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

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**ABSTRACT**

This laboratory previously showed that truncation of the gene for RluD, the *Escherichia coli* pseudouridine synthase responsible for synthesis of 23S rRNA pseudouridines 1911, 1915, and 1917, blocks pseudouridine formation and inhibits growth. We now show that RluD mutants at the essential aspartate 139 allow these two functions of RluD to be separated. In vitro, RluD with aspartate 139 replaced by threonine or asparagine is completely inactive. In vivo, the growth defect could be completely restored by transformation of an RluD-inactive strain with plasmids carrying genes for RluD with aspartate 139 replaced by threonine or asparagine. Pseudouridine sequencing of the 23S rRNA from these transformed strains demonstrated the lack of these pseudouridines. Pseudoreversion, which has previously been shown to restore growth without pseudouridine formation by mutation at a distant position on the chromosome, was not responsible because transformation with empty vector under identical conditions did not alter the growth rate.

Keywords: essential aspartate; growth defect; ribosome biosynthesis

**INTRODUCTION**

The pseudouridine (Ψ) synthase genes of *Escherichia coli* consist of 10 homologous open reading frames that have been classified into four families (Koonin, 1996). *rsuA*, *rluE*, *rluB* (formerly *ycL*), and *yjbC* belong to the *rsuA* family; *rluA*, *rluC*, *rluD*, and *truC* (formerly *yqcB*) belong to the *rluA* family; *truB* and *truA* are the sole members of their respective families. The *rsuA*, *rluA*, and *truB* families are related whereas the *truA* family is clearly distinct from all the others (Conrad et al., 1999; Ofengand & Rudd, 2000). Ψ synthase activity and the site(s) of their action have been determined for all except *yjbC* (Ofengand & Rudd, 2000; M. Del Campo & J. Ofengand, unpubl. results; Y. Kaya & J. Ofengand, unpubl. results).

Most and possibly all known and putative Ψ synthases from all organisms contain an aspartate residue in a conserved sequence motif. This aspartate has been shown, by mutation to structurally similar as well as dissimilar amino acids, to be essential for TruA synthase activity and its γ-carboxyl group has been proposed to play an essential role in the catalytic mechanism (Huang et al., 1998; Gu et al., 1999). To see if this result is also true for other Ψ synthases, a systematic investigation of all the synthases of *E. coli* has been initiated in our laboratory. So far, the same results have been reported in vivo for RsuA (Conrad et al., 1999), RluA (Raychaudhuri et al., 1999), and TruB (GutgSELL et al., 2000) and in vitro for RluA and TruB (RamaMurthy et al., 1999).

RluD is the synthase that makes Ψ1911, Ψ1915, and Ψ1917 in *E. coli* 23S rRNA (Raychaudhuri et al., 1998). Disruption of this gene not only blocked synthesis of the above-mentioned Ψ but also caused a severe growth defect, reducing the exponential growth rate to half. The growth defect could be completely relieved by trans-
formation of the RluD-minus strain with a plasmid carrying the rluD gene. However, this work left open the question of whether the growth defect was due to the absence of Ψ1911, Ψ1915, and Ψ1917 or to the absence of the synthase itself. Conceivably, the protein could have a second function needed for optimal growth. If mutation of the conserved aspartate in RluD blocked Ψ formation as it does in the above-mentioned Ψ synthases, it would be possible to use this mutant protein to attempt rescue of the growth defect in RluD-minus cells. Such an experiment would establish clearly whether or not the growth defect is related to Ψ formation or only to the presence of the RluD protein.

In this work, we show first that mutation of the conserved aspartate, D139, to threonine or asparagine blocks Ψ formation completely both in vivo and in vitro. Second, the mutant rluD gene produces a protein capable of complete reversal of the growth defect without concomitant Ψ formation.

RESULTS

D139 is necessary for in vitro Ψ formation by RluD

Santi and colleagues have shown that Asp60, in the sequence context GRTD in the tRNA Ψ synthase TruA, is an essential residue for TruA function in vitro, but not for TruA binding to its substrate (Huang et al., 1998). They further suggested that in view of the alignment of the analogous sequence motifs HRLD, GRLD, and GXLD in the Ψ synthase families RluA, RsuA, and TruB, respectively, that the aspartate residue in this sequence was essential in all Ψ synthases, and suggested a role for the γ-carboxyl of this residue in the enzyme catalytic mechanism. Although the sequence motif is not as highly conserved as first supposed, as other members of the truA family differ, and there are two known cases in which a glycine appears to replace aspartate in this sequence motif (Ofengand & Rudd, 2000), the essential nature of this aspartate has in fact been confirmed in vivo for a number of E. coli Ψ synthases. These are RsuA (Conrad et al., 1999), RluA (Raychaudhury et al., 1999), RluB and RluE (M. Del Campo & J. Ofengand, unpubl. results), RluC (S. Jean-Charles & J. Ofengand, unpubl. results), TruB (Gutgsell et al., 2000), and TruC (Y. Kaya & J. Ofengand, unpubl. results). The results for RluA and Trub were confirmed in vitro by Ramamurthy et al. (1999). Thus, for eight of the ten known E. coli Ψ synthases, this aspartate residue is essential. It is also essential in Pus5p, the synthase responsible for Ψ2819 in Saccharomyces cerevisiae 21S mitochondrial large subunit rRNA (Ansmant et al., 2000).

To determine if D139 in the HRLD sequence of the ninth Ψ synthase, RluD, was also essential, the following in vitro experiment was performed. Wild-type and both D139T and D139N mutants of RluD were over-expressed from plasmids containing the appropriate inserts, affinity purified on a Ni-containing resin by virtue of an N-terminal His tag sequence introduced from the pET28a vector, and used in an in vitro assay for Ψ formation (Fig. 1). It is clear from this experiment that whereas the wild-type RluD was capable of release of about 2.5 mol of $^3$H per mol of added 23S rRNA in 20 min, neither mutant was able to release any $^3$H at all, even when added in a 2.5-fold excess over wild type. Based on the in vivo specificity of RluD, a release of 3 mol of $^3$H per mol of RNA is expected for 100% reaction. Clearly, D139 is essential for Ψ synthase activity in vitro, in line with the in vivo results obtained for the other eight Ψ synthases.

Ψ formation activity in vivo of D139 mutants of RluD

In the previous studies on mutants of the Ψ synthases RsuA, RluA, RluC, and TruB, E. coli strains were used in which most of the chromosomal gene for the synthase had been replaced by a sequence coding for kanamycin resistance. Thus the only source of wild-type or mutant synthase genes was the plasmid used...
to transform the strain. This is not the case for RluD. We were unable to obtain an RluD deletion strain by the methods used successfully for the above-mentioned synthases, and could only obtain the RluD-minus strain described previously (Raychaudhuri et al., 1998) as a result of the serendipitous discovery (by B. Hall) of a miniTn10(Cam) insertion into the rluD gene. This occurred such that translation would yield an RluD protein lacking the C-terminal 130 amino acids out of a total of 326, and with a C-terminal extension of 12 foreign amino acids from the Tn10 insert. This mutant strain is unable to form $\Psi'$, and the overexpressed and affinity-purified truncated RluD is unable to function in vitro (S. Raychaudhuri & J. Ofengand, unpubl. results). Nevertheless, in view of the fact that $\Psi'$ synthases may function as dimers (Foster et al., 2000), there is the possibility that a full-length mutant RluD introduced by plasmid transformation could form a functionally active heterodimer with the truncated RluD present in the RluD-minus cells, which still has the essential D139 residue. Thus, in this in vivo context, although the D139T and D139N mutants may fail to form $\Psi'$, as they did in vitro, it is also possible that $\Psi'$ formation will be retained.

Recent studies on the RluD-minus strain described by Raychaudhuri et al. (1998) have revealed the presence of a second strain that was not detected previously. The second strain, termed Dust because of its very small colony size on plates, was only observed after prolonged incubation. Its growth rate is only one-third that of the previously described strain, and Dust variants were found in the Tinys will grow out three times more quickly than Tiny, which explains why it was not detected previously. The Dust strain was found in the original RluD-minus MG1655 strain described by Raychaudhuri et al. (1998) and also when three independent P1-mediated back-transductions of MH040 (the original Hall strain) to MG1655 were performed. In all four cases, both Tiny and Dust variants were found. From two of these back-transductants, four Tiny and four Dust isolates were selected for further study. All of the Dust isolates appeared identical as judged by colony morphology and also by ribosome profiles on sucrose gradients (L. Peil & J. Ofengand, unpubl. results) so only one, Dust-3, was used subsequently. Tiny-3, from the same plate, was also the only isolate used subsequently, except as noted. Neither Dust-3 nor Tiny-3 is capable of $\Psi'$1911, $\Psi'$1915, or $\Psi'$1917 formation, yet transformation with a plasmid carrying only the structural gene for RluD converts both strains to $\Psi'$-forming ability as well as to a wild-type growth rate (see Table 2 and Fig. 3). Thus, the RluD protein is capable of restoring both $\Psi'$ formation and a normal growth rate in both strain backgrounds. Both strains are still chloramphenicol-resistant and DNA sequencing established that the Tn10 insert is at the same site in both strains. Backcross of purified Tiny and Dust to MG1655 by transduction identified second-site mutations that converted Dust to Tiny. Dust-3 was backcrossed two times, yielding 0 Tinsys out of 33 and 41 Dust colonies, for a total of 74. Because the Tinsys will grow out three times more quickly, the failure to detect any is highly significant. On the other hand, from backcross of the four Tiny isolates, Dust-like transductants were found at different frequencies (Table 1). All 16 of the Dust-like colonies from experiment 1 were subcultured, and remained Dust as judged by colony morphology. The growth rates of 4 of the 16 colonies were measured, and yielded generation times of 136, 138, 145, and 148 min, consistent with the average value for Dust in Table 2. Because Dust was obtained from Tiny at frequencies ranging from 5–18%, but Tiny cannot be obtained from Dust (<1.4%), it seems

<table>
<thead>
<tr>
<th>Transduction donor</th>
<th>Colony type</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Average % Dust</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiny-1</td>
<td>Tiny</td>
<td>42</td>
<td>62</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dust</td>
<td>9</td>
<td>14</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% Dust</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Tiny-2</td>
<td>Tiny</td>
<td>39</td>
<td>39</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dust</td>
<td>2</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% Dust</td>
<td>5</td>
<td>13</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Tiny-3</td>
<td>Tiny</td>
<td>39</td>
<td>45</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dust</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% Dust</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Tiny-4</td>
<td>Tiny</td>
<td>39</td>
<td>60</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dust</td>
<td>4</td>
<td>8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% Dust</td>
<td>9</td>
<td>12</td>
<td>12</td>
<td>11</td>
</tr>
</tbody>
</table>

Backcross of Tiny isolates 1 through 4 to MG1655 was done by P1 mediated transduction as previously described (Raychaudhuri et al., 1998). Tiny colonies were visible after overnight incubation. Dust colonies were obviously smaller and only became readily detectable after a second overnight incubation of the plate. Plates were again examined after a third overnight incubation to be sure all Dust colonies had been detected.
clear that a second-site mutation of Dust is responsible for the partial growth reversion found in Tiny-1 to Tiny-4. Moreover, it appears from the results in Table 1 that at least three different sites of mutation were obtained, as three distinct frequencies were consistently observed. However, Dust need not be the original truncated RluD phenotype, as it too may arise from a pseudoreversion event that has converted a lethal or near-lethal mutation into one that is barely tolerable.

To see if the site of mutation was in the RluD promoter or structural gene, the DNA sequence of the truncated rlud in both Tiny-3 and Dust-3 isolates was determined. The published wild-type sequence was found in both strains from −203 to +640 (the A of the initiator AUG is +1 and the full-length gene is 624 nt long). Irrespective of the nature of the mutation, as is shown below, the Tiny-3 strain, but not the Dust-3 strain, proved to be a suitable host for discriminating between the role of the synthase and that of ψ1911, ψ1915, and ψ1917 in the growth inhibition caused by RluD truncation.

Wild-type and the D139N and D139T mutants of the rlud gene were transferred from pET28a to pTrc99A, and used to transform both the Dust and Tiny strains. Upon induction with IPTG, overexpression of RluD was readily observed from all three plasmids, and to approximately the same extent (Fig. 2). Therefore, the change from D139 to N or T does not appear to result in a large loss of protein, for example, by proteolysis of a partially denatured protein structure. Closer inspection of the gels shows that a small amount of RluD is present even in the uninduced lanes, compared to the lanes with no insert. This is to be expected from the leaky nature of the pTrc99A control system. Dust and Tiny host cells gave virtually the same results.

Another aliquot of the same cell pellets used above was taken for total RNA isolation and ψ sequencing (Fig. 3). When Tiny cells were the host (Fig. 3A), the results were as predicted from the in vitro results of Figure 1. Thus, in the absence of any plasmid-borne rlud gene, no strong band corresponding to ψ1911 and ψ1917 was found. [Note that ψ1915 formation cannot be determined this way because N3-methylation, which occurs in vivo on both U1915 and ψ1915 (Raychaudhuri et al., 1998), is a CMC-independent stop to reverse transcription. By the use of unmodified RNA transcripts as substrate, it was shown previously that RluD makes ψ1915 (Bakin et al., 1994).] By contrast, CMC-dependent bands at 1911 and 1917 are readily found in both the MG1655 wild-type control and when the RluD-minus strain is supplemented with the plasmid-borne wild-type rlud gene. On the other hand, when the plasmid contained the mutant genes, ψ formation was absent. The two lower panels of Figure 3A show that ψ2457 and ψ2504 were formed in all cases, demonstrating that the failure of D139T and D139N to make

![TABLE 2. Growth rate of Tiny and Dust rlud-minus E. coli MG1655 transformed with wild-type and mutant rescue plasmids](image)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling time (min)</th>
</tr>
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<tbody>
<tr>
<td>Wild-type(pTrc99A)</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>rlud-minus(pTrc99A)</td>
<td>51 ± 2</td>
</tr>
<tr>
<td>rlud-minus(pTrc99A)/D139D</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>rlud-minus(pTrc99A)/D139N</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>rlud-minus(pTrc99A)/D139T</td>
<td>32 ± 1</td>
</tr>
</tbody>
</table>

Cells were grown with aeration by shaking in LB medium plus 100 μg/mL carbenicillin at 37°C and assayed by absorption at 600 nm over three to four doublings. Doubling time was determined from a semilogarithmic plot of A600 versus time. Each plot consisted of 8–11 time points. Doubling times are the average of two determinations.

![FIGURE 2. Overexpression of wild-type and mutant rlud gene products in MG1655 rlud-minus E. coli cells. A: rlud-minus Tiny cells; B: rlud-minus Dust cells. Cells were transformed with pTrc99A carrying the indicated insert as described in Materials and Methods. O: no insert; D139N, D139T, and D139D: mutant and wild-type inserts, respectively. Cells were grown to an A600 of 0.6, induced with 1 mM IPTG for 1 h, and protein isolated and electrophoresed as described (Raychaudhuri et al., 1998). I: induced; U: uninduced; M: marker proteins with sizes in kilodaltons as indicated.](image)
1911 and 1917 was not due to some general fault in the assay, such as a failure to react with CMC correctly. This experiment was performed using the IPTG-induced cells that, according to Figure 2, clearly possess large quantities of both mutant RluD proteins. The same results were obtained from two independent cell growths and subsequent sequencing analyses.

In contrast to these findings, mutant plasmids were able to support Ψ formation in the Dust host strain even though the strain transformed with an empty pTrc99A vector did not make Ψ (Fig. 3B). Uninduced cells were used in this experiment, which, according to Figure 2, make only a marginally detectable amount of RluD. Clearly, enough was made to allow Ψ formation. The same sequencing result was obtained when induced cells were used, and when the experiment was repeated, starting from an independently grown cell culture. These surprising results will be discussed below.

### Growth properties of RluD-minus cells supplemented with mutant RluD

The original question posed in the Introduction was whether mutant forms of RluD unable to make Ψ would nevertheless rescue the growth defect of RluD-minus cells. The results shown in Table 2 demonstrate that they do. The growth defect of Tiny cells is completely overcome by transformation with either wild-type or mutant genes when compared to cells carrying only the empty vector. As D139N or D139T mutants were unable to make Ψ (Fig. 3A), this result clearly shows that rescue of the growth defect, while dependent on RluD,
is not dependent on the ability of RluD to make $\Psi$. Growth rescue by the mutants was not due to the occurrence of pseudorevertants that also generate wild-type growth rates without $\Psi$ formation (Raychaudhuri et al., 1998). This was shown by the control transformation done under exactly the same conditions but with empty vector. Growth rate rescue was also obtained in the Dust host cells, but because the RluD mutants in this host make $\Psi$, growth rescue is to be expected.

DISCUSSION

In this work, we have shown that it is possible to separate the growth-restoring activity of RluD from its $\Psi$ forming activity. By the use of RluD mutant at an amino acid known to be essential in other E. coli synthases, we were able to block $\Psi$ forming activity without affecting its growth rescue ability. This result parallels a similar finding for TruB, the $\Psi$ synthase responsible for $\Psi$55 in tRNA, although in this case, the growth defect was only detectable by competition experiments (Gutgsell et al., 2000). This result raises the possibility that the growth-retarding properties of RluA-minus and RluC-minus strains previously described (Raychaudhuri et al., 1999) may also be separable from their $\Psi$-forming ability. The appropriate mutants of RluA and RluC are available, and efforts are currently underway to extend the above results to these two additional synthases.

What might this $\Psi$-independent activity of $\Psi$ synthases be? We have previously suggested as one of a number of possibilities that the guide RNAs used in eukaryotes to identify the U residues destined for conversion to $\Psi$ might be RNA chaperones for the correct folding of ribosomal RNA (Ofengand & Fournier, 1998). In this view, $\Psi$ formation would be merely a signal that folding had occurred and it was time for the chaperone to dissociate, rather than having an intrinsic function. If this view were extended to prokaryotes, substituting the synthases for guide RNAs, the $\Psi$ synthases might then be protein chaperones of RNA folding, helping in some as yet undefined way to achieve the correct structure. However, it does not seem so likely that $\Psi$ formation is a necessary completion signal, as in its absence, when mutant RluD was used, complete growth rate restoration was observed (Table 2). More likely, RluD, and possibly the other synthases as well, have two distinct functions, one of which is related to the observed growth defects, and the other to $\Psi$ formation, the latter occurring for still unknown reasons.

There is some precedent for these ideas. First, the methyltransferase that makes m$^5$U54 in tRNA is indispensable in E. coli, yet its methyltransfer activity is not required (Persson et al., 1992). Second, Dim1p, the yeast enzyme which makes m$^5$A$^3$A at the 3’ end of the small subunit rRNA and is essential for the yeast cell (Lafontaine et al., 1994), can dispense with its methylase activity (Lafontaine et al., 1998). Third, two LSU rRNA 2’-O-methyltransferases are known whose absence perturbs ribosome assembly and results in severe or lethal growth defects. One is PET56, a yeast 2’-O-methyltransferase specific for G2251 (E. coli numbering) in yeast mitochondria (Sirum-Connolly & Mason, 1993). The other is FtsJ1, which makes Um2552 in E. coli (Bügl et al., 2000; Caldas et al., 2000a, 2000b). In the latter example, it is not known whether the 2’-O-methylation is required for proper ribosome assembly, but in the former case, recent work has shown that rescue of ribosome assembly does not require methylation (T. Mason, pers. comm.). Thus, like RluD and TruB, the protein is needed but not the product of the reaction it catalyzes. Inhibition of 50S subunit assembly in both Dust and Tiny RluD-minus strains has also recently been observed (L. Peil, N. Gutgsell, J. Ofengand, & J. Remme, unpubl. results). Considering all these results, a pattern begins to emerge in which rRNA modifying enzymes, at least the most common ones, $\Psi$ synthases and 2’-O-methyltransferases, may have an assembly function in their own right, independent of their catalytic role in modified base formation. In this regard, it is interesting to note the amino acid sequence homology between the N-terminus of RluD (residues 19–49) of this 326 amino acid protein with residues 10–40 of Hsp15, a protein highly induced by heat shock (Korber et al., 1999). Hsp15 is a 133 amino acid protein that consists almost entirely of a new type of RNA-binding fold (Staker et al., 2000) and which binds to 50S subunits carrying nascent protein chains with nanomolar affinity (Korber et al., 2000).

In this context, the occurrence of pseudorevertants should be noted. Such second-site mutants are found readily in the Dust strain, and pains must be taken to avoid them during subculturing. They occur much more rarely in the Tiny strain, possibly because the growth rate is so much better than Dust. The site of mutation in pseudorevertants from Dust has been analyzed by backcross transductions to wild type. Eight independently isolated pseudorevertants yielded 224 colonies, all of which were Dust (N. Gutgsell & J. Ofengand, unpubl. results). Because none (<0.4%) of the descendants were the original pseudorevertants as judged by colony size, the site of mutation should be distant from the RluD gene. Thus, on the one hand, mutation at a distant site can restore growth without $\Psi$, and on the other hand, mutation within RluD (D139N/T) can also restore growth without concomitant $\Psi$ formation. Because both Dust and Tiny show ribosome assembly defects (unpublished results cited above), we suggest that both RluD and the unknown site of mutation in the pseudorevertants are proteins that assist in correct rRNA folding during ribosome assembly. In this view, mutation of protein X creates a super protein able to take over the function of RluD that the truncated form can no longer perform.
How are the properties of the Dust and Tiny strains explained? The simplest interpretation is that Dust is either the original phenotype of the Tn10 insertion in \( \text{rluD} \), or is a pseudorevertant with poor growth ability and an inability to maintain RluD \( \Psi \) synthase activity. The Tins would be (further) pseudorevertants of Dust that grow three times faster, although still poorly, and still unable to make \( \Psi_{1911}, \Psi_{1915}, \) and \( \Psi_{1917} \). The sites of these mutations should be near the site of chloramphenicol resistance, as they can be separated in only 5–18% of the chloramphenicol-resistant transductants. They are not in the promoter region for RluD, as DNA sequencing to residue −203 did not reveal any deviation from wild type in either the Dust-3 or Tiny-3 strain. Nevertheless, some accessory factor could be mutated. Then, for example, if production of the truncated RluD in the Tiny strains is decreased by the mutation, and if heterodimer formation between full-length mutant protein and truncated wild type is in fact responsible for \( \Psi \) formation in the Dust strain, a decrease in the available truncated RluD in Tiny might block such heterodimer formation and hence block \( \Psi \) formation. A decrease in truncated RluD could increase the growth rate if the shortened protein inhibited the normal reaction, whatever it may be, that is performed by full-length RluD. Other scenarios are of course possible. We are currently attempting to determine the exact site of the mutations that convert Dust to Tiny in order to clarify these issues.

Despite the unclear status of the Dust strain and its interaction with plasmid-introduced full-length D139T or D139N RluD, the clear result from this work is that \( \Psi \) formation can be separated from the growth stimulating properties of this \( \Psi \) synthase. This result raises two new questions. First, what is the mechanism of the growth dependence on RluD? RluD may play a role in the mechanism of assembly of the large ribosomal subunit, possibly in a chaperone/helicase role, but this has yet to be established. Second, what are \( \Psi_{1911}, \Psi_{1915}, \Psi_{1917} \) good for? The location of these three \( \Psi \) in a loop that projects out from the interface surface of the 50S subunit (Cate et al., 1999; Merryman et al., 1999; Ban et al., 2000) and that is known to be involved in subunit–subunit interaction (Merryman et al., 1999) and to contact 16S rRNA at or near the decoding site (Mitchell et al., 1992; O’Connor & Dahlberg, 1995; Joseph et al., 1997; Wilson & Noller, 1998) suggests a direct role in subunit joining and/or in decoding. As yet, there is no evidence for or against such a role.

**MATERIALS AND METHODS**

**Wild-type and mutant rescue plasmids**

The construction of wild-type rescue plasmids pTrc99A(rluD) and pET28a(rluD) were described previously (Raychaudhuri et al., 1998). D139N and D139T mutants of \( \text{rluD} \) in pET28a and pTrc99A were created by megaprimer PCR mutagenesis as described previously for \( \text{rluA} \) (Raychaudhuri et al., 1999). The mutagenic primers were 5’-CATCGTCTGACTAAAAAGACGCCACTGCGGG-3’ for the D139T mutant or for the D139N mutant, 5’-CATCGTCTGAAATAGAAGACCC-3’ (mutation sites shown in bold), with the downstream primer 5’-GGGAAGCTTGGGTAGCAGCCACTGCGGG-3’ containing a HindIII site (underlined). The upstream primer was 5’-GGGTCGAGGCTCCGGCCACTGCGGGG-3’ with an NcoI site (underlined). Transformation of Novablue cells by the ligated plasmids yielded positive clones whose identity was confirmed by DNA sequencing. Construction of the same mutants in pET28a was done using the above constructs in pTrc99A as template. The N-terminal primer 5’-GGGGCTAGATGCACAGCAGCAAGACGCT-3’ (Nhel site underlined) and C-terminal primer 5’-GGGTCGAGGCTCCGGCCACTGCGGGG-3’ (Xhol site underlined) were used for amplification. The products were purified, digested with Nhel and Xhol, and ligated with a similarly digested pET28a vector for 16 h at 16 °C. Transformation of Novablue cells and DNA sequencing of the resultant positive clones confirmed the identity of the constructs. The \( \text{rluD} \) constructs in pTrc99A described above contained two ATG sequences separated by 1 nt, only the second of which leads to the correct sequence of RluD. The first ATG results in termination after 48 amino acids have been synthesized and is probably degraded. Despite this, the wild-type sequence in pTrc99A was able to produce enough RluD to rescue both growth and \( \Psi \) synthase in an RluD-minus strain (Raychaudhuri et al., 1998). However, for the present work, the wild-type and mutant \( \text{rluD} \) in pTrc99A were reconstructed to eliminate the extra start codon. PCR amplification used the wild-type or mutant pET28a(rluD) rescue plasmids as template with the upstream primer 5’-GGCATGATGCACAGCAGCAAGAC-3’ containing a SnaI site (underlined) to generate an overhang compatible with that produced by NcoI digestion of the pTrc99A vector. The downstream primer 5’-GGGAAGCTTGAGCAGCCACTGCGGG-3’ contained a HindIII site (underlined). The resulting 1,012-bp PCR products were digested with SnaI and HindIII, ligated with pTrc99A previously digested with NcoI and HindIII for 16 h at 16 °C, and then used to transform competent DH5α cells (Gibco BRL Life Technologies) by electroporation (Eppendorf Electroporator 2510). The absence of any wild-type \( \text{rluD} \) DNA from the putative mutant plasmids was checked by PIIMI digestion of plasmid DNA from the transformed cells. A single PIIMI restriction site is present in the wild-type \( \text{rluD} \) gene at residue 412 (CCATCGT-1CTGG). A single PIIMI site is also present in the pTrc99A vector at base 2981. Therefore, digestion of the wild-type pTrc99A(rluD) results in the appearance of two bands of 3,316 and 1,872 bp on agarose gels. Mutation of the \( \text{rluD} \) gene eliminates the PIIMI site from both mutants by replacing the 3’-terminal G by an A, yielding a single band of 5,188 bp upon PIIMI digestion. Digestion of empty pTrc99A results in a single 4,176 bp band.

**Isolation and transformation of Tiny and Dust variants of RluD-minus *E. coli***

Backcross by P1-mediated transduction of MH040 to MG1655 and plating on M9 plus medium for 19 h at 37 °C yielded chloramphenicol-resistant colonies with poor growth characteristics compared to wild type, like those previously de-
scribed (Raychaudhuri et al., 1998). These are called Tiny. Further incubation for 15 h yielded in addition even smaller colonies, called Dust. Colonies of each type were purified by sequential streaking on fresh plates until only one colony type was observed. Both variants were stored in LB-20% glycerol at −80°C. Tiny was grown overnight on LB plus 34 μg/mL chloramphenicol, diluted and regrown to an A600 of 0.6–0.7 in 50 mL, and pelleted by centrifugation. After washing with 0.1 M CaCl2, cells were suspended in 0.8 mL of 0.1 M CaCl2, and transformed with either empty pTrc99A or with wild-type or mutant rluD-containing rescue plasmids by heat shock (Sambrook et al., 1989). Overnight incubation of the transformation plates (LB plus 34 μg/mL chloramphenicol plus 0.1 mg/mL carbenicillin) yielded only very tiny colonies when the pTrc99A vector was used, indicating the absence of any pseudorevertants to wild-type growth under these conditions. Medium sized colonies were obtained when the plasmid carried either wild-type or mutant rluD constructs. Restreaking on LB plus chloramphenicol and carbenicillin plates gave tiny colonies from the empty vector and normal-sized colonies from the wild-type or mutant rescue plasmids. Analysis of plasmid DNA from the rescued strains by PflM1 digestion as described above proved the absence of any wild-type sequence in the mutant plasmid-containing strains. Transformation of Dust was done as described above for Tiny but with modifications because of the greater propensity of Dust to accumulate pseudorevertants. The glycerol stock culture was checked for pseudorevertants by plating at the same time the liquid culture was inoculated, and the culture was harvested by centrifugation at an A600 of 0.04–0.06. Preliminary experiments have shown that no pseudorevertants are ever found at a cell density <0.08. The cell pellet from 150 mL of culture was washed, resuspended in 0.6 mL of 0.1 M CaCl2, and transformed and isolated as described above for Tiny. Dust transformed with wild-type or mutant plasmids grew as wild-type colonies, whereas Dust transformed with the empty vector grew as dust-like, that is, as very small colonies. The absence of wild-type plasmid DNA from the mutant transformed cells was assayed by PflM1 digestion as described above.

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