The pseudouridine synthase RluD is required for normal ribosome assembly and function in *Escherichia coli*

NANCY S. GUTGSELL, MURRAY P. DEUTSCHER, and JAMES OFENGAND¹

Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida 33101, USA

ABSTRACT

RluD is the pseudouridine synthase responsible for the formation of Ψ 1911, Ψ 1915, and Ψ 1917 in *Escherichia coli* 23S rRNA. Previous work from our laboratory demonstrated that disruption of the *rluD* gene and/or loss of the pseudouridine residues for which it is responsible resulted in a severe growth phenotype. In the current work we have examined further the effect of the loss of the RluD protein and its product pseudouridine residues in a deletion strain lacking the *rluD* gene. This strain exhibits defects in ribosome assembly, biogenesis, and function. Specifically, there is a deficit of 70S ribosomes, an increase in 50S and 30S subunits, and the appearance of new 62S and 39S particles. Analysis of the 39S particles indicates that they are immature precursors of the 50S subunits, whereas the 62S particles are derived from the breakdown of unstable 70S ribosomes. In addition, purified mutant 70S ribosomes were found to be somewhat less efficient than wild type in protein synthesis. The defect in ribosome assembly and resulting growth phenotype of the mutant could be restored by expression of wild-type RluD and synthesis of Ψ 1911, Ψ 1915, and Ψ 1917 residues, but not by catalytically inactive mutant RluD proteins, incapable of pseudouridine formation. The data suggest that the loss of the pseudouridine residues can account for all aspects of the mutant phenotype; however, a possible second function of the RluD synthase is also discussed.

Keywords: RluD pseudouridine synthase; pseudouridine residues; ribosome assembly; 39S particles; immature precursors; protein synthesis

INTRODUCTION

Cellular RNA contains a number of post-transcriptionally modified nucleosides, the most common of which is pseudouridine (Ψ ; 5-ribosyl-uracil). Ψ is found in RNAs for which the stability of their tertiary structure is important for function, such as rRNA, tRNA, and snRNA (Ofengand and Rudd 2000). Pseudouridine synthases are the enzymes responsible for formation of the Ψ residues. In *Escherichia coli* there are 11 synthases that can be grouped into five families that account for all the known rRNA and tRNA Ψ residues. RsuA, RluE, RluB, and RluF belong to the RsuA family; RluA, RluC, RluD, and TruC are in the RluA family; and TruA, TruB, and TruD are the sole members of their respective families. Each of the synthases has proven to be site specific and no overlapping function

has been detected. The contribution to cell physiology by individual pseudouridine synthases and the Ψ residues for which each is responsible has been investigated in our laboratory by deletion of their respective genes. Mutant *E. coli* strains lacking the individual synthases, RsuA (Conrad et al. 1999), RluA (Raychaudhuri et al. 1999), RluC (Conrad et al. 1998), TruB (Gutgsell et al. 2000), RluB, RluE, RluF, and TruC (Del Campo et al. 2001), and TruD (Kaya and Ofengand 2003) have been characterized. Of these, only the loss of the *rluA* (Ψ 746 23S rRNA and Ψ 32 tRNA^{phe}) and the *truB* (Ψ 55 all tRNAs) synthase genes were shown to result in a demonstrable growth phenotype as evidenced by reduced survival in competition with wild-type *E. coli*. In the case of the *truB* mutant, the loss of the TruB synthase, but not the Ψ 55 residue, was shown to be responsible for this phenotype.

RluD is the synthase responsible for formation of Ψ 1911, Ψ 1915, and Ψ 1917, which are located in a stem-loop structure of domain IV, helix 69, of 23S rRNA. This region has been shown to be part of the peptidyltransferase center (PTC). Based on the crystal structures of *Thermus thermophilus* 70S ribosomes (Yusupov et al. 2001) and *Deinococcus radiodurans* 50S rRNA (Bashan et al. 2003; Yonath and Bashan 2004) and cryo-electron microscopy mapping of

Reprint requests to: Nancy S. Gutgsell, Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Gautier Bldg., 1011 NW 15th St., Miami, FL 33136, USA; e-mail: gutgsell@chroma.med.miami.edu; fax: (305) 243-3955.

¹Deceased December 6, 2004.

Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.2550105>.

E. coli 70S ribosomes (Agrawal et al. 2004) complexed with various substrates, domain IV and helix 69 (including Ψ 1915; Agrawal et al. 2004) are known to interact with mRNA, tRNAs, 16S rRNA, and Ribosomal Release Factor, and may be involved in proper tRNA positioning, in translocation, and in release of mRNA from the post-termination complex. Taken together, these findings suggest that helix 69 and the modified residues of 23S rRNA are important for efficient protein biosynthesis by the ribosome.

Previously, disruption of the gene encoding RluD, by insertion of a miniTn10(cam) cassette, resulted in the most profound phenotype of any synthase mutant (Raychaudhuri et al. 1998; Gutgsell et al. 2001). The resulting disruption strain was shown to lack the aforementioned Ψ modifications, and grew half as fast as wild type. However, since the disruption mutant retained the potential to produce a truncated RluD protein consisting of the N-terminal 196 amino acids, an *rluD* deletion mutant was sought (see Discussion). Here, we report on the deletion of the *rluD* gene and the resulting severe growth defect (doubling time $6\times$ that of wild type in rich media at 37°C). We further show a marked defect in ribosome assembly in the mutant strain, which likely accounts for its growth phenotype. In addition, second site suppressor revertants and transformants of the deletion strain, which still lack the three Ψ residues, either in the absence of RluD or in the presence of an inactive mutant RluD, were characterized. Taken together, these studies demonstrate that Ψ 1911, 1915, and 1917 are essential for completely wild-type growth in *E. coli*, and that their effect is likely to be at the level of ribosome assembly.

RESULTS

Generation of a Δ *rluD*::kan mutant

Deletion of the gene for the *E. coli* pseudouridine synthase, RluD, was accomplished by gene replacement resulting in a Δ *rluD*::kan mutant strain (Datsenko and Wanner 2000). As will be shown, most of the *rluD* gene in this strain is replaced by a 1015-bp *kan*^r gene insertion. However, 39 bp of the 5' and 67 bp of the 3' *rluD* gene sequence are retained. The 5' *rluD* sequence is followed by a stop codon so that at most, only a 13 amino acid RluD protein fragment could be produced. Upon introduction of this mutation into *E. coli* MG1655, only very small colonies were obtained. Twenty-seven clones of this mutant strain were isolated, all of which were kanamycin resistant.

Deletion of the *rluD* gene was confirmed by analysis of wild-type and mutant genomic DNA by PCR and by Southern hybridization. All 27 mutant isolates were examined and they showed identical properties; however, the data for only one of the strains are shown (Fig. 1). The PCR product from mutant genomic DNA was 1.1 kb

FIGURE 1. PCR, Southern transfer and hybridization, and Ψ sequence analysis of wild-type and Δ *rluD*::kan mutant *E. coli* strains. (A) PCR reaction products were generated using genomic DNA as template. Primers used contain sequences homologous to the 5' and 3' ends of the *rluD* gene retained in the Δ *rluD*::kan mutant strain (forward primer 5'-GAAGCAGTATATATGGCACAACGAGTACAG CTC and reverse primer 5'-GGGAAGCTTTCATAACCAGTCCAC TTCATC). (Lanes 1,3) wild-type MG1655; (lanes 2,4), Δ *rluD*::kan. (Lanes 1,2) undigested; (lanes 3,4) reaction products digested with 20 units of HindIII (NEB) at 37°C for ≥ 16 h. Left side of the graph, sizes (kb) of the molecular weight markers. Right side of the graph, sizes (kb) of the DNA bands for each strain. (B) Southern transfer and hybridization were performed using genomic DNA incubated with 20 units of BamHI (NEB) at 37°C for 3 h to achieve partial digestion. Probes were generated from the wild-type MG1655 PCR product by digestion with 10 units NruI (NEB) and 5 units SphI (NEB) to generate the 806-nt probe specific for the center of *rluD* and a mixed probe specific for the 5' and 3' ends of *rluD*, respectively. Wild-type MG1655 (lanes I); Δ *rluD*::kan (lanes 2); (panel I) hybridization with the 806-nt probe; (panel II) the same filter stripped; (panel III) the filter subsequently hybridized with the mixed 5' and 3' probes. (C) Pseudouridine sequencing analysis of 23S rRNA from MG1655 and Δ *rluD*::kan. Total RNA was isolated from the indicated strains and examined for Ψ (Ofengand et al. 2001).

compared to just 1.0 kb for the wild-type product (Fig. 1A, lanes 1,2). The presence of the kanamycin insert introduces a HindIII site into the mutant genome that is not present in the wild-type *rluD* gene. Consequently,

digestion of material from the PCR reactions shown in lanes 1 and 2 with HindIII resulted in two bands for the mutant product (Fig. 1A, lane 4), but only the original undigested band for the wild type (Fig. 1A, lane 3).

For Southern analysis, wild-type and mutant genomic DNAs were digested with BamHI (Fig. 1B). The probes used were derived from the center of the *rluD* gene, present only in the wild-type (806-nt probe), and from the 5' (39 nt) and 3' (67 nt) ends of the *rluD* gene that are retained in the mutant genome. As expected, the 806 nt probe hybridized only with the wild-type DNA (Fig. 1B, panel I, lane 1). After stripping this filter (Fig. 1B, panel II), the pooled 5'/3' probe reacted with both wild-type and mutant genomic DNA (Fig. 1B, panel III, lanes 1 and 2).

To confirm that the resulting mutant was not a pseudo-revertant of a still slower growing strain, a phage P1-mediated backcross to wild type was done. Of the 180 tiny colonies obtained, none were clearly smaller than any other, and all were shown to be *rluD* deletion mutants by PCR analysis, as in Figure 1A (data not shown). The cotransduction frequency for a hypothetical second site suppressor mutation would have to be >99% (<1 in 180 transductants or <1% separable), or <202 bp away from *rluD* according to the Wu formula (Bachman et al. 1976). Based on all of these data, we conclude that the Δ *rluD*::kan mutant is a deletion of *rluD*.

One isolate was chosen for further characterization. DNA sequencing was carried out using genomic DNA from the mutant strain and sequencing primers immediately 5' and 3' of the remaining *rluD* sequence (data not shown). The sequencing showed that the *kan^r* gene had inserted at the expected position in the genome. The sequencing also confirmed that a stop codon is present in the 5' *rluD* sequence at bp 40–42, and, therefore, that only a 13 amino acid RluD fragment could be produced in the mutant strain. Absence of a functional RluD protein was further assessed by loss of the Ψ residues previously shown to be made by this synthase (Raychaudhuri et al. 1998). As expected, Ψ 1911 and 1917 are missing in the deletion strain (Fig. 1C). (It was not possible to determine whether Ψ 1915 was present using the CMC modification method due to N₃ methylation of this residue in vivo; Gutsell et al. 2001.)

Consequences of the absence of RluD and Ψ 1911, 1915, and 1917

The loss of RluD synthase and/or its Ψ products results in a severe growth phenotype. This is evident from the markedly reduced colony size (Fig. 2A) and the prolonged doubling time (173 min for the mutant vs. 28 min for wild type) in rich media at 37°C. In addition, sucrose gradient analysis revealed a major ribosome defect in the deletion mutant, which likely accounts for the observed growth phenotype (Fig. 2B). Lysates prepared from wild-type cells display a typical ribosome pattern, i.e., a

FIGURE 2. Colony morphology, exponential growth rates and ribosome profiles of wild-type and Δ *rluD*::kan mutant *E. coli* strains. (A) LB plates without drug (wild type) or with 34 μ g/mL kanamycin (mutant only) were incubated 41 h at 37°C. Dilutions were prepared from suspensions of wild-type and mutant cells and 6.8×10^8 cells/mL for each strain were spread on plates and incubated. Exponential growth rates were measured by monitoring cell density at 600 nm with a Perkin Elmer Lambda 25 UV/VIS Spectrophotometer. Doubling time was determined from a semilogarithmic plot of A_{600} versus time. Each plot consisted of five to seven time points. Doubling times were the average of two determinations (wild type) or six determinations (mutant), and are shown under the plates. (B) Ribosome profiles of wild-type and Δ *rluD*::kan cells were performed as described in Materials and Methods. Wild-type and mutant cells were lysed at 6 mM Mg⁺⁺ and gradients were run at 10 mM Mg⁺⁺. Twelve A_{260} units were layered onto 14%–32% sucrose gradients and centrifuged at 22,000 rpm for 19 h at 4°C.

large peak of 70S ribosomes and minor peaks of 50S and 30S subunits. Under these same conditions, a major defect was observed in the mutant profile characterized by the accumulation of 50S and 30S subunits, the appearance of abnormal particles (62S and 39S), and the concomitant loss of 70S ribosomes. This mutant ribosome profile was consistently detected in multiple experiments, with the exception of some variation in the sedimentation position of the mutant 39S and 30S species, suggesting some degree of heterogeneity in the composition of these particles (cf. Figs. 2 and 3; 6/10 mM). The overall recovery of ribosomal material based on A_{260} was comparable across the gradient for wild type and mutant in all experiments. Therefore, the difference in the distribution of mutant particles, as compared with wild-type, was not due to the loss of ribosomal material during processing or analysis. This abnormal ribosome profile could be due to either a defect in ribosome assembly or to the accelerated breakdown of misassembled ribosomes due to their instability.

Magnesium ion concentration is known to be critical to the maintenance of ribosome structure in vitro. At 4 mM

FIGURE 3. Ribosome profiles of wild-type and $\Delta rluD::kan$ mutant strains analyzed at various Mg^{++} concentrations. Ribosome profiles were performed as described in Materials and Methods. Wild-type and mutant cells were lysed, and gradients run at the Mg^{++} concentrations (mM) shown for lysis/sedimentation. Twelve A_{260} units were layered onto 14%–32% sucrose gradients and centrifuged at 22,000 rpm for 19 h at 4°C.

Mg^{++} even the tightly coupled 70S ribosomes of wild-type MG1655 begin to separate into 50S and 30S subunits (data not shown). Therefore, analyses in which Mg^{++} concentrations are varied during both lysis and centrifugation may be useful to examine the stability of ribosome particles during processing, and may provide some insight into the nature of the $\Delta rluD::kan$ mutant defect.

The MG1655 wild-type ribosome profiles are essentially unchanged when compared at the lowest (6/10 mM lysis/sedimentation) or the highest (20/20 mM) Mg^{++} concentrations tested (Fig. 3). In contrast, the mutant ribosome profiles vary dramatically within this range of Mg^{++} concentrations, and suggest how the abnormal 62S and 39S particles may arise. First, mutant 70S ribosomes are stabilized when high Mg^{++} is present throughout or just during the 19 h of sedimentation (20/20 or 6/20 mM); 62S particles are not present. However, when Mg^{++} concentrations are decreased during centrifugation (20/10 mM) or throughout the analysis (6/10 mM) there is a destabilization of the 70S ribosomes, resulting in either a slight downward shift in sedimentation or a nearly complete conversion to 62S particles, respectively. Thus, 62S particles appear to derive from unstable 70S particles that readily dissociate when Mg^{++} concentrations are lowered to ≤ 10 mM.

On the other hand, in contrast to 62S particles, the appearance of aberrant 39S particles may not be the result of simple instability of mutant 50S ribosomes. This can be seen by a comparison of 20/20 and 20/10 mM with 6/20 and 6/10 mM Mg^{++} , which reveals that the concentration

of Mg^{++} at the time of lysis seems most important for the maintenance of mutant 50S subunits or the appearance of 39S particles. This suggests that, unlike 70S ribosomes, 50S subunits, when initially introduced into a high Mg^{++} concentration at the time of lysis (20/20 or 20/10 mM), are able to remain stably associated; however, once dissociated (6/20 mM) they cannot be easily reassembled simply by increasing the Mg^{++} concentration. Moreover, we provide evidence below that 39S particles actually are immature forms of the 50S subunit.

Further characterization of the $\Delta rluD::kan$ mutant ribosomes

The previous data indicated that particles that sediment as 70S ribosomes are stabilized in the $\Delta rluD::kan$ mutant extract by the presence of 20 mM Mg^{++} during lysis and/or centrifugation. Similar profiles are seen when lysis and sedimentation are performed at 6/15 mM and 15/10 mM Mg^{++} , except that somewhat fewer 70S particles are recovered (data not shown). However, these analyses do not address the functionality of those mutant 70S particles. Therefore, poly(U)-dependent polyphenylalanine synthesis experiments were performed to determine whether mutant 70S ribosomes were as efficient in protein synthesis as wild-type ribosomes. For these analyses, RNaseI[−] derivatives of MG1655 and $\Delta rluD::kan$ were used to decrease the probability of RNA degradation during incubation. Ribosome profiles of the MG1655/I[−]::tet and $\Delta rluD::kan$ /I[−]::tet strains were shown to be unchanged as compared to their respective parental strains (data not shown). Wild-type and mutant lysates were prepared and sedimented at 15 mM Mg^{++} and 70S particles were collected for use in polyPhe synthesis.

This analysis revealed that when equal amounts (0.5 or 1.0 A_{260} units) of 70S ribosomes were used, $\Delta rluD::kan$ /I[−]::tet ribosomes were nearly as efficient in protein synthesis ($\sim 80\%$) as wild type (Fig. 4A). In addition, the moles of polyPhe produced per mole of 70S ribosomes used were unchanged at either ribosome concentration for both the wild-type and mutant strains. Therefore, the presence of twice the amount of mutant ribosomes had no inhibitory effect on protein synthesis. These data represent the average of multiple experiments performed using six wild-type and 10 individual mutant ribosome preparations. Consequently, this result is not likely to be due to some peculiarity of any one mutant culture or ribosome preparation. In a second experiment, wild-type and mutant 70S ribosomes were collected after lysis in 6 mM Mg^{++} followed by sedimentation at 15 mM Mg^{++} , and 0.5 or 1.0 A_{260} units were again used for protein synthesis. The results revealed that both wild-type and mutant ribosomes collected under conditions that would allow the disassociation of mutant 70S particles during lysis followed by stabilization during sedimentation are functionally indistinguishable from ribo-

FIGURE 4. Polyphenylalanine synthesis by wild-type and mutant 70S ribosomes. Polyphenylalanine synthesis was performed as described in Materials and Methods. (A) 70S ribosome fractions were isolated from sucrose gradients at 15 mM Mg^{++} concentration during lysis and sedimentation. Nine individual experiments were performed using 70S fractions from four wild-type and seven mutant ribosome preparations; MG1655/ $I^{-}::tet$ 0.5 A_{260} (closed squares) or $\Delta rluD::kan/I^{-}::tet$ 0.5 A_{260} units (closed circles). Four individual experiments were performed using 70S fractions from two wild-type and four mutant ribosome preparations; MG1655/ $I^{-}::tet$ 1.0 A_{260} unit (open squares) or $\Delta rluD::kan/I^{-}::tet$ 1.0 A_{260} unit (open circles). (B) Three individual experiments were performed using 70S fractions from two wild-type and three mutant ribosome preparations (lysis 6 mM and sedimentation 15 mM Mg^{++}). Symbols are the same as for A.

somes maintained at high Mg^{++} throughout processing. Thus, though many fewer 70S particles are recovered in the mutant at the lower Mg^{++} concentrations (see Fig. 2B), those that are produced are equally efficient in protein synthesis as assessed by elongation of polyPhe peptide fragments.

The accumulation of abnormal ribosomal particles in the $\Delta rluD::kan$ mutant strain suggests defects in rRNA processing, either due to breakdown of unstable particles or to the presence of under- or unprocessed rRNA precursors. In *E. coli*, the rRNAs are cotranscribed as a single 30S transcript that is initially cleaved by the endonuclease RNase III to release precursors of the 23S, 16S, and 5S rRNAs. The p23S precursor that results from RNase III cleavage contains three to seven extra nucleotides at its 5' end and seven to nine extra nucleotides at its 3' end. The 16S precursor (17S) resulting from this primary cleavage contains 115 extra 5' nucleotides and 33 additional nucleotides at the 3' end. Previous studies have determined that final maturation of 23S rRNA occurs within polysomes (Sirdeshmukh and Schlessinger 1985; Srivastava and Schlessinger 1988). In addition, it has been shown that precursors of 16S rRNA are present in particles that cosediment with mature 30S subunits, and that these precursor particles are found only as subunits, not in 70S ribosomes (Lindahl 1975). Therefore, the rRNA content and the state of maturation of the 23S and 16S rRNA present in $\Delta rluD::kan$ mutant particles was assessed.

Lysates were prepared at 6 mM Mg^{++} and sedimentation was done at 10 mM Mg^{++} to allow for isolation of 62S and 39S particles. Fractions were then collected across the entire ribosome profile for wild-type and mutant, and A_{260} values were determined (Fig. 5A). The total amount of ribosomal material based on A_{260} units of the collected fractions was calculated for wild type and mutant and proved to be equivalent for both strains. Those fractions that represent 70S, 50S, and 30S particles for wild type and mutant, as well as mutant 62S and 39S particles, were pooled for further analysis. These pooled fractions were analyzed by slot blot hybridization using oligonucleotide probes specific for internal 23S (probe 2) and 16S (probe 4) sequences (Fig. 5B) and 5S sequences (data not shown). The same filter was hybridized with each of the oligonucleotide probes. The filter was stripped between each hybridization, and loss of signal was confirmed by autoradiography prior to hybridization with the next probe. The 23S probe revealed the presence of 23S rRNA within mutant and wild-type 70S and 50S particles as well as mutant 62S and 39S particles (Fig. 5C). The 16S probe hybridized with both wild-type and mutant 70S and 30S particles as well as mutant 62S particles (Fig. 5C). 5S rRNA was detected in wild-type and mutant 70S and 50S particles as well as mutant 62S and 39S particles (data not shown). Therefore, the additional particles detected in mutant ribosome profiles, 62S and 39S, each had the same content of rRNA as mutant and wild-type 70S and 50S particles, respectively.

To determine the state of maturation of rRNA present in mutant particles, the number of extra 5' nucleotides was determined by primer extension (Fig. 5D). The extent of maturation of 23S rRNA was determined using an oligo probe (probe 1, Fig. 5B) complementary to residues 35–55 of mature 23S rRNA (Charollais et al. 2003). Using this probe, we find that wild-type 70S and 50S particles contain mature rRNA and a +1 species, as has previously been reported (Li et al. 1999; Charollais et al. 2003). In the $\Delta rluD::kan$ mutant, 70S, 62S, and 50S particles also contain predominantly the mature and the +1 species. However, the 62S and 50S particles also contain a small amount of a +4 species as well as an unprocessed +8 species, indicating that a portion of rRNAs in these particles is immature.

Sucrose gradient analysis suggested that mutant 70S ribosomes are unstable during processing. Assuming this instability reflects the *in vivo* situation, a fraction of mutant 23S rRNA may not be part of a polysome complex long enough for final maturation to occur. It is also possible that not all mutant 70S complexes would be able to function in protein synthesis. This would be in keeping with the polyPhe synthesis experiments in which stabilized mutant 70S ribosomes were found to be 20% less efficient. Finally, mutant 39S particles contain mostly under- (+4) and unprocessed (+8) precursors (Fig. 5D). The small amount of an apparently mature species is likely due to minor contamination of the 39S fraction pool with 50S subunits

FIGURE 5. Fraction collection, slot blot, and primer extension analysis (see Materials and Methods). (A) A₂₆₀ units/mL of ribosome fractions collected from sucrose gradients (Mg⁺⁺ concentration 6 mM/10 mM at lysis/sedimentation) for wild-type MG1655/I-::tet (closed squares) and $\Delta rluD::kan/I-::tet$ mutant (open squares). (Numbers below each graph are the fractions pooled for the ribosomal subunits indicated.) (B) Open boxes represent mature rRNA species, lines represent precursor sequences, and solid bars indicate probes used for slot blot hybridization (2 and 4) and primer extension analysis (1 and 3). (C) Equal amounts (5 μ g) of RNA from pooled ribosomal fractions were transferred to nitrocellulose membranes and probed with the indicated oligonucleotides. (D) Equal amounts (0.5 μ g) of RNA from the same pooled fractions were analyzed by primer extension using the indicated oligonucleotides.

since the intensity of this band was always low and varied in individual experiments. The predominance of the +8 species indicates a complete lack of processing for most rRNA within this particle and further substantiates our conclusion that the 39S particle is an immature form of the 50S subunit (see Fig. 3).

In previous studies of a 40S particle from a $\Delta srmB$ mutant, a predominant immature species of +7 was detected. However, unexpected +8 species were also seen (Charollais et al. 2003).

The accurate numbering of the unprocessed +8 RNA species detected in our experiments was confirmed by comparison of the primer extension products with the single nucleotide step ladder and the 23S DNA sequence run on the same gel (Fig. 5D). In addition, the presence of predominantly the +8 species for the $\Delta rluD::kan$ mutant was reproducible in several experiments using two types of reverse transcriptases, AMV (data not shown) and M-MLV (Fig. 5D). The mechanism of RNase III action in maturation of rRNA has long been studied. However, its substrate specificity is still not well understood (Sirdeshmukh and Schlessinger 1985; Srivastava and Schlessinger 1990; Evguenieva-Hackenberg and Klug 2000; Lamontagne and Elela 2004). Therefore, the reaction that results in a +8 species in the 39S particles cannot be evaluated beyond indicating a complete lack of final processing for most 23S rRNA molecules.

Maturation of 16S rRNA within wild-type and $\Delta rluD$ mutant 30S particles also was assessed. Primer extension analysis was performed using an oligo probe complementary to residues 15–35 of the mature 16S sequence (probe 3, Fig. 5B). In the mutant 30S particles, many strong bands of up to +115 nt were detected, indicative of the presence of a large population of precursors to 16S rRNA (Fig. 5D). In wild-type 30S particles the same bands were detected; however, their intensity was much less, indicating that most of the rRNA contained within wild-type 30S particles is the mature 16S species. Quantification of the immature species present in wild-type and mutant 30S particles demonstrated that in both, immature species with up to five extra nucleotides are present at similar levels relative to the mature species. However, immature

RNAs with 6–115 extra 5' nucleotides are present in mutant 30S particles at much higher levels (30%) compared to wild type (1%). Therefore, high levels of the least mature 30S precursor species accumulate in the $\Delta rluD::kan$ mutant strain. In contrast, wild-type 70S, as well as mutant 70S and 62S particles, lack these additional 5' nucleotides, and contain largely mature 16S rRNA (Fig. 5D). This result provides further evidence to support the idea that mutant 62S particles are breakdown products

of unstable 70S particles, rather than precursor particles. While it is unclear why the absence of some Ψ residues in the large subunit should affect maturation of 16S rRNA, this appears to be a common finding. For example, accumulation of 16S precursor rRNA was also detected in the Δ srnB mutant, in which the primary defect is in 50S ribosome biogenesis (Charollais et al. 2003). In this case, accumulation of 16S precursors was believed to be an indirect consequence of 50S deficiency, rather than a direct result attributable to the loss of the SrmB protein.

Growth properties of the Δ rluD::kan strain supplemented with wild-type or mutant RluD

To address the question of whether the observed phenotype of the Δ rluD::kan mutant is due solely to the loss of the *rluD* gene, the mutant strain was transformed with plasmid-borne wild-type or mutated *rluD* genes. Expression of wild-type RluD restored normal growth on solid media to Δ rluD::kan cells either with or without induction (data not shown). However, expression of two RluD proteins that have been mutated at an aspartate residue previously shown to be necessary for synthase activity (D139N and D139T) had no effect on mutant colony size either with or without induction (data not shown). Exponential growth rates were determined for all transformed strains in the presence of inducer (0.05% L-arabinose). Expression of the wild-type RluD protein fully restores normal growth (doubling time 22 ± 1 min), while cells expressing the D139N (143 ± 10 min) or D139T (143 ± 4 min) proteins have generation times comparable to cells that contain empty plasmid (143 ± 7 min).

To determine whether the lack of rescue by mutant proteins might be due to lack of protein resulting from decreased expression or increased proteolysis of aberrantly folded proteins, SDS-PAGE analysis was performed. Lysates were made from cells that were scraped from the plates with or without inducer. This analysis demonstrated that induction results in the expression of equivalent amounts of wild-type and mutant proteins and that they are of the expected size, 39 kDa (data not shown). This analysis also showed that even without induction sufficient wild-type RluD protein was produced to rescue growth despite protein levels that are too low to visualize on SDS-PAGE. Pseudouridine sequence analysis revealed that the expression of wild-type RluD, which rescues growth of the Δ rluD::kan mutant, also restores synthesis of Ψ 1911, 1915, and 1917, whereas induced levels of mutant proteins that fail to rescue the mutant growth phenotype also fail to restore Ψ synthesis (data not shown). Sucrose gradient analysis further revealed that only the expression of wild-type RluD restored a normal ribosome profile to the Δ rluD::kan mutant (data not shown). The ribosome profile of the mutant carrying plasmid-borne wild-type *rluD* gene was indistinguishable from wild-type cells carrying empty plasmid. The ribosome abnormalities of the mutant

strain persist in cells expressing plasmid-borne mutant RluD proteins or empty plasmids (refer to Fig. 2B). Therefore, only the presence of the wild-type RluD protein and/or the Ψ residues synthesized by it are able to fully restore normal ribosome biogenesis and wild-type exponential growth in the Δ rluD::kan strain.

Second site suppressor mutant(s)

Due to the severity of the growth phenotype, the Δ rluD::kan mutant readily generates colonies that appear to grow normally on solid media (data not shown). Six such colonies were selected, and they proved to be pseudorevertants by molecular and functional assays including, PCR, Southern transfer, and hybridization and by their inability to form Ψ 1911, 1915, and 1917 (data not shown). In addition, these six strains retained the ability to grow on plates containing kanamycin, indicating that the kan insert was still present (data not shown). Four of these isolates were chosen for further characterization of their growth phenotype. Though apparently wild type in colony size, exponential growth rates were depressed compared with the true wild-type strain, MG1655 (doubling time 23 ± 1 min), ranging from 25 ± 2 to 29 ± 2 min in rich media at 37°C. In addition, when grown in competition with wild-type cells through all phases of the growth cycle, pseudorevertant cells were present at just 0.01%–1.0% of their original numbers after only four cycles of competitive growth (Fig. 6A). The differences in exponential growth rates and the relative competitiveness of the individual pseudorevertants suggest that separate mutations may be responsible for their improved growth. Studies to formally address what, if any, genetic difference(s) exist among the individual pseudorevertants are currently underway in our laboratory. Not surprisingly, sucrose gradient analysis demonstrated that there is also a modest and somewhat variable defect in ribosome biogenesis when compared with the true wild-type strain, MG1655, with more than normal 50S particles and fewer fully assembled 70S particles (Fig. 6B). Therefore, a mutated state that does not restore the RluD synthase or the Ψ residues for which it is responsible does not fully compensate for the loss of the *rluD* gene and the resulting phenotype of the Δ rluD::kan mutant.

DISCUSSION

In this work we demonstrate that the pseudouridine synthase RluD and/or the Ψ 1911, Ψ 1915, and Ψ 1917 residues formed by it are necessary for normal ribosome biogenesis, assembly, and function in *E. coli*. The ribosome defect and the subsequent severe growth phenotype resulting from deletion of the *rluD* gene were corrected only when plasmid-borne wild-type protein, but not D139 mutant proteins, were reintroduced into the Δ rluD::kan strain. This is in contrast to the results of previous work from our laboratory using two RluD-minus

FIGURE 6. Competition studies, exponential growth rates, and ribosome profiles of MG1655 (wild type) and four pseudorevertant isolates of the $\Delta rluD::kan$ mutant. (A) Growth competition studies were done as described previously (Gutgsell et al. 2000). MG1655 (wild type) (closed squares), four individual pseudorevertant isolates (open and closed triangles and circles). Exponential growth rates were done as described in Materials and Methods. Doubling times were determined as described in Figure 2. Each plot consisted of seven to nine time points. Doubling times were the average of two determinants. (B) Ribosome profiles were performed as described in Materials and Methods. Wild-type, pseudorevertant, and mutant cells were lysed at 6 mM Mg^{++} and gradients were run at 10 mM Mg^{++} . Twelve A_{260} units were layered onto 14%–32% sucrose gradients and centrifuged at 22,000 rpm for 19 h at 4°C.

strains (Tiny and Dust), generated by disruption of the *rluD* gene (Gutgsell et al. 2001). Expression of mutant RluD proteins, D139N and D139T, restored wild-type growth in both disruption strains, but restored Ψ formation only in the Dust strain. The restoration of growth in the absence of Ψ formation in the Tiny strain suggested a second separable function for the RluD protein. However, that disruption mutant strain was created by insertion of a mini Tn10cam cassette between residues 588 and 589 in the *rluD* gene sequence. Therefore, introduction of plasmid-borne mutated *rluD* gene sequences into the disruption mutant allowed for the potential of homologous recombination, which could have resulted in restoration of the wild-type *rluD* gene in either the genome or on the plasmid. There was also the potential for formation of functional D139/D139N or D139/D139T truncated/full-length heterodimer proteins, assuming RluD forms dimers, as was shown previously for TruA (Foster et al. 2000). The wild-type growth and Ψ formation seen with the Dust strain are in fact consistent with one of these scenarios. However,

interpretation of the results for the Tiny strain is complicated by the fact that Tiny was shown to arise from Dust as a result of an unidentified second site mutation. Without knowledge of the nature of this second site mutation, it is not yet possible to explain how Tiny cells were able to grow despite the absence of the Ψ residues. Nevertheless, in the present work, expression of the D139 mutant proteins in the background of a true *rluD* deletion strain avoided these complications, and clearly demonstrated the importance of the Ψ s.

In the current study, the lack of rescue in the absence of pseudouridine formation suggests a specific role for the Ψ 1911, 1915, and 1917 residues and argues against a second separable function for the RluD protein. If the RluD protein has multiple functions, the D139 mutant proteins might be expected to retain their ability to perform such a function secondary to Ψ formation. SDS-PAGE analysis suggests no obvious effect, such as misfolding or increased proteolysis, as a result of mutation of these RluD proteins. However, subtle changes in protein structure that could affect such a function may not be detectable by this method. Crystallization of the D139 mutant proteins is currently underway, which might help to clarify this issue (P.P. Vaidyanathan and A. Malholtra, unpubl.; Del Campo et al. 2004).

Is there then, based on current evidence, any reason to believe there is a second function for the RluD protein? There is precedence for the idea that the ribosome assembly defect seen in the $\Delta rluD::kan$ mutant could be attributed, at least in part, to the lack of the RluD protein. First, several recent studies have demonstrated that nonribosomal proteins, which do not have RNA modification functions, have a role in ribosome biogenesis. Mutation of the genes encoding the DEAD-box RNA helicases SrmB (Charollais et al. 2003) and CsdA (Charollais et al. 2004), the heat shock-induced methyltransferase RrmJ (Hager et al. 2002), as well as the DnaK chaperone (El Hage and Alix 2004) all result in ribosome and growth defects in vivo and phenotypes similar to the $\Delta rluD$ mutant. Also, other studies have demonstrated additional functions for yeast and *E. coli* modifying enzymes apart from their role in pseudouridylation or methylation. The yeast tRNA pseudouridine synthase, Rib2/Pus8p, was shown to have a second catalytic domain that is involved in a completely unrelated metabolic function (Behm-Ansmant et al. 2004). The *E. coli* *trmA* (Persson et al. 1992) and *rlmA*^I (Liu et al. 2004) gene products, but not methylation of tRNA U54 and 23S rRNA G745, respectively, are necessary for cell viability, suggesting a second important function for the TrmA and RlmA^I proteins. Interestingly, like $\Delta rluD$, the *rlmA*^I mutant strain also produces spontaneous pseudorevertants, still lacking mG745, but with wild-type growth restored, suggesting that methylation of this residue is not necessary for growth. In contrast, in the background of the $\Delta rluD::kan$ strain, the subtle defect in exponential growth, ribosome profiles, and the inability of the $\Delta rluD$ pseudorevertants to compete with true wild type suggests a role for the Ψ residues.

Taken together the results of the present study suggest the following scenario: The primary defect in the $\Delta rluD$ mutant is at the level of rRNA assembly and processing, which results in the characteristic mutant ribosome profile. Instability of mutant 70S ribosomes leads to an accumulation of 62S, 50S, and 30S particles. The accumulation of 39S particles containing precursor rRNA sequences indicates problems with large subunit assembly. The ribosome assembly and processing defect results in a severe growth phenotype reflected by a generation time sixfold that of wild type. In addition, the in vitro studies demonstrated that mutant 70S ribosomes stabilized by high Mg^{++} concentrations were reproducibly 20% less active in protein synthesis compared to wild-type ribosomes, indicating that there is a modest functional defect as well. What then can be said about the RNP species isolated from mutant lysates that sediment as 70S particles? Primer extension analysis revealed that like wild type, they contain only mature 23S rRNA and the +1 species. Such complete processing of 23S rRNA suggests that mutant 70S particles must have been engaged in protein synthesis because translation is needed to complete maturation (Sirdeshmukh and Schlessinger 1985; Srivastava and Schlessinger 1988). The fact that these mutant particles sediment like wild-type 70S ribosomes also suggests that proper folding and the association of the expected ribosomal proteins has occurred. Therefore, though few 70S particles are recovered in lysates from the mutant strain, those that are present apparently are compositionally similar to wild type. Assuming that the recovery of 70S particles closely reflects the in vivo situation, the pronounced growth defect is likely due to the low amount of functional 70S ribosomes in mutant cells. Studies to further address mutant ribosome composition and function are currently under way.

MATERIALS AND METHODS

Strains

MG1655 was the wild-type *E. coli* strain. DH5 α was the strain used for all initial transformation experiments. MG1655 $\Delta rluD::kan$ was constructed by a modification of the gene replacement method of Datsenko and Wanner (2000). A deletion allele containing the amino- and carboxy-terminal fragments of *rluD* linked to a kanamycin cassette was constructed by PCR amplification from plasmid pUC4K (Amersham). The 5' primer (5'-GCACAACGAGTACAGCTCACTGCAACGGTGTCCGAAT**AATGATTACGAATTCC**CCGGATC-3') was the *rluD* sequence of residues +4 to +39, followed by a stop codon (bold type) that overlaps the A of the initiating AUG (italics) followed by the complement of residues 1689–1670 of pUC4K (underlined). The 3' primer (5'-TTCGAAATCGGCGCGCATCAVCTCAATCAGCTCCAC**CCAATTCTGATTAGAAAACTC**-3') was the complement of *rluD* residues +951 to +916, followed by the sequence of residues 710–731 of pUC4K (underlined). After PCR amplification of the allele, the wild-type *rluD*

gene was replaced with the deletion allele as described (Datsenko and Wanner 2000).

P1-mediated backcross of four isolates of the $\Delta rluD::kan$ strain to wild type was done as described (Miller 1992). Cells were then spread on LB plates containing 34 μ g/mL kanamycin. P1 lysates were prepared from six $\Delta rluD::kan$ isolates obtained from this primary backcross into MG1655, for a second P1 mediated transductional backcross to MG1655. Cells were again spread on LB/kanamycin plates from which individual colonies were replated onto M9/kanamycin plates to avoid selection of nutritional auxotrophs.

The RNase I[−] mutation was introduced into MG1655 and $\Delta rluD::kan$ strains by P1 mediated transduction of an *rna::tet* strain, CAN 20-12E (Deutscher et al. 1984).

Transduced cells were plated on M9/tet or M9/tet, kan plates and individual colonies were transferred to a second set of M9 plates as before and subsequently streaked onto LB plates containing the same drugs. The resulting drug resistant colonies were first screened for loss of RNase I activity by Methyl Green Colorimetric Analysis as described (Wright 1971). Loss of RNase I[−] activity was confirmed for selected clones by determination of acid-soluble radioactivity released from [³H]poly(A) substrate, as described (Deutscher et al. 1984).

Liquid culture

To avoid the emergence of pseudorevertants in $\Delta rluD::kan$ cultures, all strains, including wild-type controls, were cultured by inoculating LB or YT media with single colonies. Colonies were picked with sterile pipette tips to standardize the amount of inoculum, and cultures were grown with aeration by shaking at 37°C to an $A_{600} \leq 0.5$. Aliquots were removed at the time of harvest for dilution and plating to ascertain that revertants had not accumulated at the low density to which cells were grown (<0.01% revertants was considered acceptable). As was necessary for individual experiments, cultures contained 34 μ g/mL kanamycin ($\Delta rluD::kan$); 5 μ g/mL tetracycline (RNase I[−]); 0.5% L-arabinose and/or 100 μ g/mL carbenicillin (induction and maintenance of pBAD/Myc-His A plasmids).

Plasmids and transformations

The wild-type *rluD* gene was PCR amplified from MG1655 genomic DNA using Taq DNA polymerase (Promega) and subcloned into the NcoI and HindIII sites of pBAD/Myc-His A (Invitrogen). The amino-terminal primer was (5'-GAAGAAGGGTCTCA CATGGCACAACGAGTACAGCTC-3'), incorporating a BsaI site (italics) 1 nt before the AUG start codon (underlined). The carboxy-terminal primer was (5'-GGGAAGCTTTCATAACCAGTCCACTTCATC-3'), incorporating a HindIII site (italics) followed by the complement of residues 981–961 (numbering begins with the TGA stop codon). As a result of inclusion of the *rluD* stop codon, the C-terminal polyhistidine tag is not expressed from this plasmid.

To make the D139N and D139T mutants of *rluD*, the wild-type *rluD* gene was amplified from MG1655 genomic DNA as described above and subcloned into the NcoI and HindIII sites of pTrc99A (Pharmacia). The pTrc99A/*rluD* construct was used as template in PCR reactions using pfu turbo polymerase

(Stratagene) to incorporate the mutation and amplify the mutated gene. For *rluD*(D139N) the mutagenic primers were (5'-CATCG TCCATCGTCTGAATAAAGACACCACTGGCC-3') and (5'-GGCC AGTGGTGTCTTTATTTCAGACGATGGACGATG-3'), which incorporated the necessary base change (underlined in the primer sequences) to mutate the aspartate residue to asparagine. For *rluD*(D139T), the mutagenic primers were (5'-GGATCGCCAT CGTCTGACTAAAGACACCACTGGCC-3') and (5'-GGCCAGTGG TGTCTTTAGTCAGACGATGGACGTGCC-3'), which incorporated the necessary base changes (underlined in the primer sequences) to mutate the aspartate residue to threonine. The pTrc99A*rluD*/D139N and pTrc99A*rluD*/D139T plasmids were used as template in PCR reactions to amplify mutated *rluD* genes as described above for the wild-type *rluD* gene for subcloning into the NcoI and HindIII sites of pBAD/Myc-His A. All of the mutant plasmids were verified by DNA sequencing.

The Δ *rluD*::kan mutant was transformed with empty pBAD/Myc-His A plasmid or with wild-type or mutant *rluD*-containing plasmids by 42°C heat shock (Sambrook et al. 1989) with modifications in order to avoid pseudorevertants as described (see Liquid culture). The following day, colonies were picked from the transformation plates and inoculated into 1 mL medium containing LB kanamycin (34 µg/mL) and carbenicillin (100 µg/mL) for incubation $\leq$ 4 h at 37°C. From these short-term cultures glycerol stocks were prepared and LB (kan/carb) plates were streaked in order to determine colony size of transformants and to again ensure that no revertants had emerged. From each 4-h culture, 0.1 mL was retained and 4 mL LB (kan/carb) were added for overnight incubation for plasmid DNA isolation and characterization to insure the presence of the expected construct. Briefly, the pBAD/Myc-His A plasmid and the wild-type *rluD* gene contain a single PflMI site, while both the D139N and D139T mutants have lost this site as a direct consequence of the nucleotide change(s) that create(s) the aspartic acid mutations. Therefore, upon PflMI digestion, mutant plasmids are linearized and wild-type plasmids appear as two bands on agarose gels.

Ribosome analysis

All strains tested were grown in YT or LB medium as described (see Liquid culture). Cells were cooled by incubating 10 min on ice in precooled centrifuge bottles or by pouring cultures into centrifuge bottles that contained frozen, crushed medium stored at -20°C. Cells were collected by centrifugation and pellets resuspended in lysis buffer (50 mM Tris-HCl at pH 8, 60 mM KCl, 60 mM NH₄Cl, 1 mM DTT, 16% sucrose, and various concentrations [6–20 mM] MgOAc) plus 1 mg/mL lysozyme (Sigma) and 40 U/mL DNaseI (Sigma) to begin three freeze-thaw cycles. After the third thaw cycle, 0.5% Brij 58 (Sigma) and 0.5% deoxycholic acid (MC & B) were added, and the sample was incubated on ice for 20 min. All lysates were clarified by centrifugation at 14,000 rpm for 20 min at 4°C. The extract concentration was determined by measuring the A₂₆₀. To analyze ribosome profiles, 12 A₂₆₀ units of lysate were layered onto 14%–32% sucrose gradients in buffer (20 mM Tris-HCl at pH 8; 60 mM KCl, 60 mM NH₄Cl, 1 mM DTT, and various concentrations [10–20 mM] MgOAc,) and centrifuged at 22,000 rpm for 19 h at 4°C in a Beckman SW 28 rotor. Gradients were analyzed with a Gilson

Holochrome with continuous monitoring at A₂₆₀ nm and a Bromma LKB 2 channel recorder.

To prepare S150 enzymes, 1 or 2 L of LB_{tet} or LB_{tet/kan} medium were inoculated with single colonies of MG1655/I⁻::tet or MG1655 Δ *rluD*::kan/I⁻::tet (RNase I⁻ strains), respectively, and grown as described (see Liquid culture). Cells were harvested and lysates were prepared and fractionated to obtain the S150 enzymes fraction as described (Nierhaus and Dohme 1979).

Polyphenylalanine synthesis experiments were performed as described with some modifications (Cunningham et al. 1992). The reaction mixture for polyPhe synthesis contained 45 mM Bicine (pH 8.5), 5 mM HEPES (pH 7.5), 50 mM NH₄Cl, 15 mM Mg acetate, 10 mM phosphoenolpyruvate, 2 mM ATP, 5 mM DTT, 1 mM GTP, 80 µg/mL pyruvate kinase, 0.022 mM cold phenylalanine, and 0.25 µCi [³H] phenylalanine. Reaction mixtures (100 µL) contained 53 µL of this master mix plus S150 enzymes and tRNA (Sigma, *E. coli* Strain W) at concentrations calculated to provide 100 pmol tRNA^{Phe} based on 2% representation in a total tRNA pool. This reaction mix was incubated 7 min at 37°C to precharge the tRNA. Poly(U) (100 µg/mL) and ribosomes (0.5 or 1.0 A₂₆₀ units) were added and incubation at 37°C continued. At the indicated times, 35-µL aliquots of the reaction mixture were removed to 13 × 100 mm glass tubes. A solution containing 10% TCA, 2% Casamino Acids was added to ~1/4 tube volume, vortexed briefly, and incubated 15 min at 90°C. A solution containing 5% TCA, 2% Casamino Acids was added to ~1/2 tube volume. Solutions were vortexed briefly, cooled, and passed through nitrocellulose filters (Schleicher & Schuell Grade BA85) with vacuum. Filters were dried, dissolved in scintillation fluid (Filtron X), and counted (Beckman LS 3801 Liquid Scintillation System). Values were corrected for the small amount of reaction found in the absence of ribosomes.

RNA analysis

Wild-type MG1655/I⁻::tet and mutant Δ *rluD*::kan/I⁻::tet lysates were prepared as described for ribosome analysis. rRNA fractions (1 mL) were collected from across the resulting sucrose gradients and A₂₆₀ measurements were taken (PerkinElmer Lambda 25 spectrophotometer). Fractions that represent 70S, 50S, and 30S for wild-type and mutant cells and fractions for mutant 62S and 39S were pooled. The pooled fractions were extracted with phenol:chloroform (1:1), and chloroform:isoamyl alcohol (24:1), and the isolated RNA was precipitated with ethanol and dissolved in distilled H₂O. For further analysis, RNA (5 µg) was mixed with 2.5% Ficoll, 0.075% bromophenol blue, 48% formide, 6.5% formaldehyde, 1× MOPS gel-loading buffer. Samples were heated at 65°C for 5 min, mixed with an equal volume of 20× SSC, and placed on ice. RNAs were transferred to Nytran nylon membranes (Schleicher & Schuell) using a slot blot manifold (Bethesda Research Laboratories). RNA was fixed to the membrane by cross-linking (Stratalinker) and membranes were prehybridized $\geq$ 4 h at 50°C in hybridization buffer (Clontech ExpressHyb Hybridization Solution) followed by overnight hybridization at 50°C with [³²P]-labeled probes. After hybridization, membranes were washed first in 6× SSC for 30 min at 30°C followed by a second wash in 3× SSC 0.5% SDS for 15 min at 50°C. Membranes were analyzed by autoradiography (Kodak Biomax MR film). The filters were hybridized with multiple probes and were stripped

between each hybridization by washing $\geq 3\times$ in boiling $0.1\times$ SSC 0.1% SDS. Complete stripping was verified by autoradiography. Oligonucleotide probes used were 23S (5'-GCACCTGCTCGCGCCGTC-3'), complementary to residues 2359–2376 of mature 23S rRNA; 16S (5'-GGTTGAGCCCGGG GATTTCAC-3'), complementary to residues 604–624 of 16S rRNA; and a pool of two 5S (5'-AGGTGGGACCACCTCTC TAC-3') and (5'-GGCGCTACGGCGTTTCACTTC-3'), complementary to residues 14–35 and 54–75 of mature 5S rRNA, respectively.

The same RNAs (0.5 μ g of each) were used for primer extension using oligonucleotides 23S (5'-CCTTCATCGCCTCTGACTGCC-3') (Charollais et al. 2003) and 16S (5'-CGTTCAATCTGAGCCAT GATC-3') (Sigma Genosys), complementary to residues 39–58 and 15–35 of 23S and 16S rRNAs, respectively (numbering is from the mature 5' end). Oligonucleotide primers were [32 P] 5' end labeled using T4 polynucleotide kinase (NEB). 23S primer extensions were done using Superscript II RNase H⁻ M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's specifications. 16S and 23S primer extensions were done using AMV reverse transcriptase (Promega) as described previously (Ofengand et al. 2001) except that annealing was at 50°C. The extension products were separated on 8% polyacrylamide 7 M urea gels and detected by autoradiography. The 23S DNA sequencing ladder and the single nucleotide step ladder were obtained using the same primer and the plasmid pCW1 as template (Weitzmann et al. 1990). The 16S sequence was done in parallel with the primer extension reactions using the same primer and MG1655/I⁻ :: tet total RNA as template. Intensity of the bands resulting from the 16S primer extension reactions was determined using a PhosphorImager (Molecular Dynamics).

Other methods and materials

Isolation of total RNA and Ψ sequencing were performed as described (Ofengand et al. 2001). Primers were obtained from Gibco-BRL and used without further purification. SDS polyacrylamide gel electrophoresis was performed as described previously (Raychaudhuri et al. 1998). Competition studies were done as described previously (Guttsell et al. 2000). DNA sequencing was done with a Sequenase Version 2.0 DNA Sequencing Kit (USB Corporation) according to the manufacturer's directions using primers described for amplification and subcloning of the *rluD* gene.

ACKNOWLEDGMENTS

We are grateful to professors Kenn E. Rudd and Chaitanya Jain for helpful discussions and editorial assistance. We thank Yusuf Kaya for technical assistance with the primer extension analysis shown in Figure 5D.

Received March 10, 2005; accepted April 15, 2005.

REFERENCES

Agrawal, R.K., Sharma, M.R., Kiel, M.C., Hirokawa, G., Booth, T.M., Spahn, C.M., Grassucci, R.A., Kaji, A., and Frank, J. 2004. Visualization of ribosome-recycling factor on the *Escherichia coli* 70S

- ribosome: functional implications. *Proc. Natl. Acad. Sci.* **101**: 8900–8905.
- Bachman, B.J., Low, K.B., and Taylor, A.L. 1976. Recalibrated linkage map of *Escherichia coli* K-12. *Bacteriol. Rev.* **40**: 116–167.
- Bashan, A., Agmon, I., Zarivach, R., Schlutzen, F., Harms, J., Berisio, R., Bartels, H., Franceschi, F., Auerach, T., Hansen, H.A.S., et al. 2003. Structural basis of the ribosomal machinery for peptide bond formation, translocation, and nascent chain progression. *Mol. Cell* **11**: 91–102.
- Behm-Ansmant, I., Grosjean, H., Massenet, S., and Motorin, Y. 2004. Pseudouridylation at position 32 of mitochondrial and cytoplasmic tRNAs requires two distinct enzymes in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **279**: 52998–53006.
- Charollais, J., Pflieger, D., Vinh, J., Dreyfus, M., and Iost, I. 2003. The DEAD-box RNA helicase SrmB is involved in the assembly of 50S ribosomal subunits in *Escherichia coli*. *Mol. Microbiol.* **48**: 1253–1265.
- Charollais, J., Dreyfus, M., and Iost, I. 2004. CsdA, a cold-shock RNA helicase from *Escherichia coli*, is involved in the biogenesis of 50S ribosomal subunit. *Nucleic Acids Res.* **32**: 2751–2759.
- Conrad, J., Sun, D., Englund, N., and Ofengand, J. 1998. The *rluC* gene of *Escherichia coli* codes for a pseudouridine synthase that is solely responsible for synthesis of pseudouridine at positions 955, 2504, and 2580 in 23S ribosomal RNA. *J. Biol. Chem.* **273**: 18562–18566.
- Conrad, J., Niu, L., Rudd, K., Lane, B.G., and Ofengand, J. 1999. 16S ribosomal RNA pseudouridine synthase RsaA of *Escherichia coli*: Deletion, mutation of the conserved Asp102 residue, and sequence comparison among all other pseudouridine synthases. *RNA* **5**: 751–763.
- Cunningham, P.R., Nurse, K., Bakin, A., Weitzmann, C.J., Pflumm, M., and Ofengand, J. 1992. Interaction between the two conserved single-stranded regions at the decoding site of small subunit ribosomal RNA is essential for ribosome function. *Biochemistry* **31**: 12012–12022.
- Datsenko, K.A. and Wanner, B.L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci.* **97**: 6640–6645.
- Del Campo, M., Kaya, Y., and Ofengand, J. 2001. Identification and site of action of the remaining four putative pseudouridine synthases in *Escherichia coli*. *RNA* **7**: 1603–1615.
- Del Campo, M., Ofengand, J., and Malhotra, A. 2004. Crystal structure of the catalytic domain of RluD, the only rRNA pseudouridine synthase required for normal growth of *Escherichia coli*. *RNA* **10**: 231–239.
- Deutscher, M.P., Marlor, C.W., and Zaniewski, R. 1984. Ribonuclease T: New exoribonuclease possibly involved in end-turnover of tRNA. *Proc. Natl. Acad. Sci.* **81**: 4290–4293.
- El Hage, A. and Alix, J.-H. 2004. Authentic precursors to ribosomal subunits accumulate in *Escherichia coli* in the absence of functional DnaK chaperone. *Mol. Microbiol.* **51**: 189–201.
- Evgenieva-Hackenberg, E. and Klug, G. 2000. RNase III processing of intervening sequences found in helix 9 of 23S rRNA in the α subclass of *Proteobacteria*. *J. Bacteriol.* **182**: 4719–4729.
- Foster, P.G., Lixuan, H., Santi, D.V., and Stroud, R.M. 2000. The structural basis for tRNA recognition and pseudouridine formation by pseudouridine synthase I. *Nat. Struct. Biol.* **7**: 23–27.
- Guttsell, N.S., Englund, N., Niu, L., Kaya, Y., Lane, B.G., and Ofengand, J. 2000. Deletion of the *Escherichia coli* pseudouridine synthase gene *truB* blocks formation of pseudouridine 55 in tRNA in vivo, does not affect exponential growth, but confers a strong selective disadvantage in competition with wild-type cells. *RNA* **6**: 1870–1881.
- Guttsell, N.S., Del Campo, M., Raychaudhuri, S., and Ofengand, J. 2001. A second function for pseudouridine synthases: A point mutant of RluD unable to form pseudouridines 1911, 1915, and 1917 in *Escherichia coli* 23S ribosomal RNA restores normal growth to an RluD-minus strain. *RNA* **7**: 990–998.
- Hager, J., Staker, B.L., Bügl, H., and Jakob, U. 2002. Active site in RrmJ, a heat shock-induced methyltransferase. *J. Biol. Chem.* **277**: 41978–41986.
- Kaya, Y. and Ofengand, J. 2003. A novel unanticipated type of pseudouridine synthase with homologs in bacteria, archaea, and eukarya. *RNA* **9**: 711–721.

- Lamontagne, B. and Elela, S.A. 2004. Evaluation of the RNA determinants for bacterial and yeast RNase III binding and cleavage. *J. Biol. Chem.* **279**: 2231–2241.
- Li, Z., Pandit, S., and Deutscher, M.P. 1999. Maturation of 23S ribosomal RNA requires the exoribonuclease RNase T. *RNA* **5**: 139–146.
- Lindahl, L. 1975. Intermediates and time kinetics of the in vivo assembly of *Escherichia coli* ribosomes. *J. Mol. Biol.* **92**: 15–37.
- Liu, M., Novotny, G.W., and Douthwaite, S. 2004. Methylation of 23S rRNA nucleotide G745 is a secondary function of the RlmA¹ methyltransferase. *RNA* **10**: 1–8.
- Miller, J.H. 1992. *A short course in bacterial genetics: A laboratory manual and handbook for Escherichia coli and related bacteria*, Chapter 28, pp. 357–364. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Nierhaus, K.H. and Dohme, F. 1979. Total reconstitution of 50S subunits from *Escherichia coli* ribosomes. *Methods Enzymol.* **LIX**: 443–449.
- Ofengand, J. and Rudd, K.E. 2000. The ribosome: Structure, function, antibiotics and cellular interactions. In *The ribosome: Structure, function, antibiotics, and cellular interactions* (eds. R.A. Garrett et al.), pp. 175–189. ASM Press, Washington, DC.
- Ofengand, J., Del Campo, M., and Kaya, Y. 2001. Mapping pseudouridines in RNA molecules. *Methods* **25**: 365–373.
- Persson, B.C., Gustafsson, C., Berg, D.E., and Bjork, G.R. 1992. The gene for a tRNA modifying enzyme, m⁵U54-methyltransferase, is essential for viability in *Escherichia coli*. *Proc. Natl. Acad. Sci.* **89**: 3995–3998.
- Raychaudhuri, S., Conrad, J., Hall, B.G., and Ofengand, J., 1998. A pseudouridine synthase required for the formation of two universally conserved pseudouridines in ribosomal RNA is essential for normal growth of *Escherichia coli*. *RNA* **4**: 1407–1417.
- Raychaudhuri, S., Niu, L., Conrad, J., Lane, B.G., and Ofengand, J. 1999. Functional effect of deletion and mutation of the *Escherichia coli* ribosomal RNA and tRNA pseudouridine synthase RluA. *J. Biol. Chem.* **274**: 18880–18886.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: A laboratory manual*, 2d ed., pp. 1.82–1.83. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sirdeshmukh, R. and Schlessinger, D. 1985. Ordered processing of *Escherichia coli* 23S rRNA in vitro. *Nucleic Acids Res.* **13**: 5041–5054.
- Srivastava, A.K. and Schlessinger, D. 1988. Coregulation of processing and translation: Mature 5' termini of *Escherichia coli* 23S ribosomal RNA form in polysomes. *Proc. Natl. Acad. Sci.* **85**: 7144–7148.
- . 1990. Mechanism and regulation of bacterial ribosomal RNA processing. *Annu. Rev. Microbiol.* **44**: 105–129.
- Weitzmann, C.J., Cunningham, P.R., and Ofengand, J. 1990. Cloning in vitro transcription, and biological activity of *Escherichia coli* 23S ribosomal RNA. *Nuc. Acids Res.* **18**: 3515–3520.
- Wright, M. 1971. Mutants of *Escherichia coli* lacking endonuclease I, ribonuclease I, or ribonuclease II. *J. Bacteriol.* **107**: 87–94.
- Yonath, A. and Bashan, A. 2004. Ribosomal crystallography: Initiation, peptide bond formation, and amino acid polymerization are hampered by antibiotics. *Annu. Rev. Microbiol.* **58**: 233–251.
- Yusupov, M.M., Yusupova, G.Z., Baucom, A., Lieberman, K., Earnest, T.N., Cate, J.H.D., and Noller, H.F. 2001. Crystal structure of the ribosome at 5.5 Å resolution. *Science* **292**: 883–896.

RNA

A PUBLICATION OF THE RNA SOCIETY

The pseudouridine synthase RluD is required for normal ribosome assembly and function in *Escherichia coli*

NANCY S. GUTGSELL, MURRAY P. DEUTSCHER and JAMES OFENGAND

RNA 2005 11: 1141-1152

References

This article cites 36 articles, 23 of which can be accessed free at:
<http://rnajournal.cshlp.org/content/11/7/1141.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

To subscribe to *RNA* go to:
<http://rnajournal.cshlp.org/subscriptions>