase E deficiency causes cell division defects due to decreased synthesis of the essential cell division protein FtsZ. Since lack of RNase E causes cell death, it is difficult to identify other physiological effects caused by RNase E deficiency. Here we first demonstrated that the rne-1 mutant strain showed decreased survival under acidic conditions. This is the first apparent (visible) phenotype of rne mutant strains besides cell division defects.

An acid resistance mechanism is needed for pathogenic as well as commensal E. coli strains to survive through the stomach. It has also been reported that Hfq is required for the expression of acid resistance in Brucella abortus. A B. abortus hfq mutant showed decreased survival under acidic conditions during the stationary phase of growth, although the regulatory mechanism of Hfq in B. abortus is as yet unknown. In several other gram-negative pathogens, including Yersinia enterocolitica, Pseudomonas aeruginosa, and a gram-positive pathogen, Listeria monocytogenes, Hfq has been implicated in their virulence. Here we found that RNase E is involved in the expression of acid resistance in E. coli. Hence the Hfq- and RNase E-mediated regulatory mechanism of gene expression might be a target of antibacterial agents against these pathogenic bacteria.

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