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Characterization of Ribonuclease HII from *Escherichia coli* Overproduced in a Soluble Form

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***Escherichia coli* RNase HII is composed of 198 amino acid residues. The enzyme has been overproduced in an insoluble form, purified in a urea-denatured form, and refolded with poor yield [M. Itaya (1990) *Proc. Natl. Acad. Sci. USA* 87, 8587–8591]. To facilitate the preparation of the enzyme in an amount sufficient for physicochemical studies, we constructed an overproducing strain in which *E. coli* RNase HII is produced in a soluble form. The enzyme was purified from this strain and its biochemical and physicochemical properties were characterized. The good agreement in the molecular weights estimated from SDS-PAGE (23,000) and gel filtration (22,000) suggests that the enzyme acts as a monomer. From the far-UV circular dichroism spectrum, its helical content was calculated to be 23%. The enzyme showed Mn^{2+}-dependent RNase H activity. Its specific activity determined using ^{3}H-labeled M13 RNA/DNA hybrid as a substrate was comparable to but slightly higher than that of the refolded enzyme, indicating that the enzyme overproduced and purified in a soluble form is more suitable for structural and functional analyses than the refolded enzyme.**

Key words: amino acid sequence, enzymatic activity, *Escherichia coli*, overproduction, RNase H.

Ribonuclease H (RNase H) [EC 3.1.26.4] specifically cleaves the RNA strand of RNA/DNA hybrids (*1*). This activity is present in almost all organisms. In addition, single bacterial and eukaryotic cells usually contain multiple RNases H. Based on the sequence information, the enzymes have been classified into two major families, Type 1 and Type 2 RNases H (*2*). Bacterial RNases HI, eukaryotic RNases H1, and retroviral RNase H domains of reverse transcriptases are Type 1 enzymes. Bacterial RNases HII and HIII, archaeal RNases HII, and eukaryotic RNases H2 are Type 2 enzymes. The Type 2 enzymes are universally present in all three domains of living organisms, bacteria, archaea, and eukaryotes, whereas the Type 1 enzymes are present only in bacteria and eukaryotes (*2–4*). Nevertheless, little is known about the structures and functions of the Type 2 enzymes, although biochemical characterizations of *Escherichia coli* RNase HII (*5*), *Bacillus subtilis* RNases HII and HIII (*6*), and *Pyrococcus kodakaraensis* RNase HII (*7*) have suggested that the Type 2 enzymes are functionally related to the Type 1 enzymes.

Among RNase H genes, the *rnhA* (*8*) and *rnhB* (*5*) genes encoding *E. coli* RNases HI (Type 1) and HII (Type 2), respectively, were cloned first, and these enzymes provided a starting point for comparison with similar proteins from other sources. However, between them, only the RNase HI protein has been extensively studied for structure-function relationships (*9–11*). The main reason for this is that *E. coli* RNase HI can be overproduced in a soluble form (*12*), whereas *E. coli* RNase HII was overproduced in an insoluble form (*5*). This RNase HII protein was solubilized in the presence of 7 M urea, purified in the presence of 4 M urea, and refolded with poor yield by removing the urea in the presence of 50% glycerol and 0.1% Triton X-100 (*5*). Therefore, we decided to construct overproducing strains in which *E. coli* RNase HII can be produced in a soluble form, to facilitate the preparation of the enzyme in an amount sufficient for physicochemical studies. Construction of this system would also allow us to answer the question as to whether the enzymatic properties of the refolded enzyme reflect those of the native enzyme.

EXPERIMENTAL PROCEDURES

Cells and Plasmids—The *E. coli* strains, *rnhA* mutant MIC3009 [F^-, *supE44 supF58 lacY1 or Δ(lacIZY)6 trpR55 galK2 galT22 metB1 hsdR14*($r_K^-m_K^+$) *rnhA339::cat*] (*13*), *rnhA* and *rnhB* double mutant MIC2067 [F^- λ^-, IN(*rrn*D-*rrn*E)1 *rnhA339::cat rnhB716::kam*] (*14*), and plasmid pMIC2721 for the overproduction of *E. coli* RNase HII (*5*) were kindly donated by M. Itaya. *E. coli* MIC2067(DE3) was constructed by lysogenizing *E. coli* MIC2067 with λDE3, which is a recombinant phage carrying the cloned gene for T7 RNA polymerase under *lacUV5* control, using a λDE3 Lysogenization Kit (Novagen). λDE3 lysogens can be used to express the target genes cloned into pET vectors. *E. coli* BL21(DE3) [F^- *ompT hsdS*$_B$($r_B^-m_B^-$) *gal* (*λcI857 ind1 Sam7 nin5 lacUV5-T7gene1) dcm* (DE3)], HMS174(DE3) [F^- *recA1 hsdR*($r_{K12}^-m_{K12}^+$) Rif^R (DE3)], HMS174(DE3)-pLysS, and plasmid pET-25b were from Novagen. Plasmid

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Abbreviation: RNase H, ribonuclease H.

pJLA503 and pTYB1 were from Medac Gentechnologie and New England Biolabs, respectively. The transformants were grown in Luria-Bertani medium containing 50 mg/liter ampicillin.

Overproduction—The *rnhB* gene encoding *E. coli* RNase HII was amplified by PCR so that it can be ligated to the *Nde*I–*Sal*I sites of pJLA503 and pET-25b, or the *Nde*I–*Sap*I site of pTYB1. The resultant plasmids pJAL600E and pET600E contain the *rnhB* gene under the control of the λP_R and P_L promoters and the T7 promoter, respectively. Another plasmid, pTYB600E, contains the gene encoding a fusion protein, in which *E. coli* RNase HII, self-cleavable intein, and the chitin binding domain are linked in this order, under the control of the T7 promoter. These plasmids were used to transform various *E. coli* strains. Overproduction with MIC3009/pJAL600E was performed as described previously for *E. coli* RNase HI (*12*). Overproductions with BL21(DE3)/pET600E, MIC2067(DE3)/pET600E, HMS-174(DE3)pLysS/pET600E, and MIC2067(DE3)/pTYB600E were performed as described previously for *B. subtilis* RNase HIII (*6*), except that MIC2067(DE3) transformants were grown at 30°C. Cells were harvested by centrifugation at 6,000 ×*g* for 10 min, and suspended in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, disrupted by sonication, and centrifuged at 15,000 ×*g* for 30 min. For the estimation of the production level, and the amount of enzymes in soluble and insoluble forms, whole cell extracts, and soluble and insoluble fractions obtained after sonication lysis were analyzed by SDS-PAGE on a 15% polyacrylamide gel (*15*), followed by staining with Coomassie Brilliant Blue.

Purification—Because of the ease of the purification procedures, the enzyme was overproduced as the fusion protein with intein and the chitin binding domain in MIC-2067(DE3) harboring pTYB600E as described above. Cells were harvested, suspended in 20 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl, 0.1 mM EDTA, and 0.1% Triton X-100, disrupted by sonication, and centrifuged at 15,000 ×*g* for 30 min. Binding of the fusion protein to chitin beads (New England Biolabs), self-cleavage at the peptide bond between the C-terminal serine residue of *E. coli* RNase HII and the N-terminus of the intein, and elution of the RNase HII from chitin beads were done according to the instructions of the manufactures. The fraction containing the pure enzyme was dialyzed against 20 mM sodium acetate (pH 5.5) containing 150 mM NaCl and used for further characterizations. The purity of the enzyme was analyzed by SDS-PAGE (*15*). The protein concentration was determined from the UV absorption with an $A_{280}^{0.1\%}$ value of 0.61, which was calculated from ε values of 1,576 M^{-1} cm^{-1} for Tyr and 5,225 M^{-1} cm^{-1} for Trp at 280 nm (*16*).

The DNA sequence was determined with a Prism 310 DNA sequencer (PE Applied Biosystems).

Biochemical Characterizations—Gel filtration was performed on a column (1.6 × 60 cm) of HiLoad 16/60 Superdex 200 pg (Pharmacia LKB Biotechnology) equilibrated with 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl. For the estimation of molecular mass, blue dextran 2000, bovine serum albumin (BSA), ovalbumin, chymotrypsinogen A, and ribonuclease A were used as standard proteins. The far-UV CD spectra were measured on a J-725 spectropolarimeter (Japan Spectroscopic) as described previously (*6*).

Enzymatic Activity—RNase H activity was determined at 30°C for 15 min by measuring the radioactivity of the acid-soluble digestion product from the substrate, ^{3}H-labeled M13 RNA/DNA hybrid, as previously described (*17*). The buffer for *E. coli* RNase HII was 10 mM Tris-HCl (pH 8.5) containing 5 mM $MnCl_2$, 50 mM NaCl, 1 mM 2-mercaptoethanol (2-Me), and 50 μg/ml BSA. The buffer for *E. coli* RNase HI was previously described (*17*). One unit was defined as the amount of enzyme producing 1 μmol of acid-soluble material per minute at 30°C. The specific activity was defined as the enzymatic activity per milligram of protein. For the determination of kinetic parameters, the substrate concentration was varied from 0.15 to 1.3 μM. The hydrolysis of the M13 RNA/DNA hybrid by the enzyme followed Michaelis-Menten kinetics, and the kinetic parameters were determined from the Lineweaver-Burk plot. For the analysis of pH dependence, 10 mM BisTris-HCl (pH 5.7–7.1), 10 mM Tris-HCl (pH 7.1–8.8), or 10 mM glycine-NaOH (pH 8.3–9.8) was used as the assay buffer. For analyses of the dependence on divalent cation concentration, the enzymatic activity was determined in 10 mM Tris-HCl (pH 8.0) containing 50 mM NaCl, 1 mM 2-Me, 10 μg/ml BSA, and various concentrations (50 μM–100 mM) of $MnCl_2$ or $MgCl_2$.

RESULTS AND DISCUSSION

Amino Acid Sequence—Itaya has reported that *E. coli* RNase HII is composed of 213 amino acid residues (*5*) (accession number of QQECBE). However, according to the entire genome sequence of *E. coli* determined by Blattner *et al.* (*18*) (accession numbers of AAB08612 and P10442), it is composed of 198 amino acid residues. These two sequences differ only in the C-terminal regions. One has the amino acid sequence LAS at positions 196–198, while the other has TCVLILVSRLSKPESEDV at positions 196–213. When the nucleotide sequences of the *rnhB* genes were compared, the insertion of a single thymine base between the second and third codon for Leu^{194} resulted in an extension of the position of the C-terminal residue from 198 to 213. Therefore, we reexamined the nucleotide sequence of the *rnhB* gene contained in plasmid pMIC2721, which was constructed by Itaya (*5*), and found that the thymine base was not inserted within the codon for Leu^{194}. Thus, we conclude that *E. coli* RNase HII is composed of 198 rather than 213 amino acid residues with the C-terminal sequence LAS.

Overproduction and Purification—Most of the *E. coli* RNase HII protein overproduced in strain BL21(DE3) harboring pMIC2721 accumulated intracellularly in an insoluble form (*5*). When strain BL21(DE3) harboring pET600E was used to overproduce the RNase HII protein, similar results were obtained (Fig. 1, lanes 5–7). Despite the poor production level (2–3 mg/liter culture), most of the RNase HII protein was recovered in an insoluble form. Likewise, when strain MIC3009 harboring pJAL600E was used to overproduce the RNase HII protein, most of the protein was recovered in an insoluble form, although the production level of the protein in this overproducing strain was dramatically improved (Fig. 1, lanes 2–4). However, when strains HMS174(DE3)pLysS, HMS174(DE3), and MIC-2067(DE3) harboring pET600E were used to overproduce the RNase HII protein, the yield of the protein in a soluble form was dramatically improved (Fig. 1, lanes 8–16). The ratio of the amount of protein recovered in a soluble form to

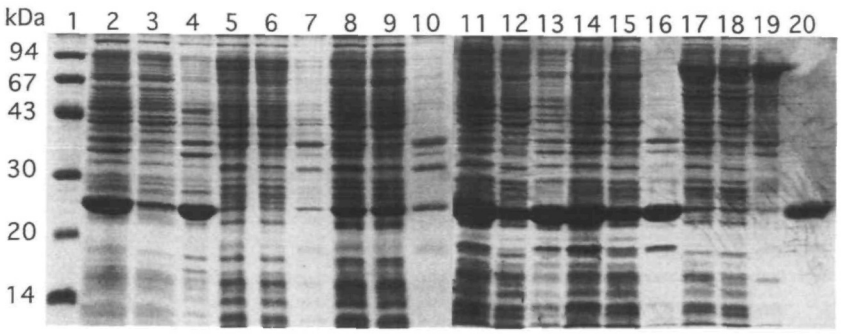

Fig 1 **Estimation of the production level and amount of the enzymes in soluble and insoluble forms by SDS-PAGE.** Whole cell extracts (lanes 2, 5, 8, 11, 14, 17), and soluble (lanes 3, 6, 9, 12, 15, 18) and insoluble (lanes 4, 7, 10, 13, 16, 19) fractions after sonication lysis were analyzed by 15% SDS-PAGE The overproducing strains for *E coli* RNase HII were MIC3009/pJAL600E (lanes 2–4), BL21(DE3)/pET600E (lanes 5–7), HMS174-(DE3)pLysS/pET600E (lanes 8–10), HMS174-(DE3)/pET600E (lanes 11–13), MIC2067-(DE3)/pET600E (lanes 14–16), and MIC2067-(DE3)/pTYB600E (lanes 17–19) The gel was stained with Coomassie Brilliant Blue. Lane 1, low molecular weight marker kit (Pharmacia LKB Biotechnology) containing phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin; lane 20, purified *E coli* RNase HII Numbers along the gel represent the molecular weights of individual standard proteins.

that overproduced in cells was roughly 70% for HMS174-(DE3)pLysS/pET600E and 30–40% for HMS174(DE3)/pET600E and MIC2067(DE3)/pET600E. In addition, the production levels of the protein were increased by roughly 10–20 times as compared to that in BL21(DE3) harboring pET600E. As a result, strains HMS174(DE3) and MIC-2067(DE3) harboring pET600E gave the highest production level, which was estimated to be 80 mg/liter culture. Thus, both the solubility and the production level of *E. coli* RNase HII varies greatly among different overproducing strains. We used MIC2067(DE3) as a host strain for the overproduction of *E. coli* RNase HII to avoid contamination with *E. coli* RNase HI. It remains to be determined why strain HMS174(DE3)pLysS, which is a λDE3 lysogen carrying plasmid pLysS, is less effective than strain HMS174-(DE3) or MIC2067(DE3), which do not carry pLysS, as a host for the overproduction of *E. coli* RNase HII. Plasmid pLysS contains the gene encoding T7 lysozyme, which inhibits RNA polymerase and thus reduces the ability of the plasmid to transcribe target genes in uninduced cells.

For the purification of *E. coli* RNase HII, the fusion protein, in which a chitin binding domain is attached to the C-terminus of *E. coli* RNase HII through a self-cleavable intein, was overproduced in strain MIC2067(DE3) harboring pTYB600E. The production level was estimated to be 60 mg/liter culture, and roughly 40% of the fusion protein produced in the cells was recovered in a soluble form (Fig. 1, lanes 17–19). Binding of the fusion protein to an affinity column with chitin beads, intein cleavage, and elution from the affinity column, yielded pure RNase HII protein, as judged from SDS-PAGE (Fig. 1, lane 20). Using this system, approximately 5 mg of *E. coli* RNase HII was obtained from one liter of culture.

Molecular Properties—The molecular weight of *E. coli* RNase HII is calculated to be 21,525 from the amino acid sequence, and was estimated to be 23,000 from SDS-PAGE (Fig. 1) and 22,000 from gel filtration column chromatography (data not shown). These results suggest that *E. coli* RNase HII acts as a monomer. The far-UV CD spectrum of *E. coli* RNase HII is shown in Fig. 2 in comparison with that of *E. coli* RNase HI. It is considerably different from the spectrum of *E. coli* RNase HI, exhibiting a broad trough with a minimum [θ] value of –10,840 at 209 nm, accompanied by a shoulder with a [θ] value of –10,490 at 217 nm. The helical content of *E. coli* RNase HII was calculated to

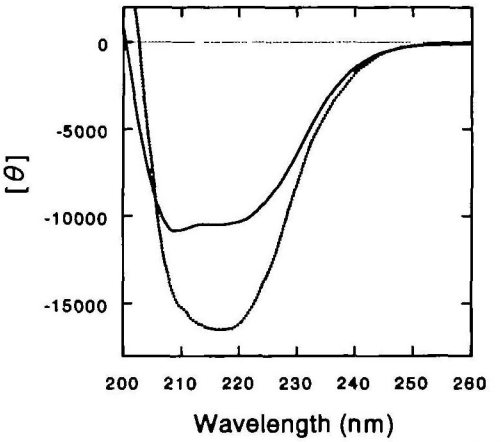

Fig. 2 **Far-UV CD spectra of *E. coli* RNases HI and HII.** The far-UV CD spectrum of *E coli* RNase HII (solid line) is shown in comparison with that of *E coli* RNase HI (dotted line). The spectra were recorded at 25°C a solution containing 0.12 mg/ml enzyme in 20 mM sodium acetate (pH 5.5) containing 150 mM NaCl in a cell with an optical path length of 2 mm. The mean residue ellipticity, with units of deg cm^2 $dmol^{-1}$, was calculated using an average amino acid molecular weight of 110.

be 23% by the method of Wu *et al.* (*19*), which is lower than that of *E. coli* RNase HI. The helical content of *E. coli* RNase HI is calculated to be 34%, which is comparable to that (42%) calculated from its crystal structure (*20*).

Enzymatic Activities—E. coli RNase HII exhibited an RNase H activity at alkaline pH in the presence of 10 mM $MnCl_2$ or $MgCl_2$, whereas it did not exhibit activity in the absence of these metal ions or in the presence of 10 mM $BaCl_2$, $CaCl_2$, $CoCl_2$, $ZnCl_2$, $CuCl_2$, $FeCl_2$, or $SrCl_2$ (data not shown). In addition, it prefers Mn^{2+} to Mg^{2+} for activity. These results are consistent with those reported for the refolded enzyme (*6*, *7*). Therefore, it seems likely that the refolded enzyme assumes a native conformation and its enzymatic properties previously reported for the hydrolyses of oligomeric substrates (*6*) represent those of the native enzyme. However, the specific activity of the refolded enzyme, which has been reported to be 0.31 unit/mg at pH 8.0 in the presence of 10 mM $MnCl_2$ (*6*, *7*), is comparable to but slightly lower than that of the enzyme purified in these experiments, which was determined to be 0.41 unit/mg under

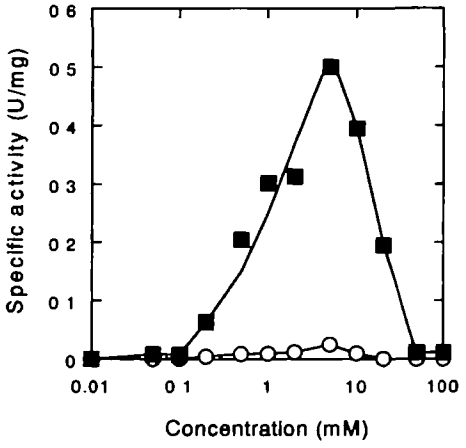

Fig. 3. **Dependence of *E. coli* RNase HII activity on the concentration of $MnCl_2$ or $MgCl_2$.** The enzymatic activity of *E coli* RNase HII was determined at 30°C in 10 mM Tris-HCl (pH 8 0) containing 50 mM NaCl, 1 mM 2-Me, 10 μg/ml BSA, and various concentrations (50 μM–100 mM) of $MnCl_2$ (■) or $MgCl_2$ (○), using M13 RNA/DNA hybrid as a substrate.

the same conditions. The RNase HII protein overproduced in an insoluble form and solubilized in the presence of 7M urea was probably refolded into the native enzyme, but with a yield less than 100%.

The dependence of the *E. coli* RNase HII activity on the divalent cation concentration was analyzed for Mn^{2+} and Mg^{2+} in the range of 50 μM and 100 mM. The concentrations required for optimal enzymatic activities were 5 mM for both $MnCl_2$ and $MgCl_2$ (Fig. 3). The specific activity determined in presence of 5 mM $MgCl_2$ was 3% of that determined in the presence of 5 mM $MnCl_2$. It has been reported that *E. coli* RNase HI shows Mn^{2+}- and Mg^{2+}-dependent RNase H activities at metal ion concentrations of 2–20 μM and >1 mM, respectively (*21*, *22*). The specific activity of this enzyme determined in the presence of 5 mM $MnCl_2$ was ~20% of that determined in the presence of 5 mM $MgCl_2$ (*21*). These results suggest that the Mn^{2+} ion binds to *E. coli* RNase HI more tightly than the Mg^{2+} ion, whereas these metal ions bind weakly to *E. coli* RNase HII with similar dissociation constants. The *E. coli* RNase HII activity was inhibited by Mn^{2+} or Mg^{2+} at concentrations higher than 5 mM (Fig. 3), probably because the enzyme is activated by metal ion(s) but can be inhibited upon subsequent binding of additional metal ion(s).

The dependence of the *E. coli* RNase HII activity on the concentration of NaCl or KCl was analyzed at pH 8.0 in the presence of 5 mM $MnCl_2$. The results indicate that this activity responds equally to NaCl and KCl (data not shown). The enzyme exhibited the highest activity in the presence of 50–100 mM NaCl or KCl, and ~50 and ~10% of the maximal activity in the absence of these salts and the presence of 200 mM NaCl or KCl, respectively. The influence of pH on the *E. coli* RNase HII activity was analyzed in the presence of 5 mM $MnCl_2$ and 50 mM NaCl. Activity was detected only at pH > 7.0 and increased as the pH increased from 7.0 to 9.8 (data not shown). However, because the solubility of the Mn^{2+} ion decreases as the pH increases, and both RNA/DNA substrate and enzyme may be unstable at highly alkaline pH, we decided to measure the *E. coli* RNase HII activity at relatively mild pH 8.5.

TABLE I. **Specific activities and kinetic parameters of *E. coli* RNases HI and HII.** Hydrolysis of the M13 RNA/DNA hybrid by the enzyme was carried out at 30°C for 15 min under the conditions described in "EXPERIMENTAL PROCEDURES." Errors, which represent 67% confidence limits, are all at or below ±20% of the values reported

Enzyme	Specific activity (units/mg)	K_m (μM)	V_{max} (units/mg)
RNase HI	9 5	0 088	9 7
RNase HII	0 75	0 26	1.8

The specific activity of *E. coli* RNase HII determined under optimal conditions was 0.75 unit/mg, which is 7.9% that of *E. coli* RNase HI (Table I). A comparison of the kinetic parameters for *E coli* RNase HII with those for *E. coli* RNase HI suggests that the binding-affinity to the substrate and the catalytic efficiency of *E. coli* RNase HII are lower than those of *E. coli* RNase HI by 3-fold and 5-fold, respectively.

Comparison with Other RNase HII Enzymes—Among various RNase HII enzymes, RNases HII from *B. subtilis* (*6*), and *P. kodakaraensis* KOD1 (*7*) have been biochemically and enzymatically characterized. As expected from the high amino acid sequence identity (44.9%) between *B. subtilis* and *E. coli* RNases HII, these enzymes share most enzymatic and biochemical properties. However, they show different salt and metal ion dependencies. The *E. coli* enzyme did not exhibit RNase H activity in the presence of Co^{2+} ion, whereas the *B. subtilis* enzyme did. The *E. coli* RNase HII activity responded equally to NaCl and KCl, whereas the *B. subtilis* RNase HII activity preferred KCl to NaCl. It would be informative to identify the amino acid substitutions responsible for these differences with protein engineering techniques.

It is noted that the *B. subtilis* RNase HII sequence has an N-terminal extension of ~60 amino acid residues as compared to the *E. coli* RNase HII sequence. The observation that *E. coli* RNase HII is as active as *B. subtilis* RNase HII suggests that this sequence is unrelated to the enzymatic function. In addition, *B. subtilis* RNase HII seems to be more unstable than *E. coli* RNase HII and gradually loses enzymatic activity during storage (Ohtani, N., unpublished result). Therefore, the *E. coli* enzyme may be more favorable than the *B. subtilis* enzyme for further structural and functional analyses.

Physiological Function—The physiological functions of *E. coli* RNase HII remain to be determined. However, *E. coli* strain MIC2067 with the *rnhA* and *rnhB* double mutations shows a temperature-sensitive growth phenotype (*14*). In addition, the *rnhB* and *rnhC* double mutations make *B. subtilis* unable to grow (*14*). The *rnhB* and *rnhC* genes of *B. subtilis* encode RNases HII and HIII, respectively, which are the only functional RNase H enzymes in *B. subtilis*. These results suggest that the RNase HII activity is involved in biological processes that are important for cell growth. Studies using an *rnhB* mutant strain of *E. coli* would provide valuable information about the physiological functions of RNase HII activity.

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