Regulation of RraA, a Protein Inhibitor of RNase E-Mediated RNA Decay

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The recently discovered RraA protein acts as an inhibitor of the essential endoribonuclease RNase E, and we demonstrated that ectopic expression of RraA affects the abundance of more than 700 transcripts in *Escherichia coli* (K. Lee, X. Zhan, J. Gao, J. Qiu, Y. Feng, R. Meganathan, S. N. Cohen, and G. Georgiou, Cell 114:623–634, 2003). We show that *rraA* is expressed from its own promoter, *PrraA*, located in the menA-rraA intergenic region. Primer extension and lacZ fusion analysis revealed that transcription from *PrraA* is elevated upon entry into stationary phase in a σ70-dependent manner. In addition, the stability of the *rraA* transcript is dependent on RNase E activity, suggesting the involvement of a feedback circuit in the regulation of the RraA level in *E. coli*.

RNase E is an essential protein that plays a crucial role in global mRNA metabolism as well as in the maturation of functional RNAs such as rRNAs, tRNAs, tmRNA, and small regulatory RNAs (3, 9, 18, 19, 21, 28). To date, RNase E homologs have been found in more than 50 eubacteria, archaea, and plants (16). The cellular level and activity of RNase E are subjected to multiple environmental controls. At one level, RNase E synthesis is autoregulated by modulating the half-life of its own mRNA (12, 26). In addition, recent studies have revealed that 5′-monophosphorylated RNA serves as an allosteric activator of the endonuclease activity (13). Furthermore, the degradation of *rraA* is elevated upon entry into stationary phase in a σ70-dependent manner. In addition, the stability of the *rraA* transcript is dependent on RNase E activity, suggesting the involvement of a feedback circuit in the regulation of the RraA level in *E. coli*.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and phage vectors.** The strains, plasmid, and phage vectors used in this study are listed in Table 1. The *rpoS:kan* mutation from strain ZK1000 was introduced into various strains by P1 transduction as described by Miller (24). The transduction of the *rpoS* disruption was confirmed by streaking single kanamycin-resistant transductants onto Luria-Bertani broth (LB)-kanamycin agar plates and testing the ability of catalase to hydrolyze hydrogen peroxide.

**Growth conditions.** LB and M9 minimal medium (23) containing thiamine (50 μg/ml), 0.4% (wt/vol) glucose, and 0.2% (wt/vol) casein were supplemented with antibiotics, as required (50 μg/ml ampicillin, 25 μg/ml kanamycin, or 15 μg/ml tetracycline). Unless otherwise stated, cells were grown in LB medium under aeration at 37°C, and the growth was monitored by measuring the absorbance at 600 nm.

**RNA methods.** For reverse transcriptase-PCR (RT-PCR) analysis, total RNA was isolated with the RNeasy kit (Qiagen, Valencia, CA) and treated with RNase-free DNase (Ambion, Austin, TX). Fifty ng of total RNA was subjected to RT-PCR analysis using the One Step RNA PCR kit (TaKaRa, New York, NY). Northern blot assays were performed using total RNA isolated from *E. coli* JCB570 grown as described above. Samples were collected at 1-hour intervals throughout the exponential and stationary phases. Five μg of total RNA per lane...
TABLE 1. Strains, plasmids, and phage vectors

<table>
<thead>
<tr>
<th>Strain, plasmid, or phage vector</th>
<th>Description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
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<td>MC1000; proR zih12::Tn10</td>
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<td>ZK126</td>
<td>W3110; ΔlacU16 mua-2</td>
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<tr>
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<td>pBR ori Amp'</td>
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<td>pMZ002</td>
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<td>λM4</td>
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was loaded onto a denaturing gel containing formaldehyde and then transferred to a positively charged nylon membrane (Hybond N +; Amersham, United Kingdom). The AlkPhos direct nucleic acid labeling and detection system (Amer- sham, United Kingdom) was used for probe synthesis. Hybridization, washing of the membranes, and detection of signals were carried out according to the manufacturer's protocol.

For primer extension, a 5'-32P-labeled oligonucleotide (5'-CCCTCCACCCGACGAATACATCTTCTTGA-3' or 5'-CCCTCCACCCGACGAATACATCTTCTTGA-3') was used as a primer for the RT reaction with 5 µg of total RNA (SuperScript III RNase H - reverse transcriptase; Invitrogen, Carlsbad, CA). The primer extension products were separated on 6% polyacrylamide-7 M urea gels. The dideoxy-DNA sequence ladder from the same primer was prepared using the fmol DNA Cycle Sequencing System (Promega, Madison, WI).

For RNase protection assays, E. coli CH1827 (MC1061; zee-726::Tn10) or CH1828 (CH1827; mcl-1) was grown in LB at 30°C to an A600 of 0.4 and then half of the culture was transferred to 43.5°C and incubated for an additional 20 min. After the addition of rifampin (500 µg/ml), aliquots were withdrawn every 45 s, immediately chilled, and stored in liquid nitrogen. Total RNA was prepared from the frozen samples by using the RNaseasy kit (QIAGEN, Valencia, CA), and RNase protection assays were performed using the RPA III kit (Ambion, Austin, TX) with the in vitro transcript from the complementary strand of the rraA gene (nucleotides [nt] +13 to +318 of rraA) as the probe. The band intensity was quantified using Quantity One software.

**RESULTS AND DISCUSSION**

**Identification of the P"rraA promoter.** Meganathan had proposed that rraA is transcribed as a dicistronic mRNA from the

![FIG. 1. RT-PCR analysis. A. The annealing positions of the reverse primer (P-RT-stop) and forward primer (P4) used in the RT-PCR analysis of the menA-rraA region. B. RT-PCR analysis of rraA transcript using the primers P4 and P-RT-stop. – RT, the negative control containing the same amounts of RNA, primers, and Taq polymerase but no reverse transcriptase. Lane Mks, molecular size markers.](http://jb.asm.org/Downloaded from http://jb.asm.org/)

plasmids pMZ002 ([nt -1076 to -1]-lacZ), pMZ003 ([nt -92 to -1]-lacZ), and pMZ004 ([nt -1076 to -93]-lacZ); pMZ001 was the negative-control vector. The lacZ fusions in pMZ002, pMZ003, and pMZ004 were transferred onto the chromosome using the transducing lambda phage system (33). The fusions were transferred into λRS45, whereas the negative-control fusion in pMZ001 was transferred into λRS74 via a double recombination event. Plaques containing the recombinant lambda phages were isolated based on their blue plaque phenotype. The recombinant lambda phages were used to lysogenize EC-O [Δpro-lacZ] and generated the following strains: MZ001 (EC-O; λ p-lacZ), with a chromosomal promoterless lacZ gene; MZ002 (EC-O; λ [nt -1076 to -1]-lacZ), harboring nt -1076 to -1 of the rraA upstream region fused to lacZ; MZ003 (EC-O; λ [nt -92 to -1]-lacZ), harboring nt -92 to -1 of the menA-rraA intergenic region fused to lacZ; and MZ004 (EC-O; λ [nt -1076 to -93]-lacZ), harboring nt -1076 to -93 of the rraA upstream region fused to lacZ. All lysogens were tested for monolysogenization by PCR (30).

**β-Galactosidase assays.** Cultures grown with aeration at 37°C, in rich or M9 medium overnight, were subcultured into the fresh medium. Samples were collected at exponential and stationary phases. The samples were collected at 4°C and resuspended in an appropriate volume of ice-cold Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM M KCl, 1 mM MgSO4; pH 7.0) (23) to give an A600 in the range from 0.6 to 0.9. β-Galactosidase activities were determined from at least three independent experiments, as previously described (24).

**Western immunoblotting.** The cells were harvested by centrifugation, resuspended in phosphate-buffered saline, and lysed by passage through a French press (2,000 lb/in²). The lysate was centrifuged at 15 min for 4°C to remove cell debris, and the protein concentration in the supernatant was determined by the Bradford assay (Brad-Rad Laboratories, Hercules, CA). One µg of total protein was separated by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis. Western blot assays were performed by standard methods using monoclonal anti-FLAG-M2-peroxidase (horseradish peroxidase) antibody conjugate (Sigma, St. Louis, MO). Signal intensities were measured, quantified by molecular analysis software (Quantity One; Bio-Rad), and displayed below the blot image.
The transcription initiation site for this putative dicistronic transcript was identified at 57 bp upstream of the \( \text{menA} \) translation start site (34). Consistent with this hypothesis, a \( \text{menA-rraA} \) transcript of the expected size was detected by RT-PCR analysis (Fig. 1), suggesting that \( \text{rraA} \) is transcribed together with the preceding \( \text{menA} \) as a dicistronic mRNA. However, Northern blot analysis with two different \( \text{rraA} \)-specific probes revealed that the predominant RNA in samples in either exponential or stationary phase corresponds to a shorter species, ca. 550 bases in length (data not shown). Primer extension of RNA isolated from \( E. \ coli \) JCB570 using the \( 5'-\) labeled oligonucleotide probe revealed an \( \text{rraA} \) transcript that is initiated at an A residue 28 nt upstream from the ATG codon (Fig. 2A). In addition to this major transcript, the data in Fig. 2A also showed a faint band at the position of the initiation codon of \( \text{rraA} \) (indicated by an open arrowhead). However, this band was not detected in a primer extension.
assay using an alternative primer (\(^{2}P\)-CCGTCGCCCAAGGTTGAGAACAGC) that anneals to a different location within the rraA RNA (located 23 bp upstream from the first primer), suggesting that the minor band likely corresponds to a premature stop product of the reverse transcriptase.

DNA sequence analysis of the menA-rraA intergenic region suggested the existence of a putative promoter (\(P_{\text{menA}}\)) (Fig. 2B). rraA and menA are separated by a 92-bp intergenic region that shows a significant degree of conservation among closely related bacteria such as Shigella flexneri (92/92 bp, 100% identity in the intergenic region) and Salmonella enterica (86/92 bp, 93% identity in the intergenic region). A lower degree of sequence identity was observed for phylogenetically more distant bacteria, Yersinia pestis, Erwinia carotovora, and Photorhabdus luminescens. Analysis using GENETYX-MAC 11.2.5 identified regions that match the \(\sigma^{70}\) consensus –35 and –10 sequences, centered at 32 and 8 nucleotides, respectively, upstream of the transcription initiation site (Fig. 2B). Multiple sequence alignment, using CLUSTAL X 1.8, among the bacterial species which have close homologs (>80%) of E. coli K-12 rraA revealed that the sequence of the \(P_{\text{menA}}\) promoter is conserved among gammaproteobacteria. As shown in Fig. 2B, the –35 and –10 regions are identical, except for Photorhabdus luminescens, whose –35 region differs from that of E. coli K-12 by 1 bp.

Genetic analysis of expression of \(P_{\text{menA}}\) using \(\text{lacZ}\) transcriptional fusions. We used PCR amplification to generate DNA fragments containing different regions upstream of rraA extending up to, and including, the menA promoter, as shown in Fig. 3A, and cloned each fragment upstream of the \(\text{lacZ}\) gene in a multicopy transcriptional fusion vector, pSP417 (29). In this way, we generated plasmids pMZ002 (nt –1076 to –1-\(\text{lacZ}\)), pMZ003 (nt –92 to –1-\(\text{lacZ}\)), and pMZ004 (nt –1076 to –93-\(\text{lacZ}\)); pMZ001 was the negative-control vector.

To rule out the possibility that differences in the \(\beta\)-galactosidase activity expressed from the above transcriptional fusions might be partially due to plasmid copy number effects, we then made single chromosomal copy isolates of each construct using the transducing lambda phage system (33). The \(\text{E. coli}\) K-12 strain used for all the experiments was EC-O; H11002 was lysogenized with the recombinant phage. The transducing lambda phage system (33) was used to generate \(\text{P}_{\text{menA}}\), \(\text{P}_{\text{rraA}}\), and \(\text{P}_{\text{menA-rraA}}\) transcriptional fusions in \(\text{E. coli}\) K-12 by 1 bp.

**rraA expression in stationary phase.** Earlier microarray studies had shown that the \(\text{rraA}\) mRNA level is increased upon entrance to stationary phase (https://asap.ahabs.wisc.edu/annotation/php/ASAP1.htm). The abundance of the RraA protein in exponential- and stationary-phase cells was examined by Western blotting using strain DY330-\(\text{rraA}\)SP4 (5, 39), in which sequential peptide

![FIG. 3. \(\text{lacZ}\) transcriptional fusions. A. Schematic of the transcriptional \(\text{rraA}\)-\(\text{lacZ}\) fusions used in this study. B. \(\beta\)-Galactosidase activities in MZ001, MZ002, MZ003, and MZ004 cells. MZ001 (EC-O; \(\lambda\) \(\text{pO-lacZ}\)) has a chromosomal promoterless \(\text{lacZ}\) gene; MZ002 (EC-O; \(\lambda\) [nt –1076 to –1-\(\text{lacZ}\)] has nt –1076 to –1 of the \(\text{rraA}\) upstream region fused to \(\text{lacZ}\); MZ003 (EC-O; \(\lambda\) [nt –92 to –1-\(\text{lacZ}\)] has nt –92 to –1 of the \(\text{menA-rraA}\) intergenic region fused to \(\text{lacZ}\); MZ004 (EC-O; \(\lambda\) [nt –1076 to –93-\(\text{lacZ}\)] has nt –1076 to –93 of the \(\text{rraA}\) upstream region fused to \(\text{lacZ}\). Cells were grown in LB under aeration at 37°C and harvested in log phase (\(A_{600}\) = 0.5 to 0.8). Samples were normalized by optical density, and enzymatic activities were measured in Miller units. The data presented are averages of at least three independent determinations, and error bars correspond to the standard deviations.
phase cultures was observed in theSS mutant strain MZ100 (JCB570; rpoS:kan). Collectively, these results indicate that transcription from the P_{rraA} promoter increases in stationary phase and this effect is dependent on the transcription factor σ^e.

The effect of rpoS on the transcriptional activity of the P_{rraA} promoter was also evaluated using lacZ fusions transcribed from the P_{rraA} promoter. Specifically, β-galactosidase activity was determined in strains MZ003, MZ004, MZ013 (MZ003; rpoS:kan), and MZ014 (MZ004; rpoS:kan) grown to either exponential or stationary phases. As shown in Fig. 4C, in strain MZ003, the β-galactosidase activity increased more than two-fold in stationary-phase cultures (A_600 = 2.5) compared with log-phase cells (A_600 = 0.6). In the isogenic rpoS mutant MZ013, the β-galactosidase activity was comparable to the level observed in parental strain MZ003 grown in exponential phase, but the induction of lacZ expression in stationary phase was abolished. As a control, the β-galactosidase activity of MZ004 and MZ014 cells where lacZ is transcribed from the upstream menA promoter was neither growth phase dependent nor affected by rpoS. These results clearly suggest that σ^e is responsible for the increased transcription from P_{rraA} upon entrance into stationary phase. Recently, using microarray analysis Hengge-Aronis’s group identified over 400 genes which are positively controlled by σ^e in E. coli. While rraA was not recognized as a σ^e-dependent gene in this study, we note that 33 out of the 87 genes which had been experimentally shown to be σ^e controlled in earlier studies also failed to be detected in the microarray experiments (37).

Many σ^e promoters contain an extended −10 region, KCT AYRCTTAA (nucleotides −14 to −4; K stands for T or G, Y stands for T or C, and R stands for A or G) (37). Particularly, C at −13 has been shown to interact directly with lysine 173 in the 2.5 region of σ^e (2, 7, 31, 37). Nevertheless, despite its

**TABLE 1.** Western blot analysis of RraA level in exponential and stationary phases. Strain DY330-rraA::SPA, bearing a sequential peptide affinity (SPA) tag at the C terminus of the rraA open reading frame, was cultured in LB medium at 37°C and harvested during exponential growth (A_600 = 0.4) and stationary phase (A_600 = 3.5). RraA is expressed at an endogenous level from its chromosomal promoter and detected by the highly specific anti-FLAG antibody. Blots were replicated from three independent protein preparations. Replicate measurements were made on the same membrane to determine the reproducibility of the analysis. The intensity of the signal was observed to be linear by using a dilution series of total protein. The data are the means and errors of three separate blots.

**FIG. 4.** rraA expression in stationary phase. A. Western immunoblot analysis of RraA level in exponential and stationary phases. Strain DY330-rraA::SPA, bearing a sequential peptide affinity (SPA) tag at the C terminus of the rraA open reading frame, was cultured in LB medium at 37°C and harvested during exponential growth (A_600 = 0.4) and stationary phase (A_600 = 3.5). RraA is expressed at an endogenous level from its chromosomal promoter and detected by the highly specific anti-FLAG antibody. Blots were replicated from three independent protein preparations. Replicate measurements were made on the same membrane to determine the reproducibility of the analysis. The intensity of the signal was observed to be linear by using a dilution series of total protein. The data are the means and errors of three separate blots. B. Primer extension analysis of the transcription from P_{rraA} in growth phases. Total RNA was prepared from isogenic strains JCB570 (WT, wild type) and MZ100 (JCB570; rpoS:kan) grown to log phase (A_600 = 0.6) and stationary phase (A_600 = 2.5). Equivalent amounts of RNA (5 µg) were reverse transcribed using the primer (5'-GGGTTCACGCAGGTAAACATCGTTGAC-3') complementary to nucleotides +36 to +64 of rraA. The position of the reverse transcription product is shown by the arrowhead. C. Effect of σ^e level on the transcriptional activity of the various P_{rraA}-lacZ fusions in growth phases. Strains MZ003 (EC-O; λ[n = −92 to −1]lacZ), MZ004 (EC-O; λ[n = −1076 to −93]lacZ), MZ013 (MZ003; rpoS:kan), and MZ014 (MZ004; rpoS:kan) were grown in log (A_600 = 0.6) and stationary (A_600 = 2.5) phases in M9 medium supplemented with 0.2% casitone with aeration, at 37°C. Samples were normalized by optical density, and enzymatic activities were measured in Miller units. The data presented are averages of at least three independent determinations, and error bars correspond to the standard deviations.
RNase E plays a role in the decay of the activity of RNase E, it was of interest to examine whether since the function of RraA is to modulate the endonuclease temperature resulted in a significant (twofold) stabilization of because RNase E is an essential protein, the stability of the sequence, GATATTTT) (20, 38) and requires cyclic H11002/AMP-cAMP receptor protein for sigma factor sele-

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REFERENCES


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**FIG. 5.** Degradation profile of the rraA mRNA in wild-type and rne-1 strains. RNase protection assays and total RNA isolation were done as described in Materials and Methods on strains CH1827 (MC1061; cec-726::Tn10) and CH1827 (CH1827·rne-1). Time points in seconds were sampled after rifampin addition. Equivalent amounts of RNA (5 μg) were used in RNase protection assays and loaded into each lane of a 6% polyacrylamide-7 M urea gel. The band intensity was quantified using ImageQuant software. Degradation rates were determined by fitting the linear regression model and represent the averages of two independent determinations.


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