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A pseudouridine synthase required for the formation of two universally conserved pseudouridines in ribosomal RNA is essential for normal growth of *Escherichia coli*

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ABSTRACT

***Escherichia coli* rRNA contains 10 pseudouridines of unknown function. They are made by synthases, each of which is specific for one or more pseudouridines. Here we show that the *sfhB* (*yfil*) ORF of *E. coli* is a pseudouridine synthase gene by cloning, protein overexpression, and reaction in vitro with rRNA transcripts. Gene disruption by miniTn10(cam) insertion revealed that this synthase gene, here renamed *rluD*, codes for a synthase which is solely responsible in vivo for synthesis of the three pseudouridines clustered in a stem-loop at positions 1911, 1915, and 1917 of 23S RNA. The absence of RluD results in severe growth inhibition. Both the absence of pseudouridine and the growth defect could be reversed by insertion of a plasmid carrying the *rluD* gene into the mutant cell, clearly linking both effects to the absence of RluD. This is the first report of a major physiological defect due to the deletion of any pseudouridine synthase. Growth inhibition may be due to the lack of one or more of the 23S RNA pseudouridines made by this synthase since pseudouridines 1915 and 1917 are universally conserved and are located in proximity to the decoding center of the ribosome where they could be involved in modulating codon recognition.**

Keywords: Ψ1911; Ψ1915; Ψ1917; 23S RNA; gene disruption; growth inhibition; *rluD*; *sfhB(yfil)*

INTRODUCTION

Although the structure of pseudouridine (Ψ) was determined almost 40 years ago (Cohn, 1960), its role in RNA remains an enigma. Despite the fact that Ψ is found in all classes of RNA that must maintain a tertiary structure for proper function, namely tRNA (Sprinzl et al., 1998), rRNA (Maden, 1990), and sn(o)RNA (Gu et al., 1998; Massenet et al., 1998), the function of Ψ in these molecules has remained elusive. The presence of Ψ in rRNA, especially in the large subunit (LSU) RNA, is noteworthy. There, the Ψ residues cluster in or near the peptidyl transferase center of all organisms studied, despite large variations in the total number of Ψ found in the RNA (Ofengand & Bakin, 1997).

Ψ is formed at the polynucleotide level by isomerization of selected uridines in an enzyme-catalyzed reaction requiring neither added cofactors nor an energy source (reviewed in Ofengand & Fournier, 1998). Locating and disrupting the genes coding for Ψ synthases should, therefore, result in the absence of specific Ψ residues, assuming that individual or subsets of Ψ are formed by distinct synthases. This is true for the cloned synthases for tRNA (Kammen et al., 1988; Nurse et al., 1995; Becker et al., 1997; Grosjean et al., 1997; Lecointe et al., 1998), and appears to be the case for rRNA as well (Wrzesinski et al., 1995a,b).

In *Escherichia coli*, there are 9 Ψ in the LSU RNA (Bakin & Ofengand, 1993; Bakin et al., 1994b), and one in the small subunit (SSU) RNA (Bakin et al., 1994a). To understand the function of Ψ in rRNA, we have embarked on a program to block the formation of specific Ψ residues by interfering with production of the enzymes required for their biosynthesis. We previously identified a synthase, RsuA, specific for the single Ψ in SSU RNA (Wrzesinski et al., 1995a) and another, RluA, specific for Ψ746 in LSU RNA (Wrzesinski et al., 1995b). We recently identified an additional synthase, RluC,

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which forms Ψ955, Ψ2504, and Ψ2580 (Conrad et al., 1998; see Table 1). Here, we describe another synthase, RluD, the product of the *sfhB* (previously known as *yfiI*) gene, renamed *rluD*, which is solely responsible in vivo for synthesis of Ψ1911, Ψ1915, and Ψ1917. Two of these, Ψ1915 and Ψ1917, are found in the equivalent location in the LSU RNA of all organisms examined, which include representatives from the Prokarya, Eukarya, Archaea, mitochondria, and chloroplasts (Ofengand & Bakin, 1997). Disruption of the *rluD* gene and consequent lack of these three Ψ causes a severe growth inhibition under laboratory growth conditions. A preliminary account of part of this work has appeared (Ofengand et al., 1997).

RESULTS

Identification of RluD as a pseudouridine synthase

During the purification of the RluA synthase from *E. coli*, a second peak of pseudouridine synthase activity was found. This activity was purified and shown to be mainly specific for formation of the naturally occurring Ψ1911, Ψ1915, and Ψ1917 on in vitro transcripts of 23S ribosomal RNA (J. Wrzesinski, A. Bakin, & J. Ofengand, unpubl. results). Although 30 residues of the N-terminal amino acid sequence were determined for this synthase, AQRVQLTATVSENQLGQRLDQALAEM FPDY, no such sequence could be located in the databank at the time, when only ~50% of the genome was known. There was, however, a 57% identity to the first 30 residues coded by an ORF in *Hemophilus influenzae*, HI0176 (P44445), which in turn was homologous to the *E. coli* protein YfiI (P33643) whose N-terminus had not yet been determined. (We thank Guy Plunkett III, University of Wisconsin, who pointed this relationship out to us.) Subsequently, when the remainder of the yfiI gene sequence was determined, it coded for a perfect match to the N-terminal sequence given above. It turned out that there was only an eight-amino-acid gap between the 30 residues we had sequenced and the 5′ end of the YfiI protein that was in the databank at the time. yfiI was shown to be a suppressor of a ts mutant in *ftsH* and was given the designation of *sfhB* (cited in Myler et al., 1994). In light of the above findings and those detailed below, we have renamed the gene *rluD*, ribosomal large subunit pseudouridine synthase D.

The *rluD* gene was cloned into pET28a following procedures we had established previously using pET15b (Nurse et al., 1995; Wrzesinski et al., 1995a,b), and the protein was overexpressed and purified on a Ni^{2+} column by affinity chromatography. Using a 5-[^{3}H]uridine-labelled in vitro transcript of 23S RNA as substrate (Weitzmann et al., 1990), the protein was shown to possess synthase activity, confirming that RluD was indeed a pseudouridine synthase for 23S RNA. As shown in Figure 1, there was an approximately linear increase in ^{3}H release with time reaching a molar yield of 1.1 mol ^{3}H released per mole of RNA added at the time the reaction was terminated (30 min). Ψ sequence analysis of the in vitro product taken at 30 min (Fig. 2) showed formation of Ψ1915 and Ψ1917, but not of Ψ1911. Both Ψ appeared to be formed at approximately equivalent rates since both band intensities were about the same despite the fact that, according to the extent of ^{3}H release, the overall reaction had only gone halfway. Based on the earlier (1995) experiments mentioned above with native synthase, U1911 was also expected to react. However, many factors could influ-

TABLE 1. Known and putative *E. coli* rRNA Ψ synthases.

Name	Gene locus (min)	RNA substrate	Site no.	Specificity class[a]	ORF MW (kD)	SWISS-PROT no.	Reference
RsuA	49.12	16S rRNA	516	I	25.9	P33918	Wrzesinski et al., 1995a
RluA	1.30	23S rRNA	746	IV	24.9	P39219	Wrzesinski et al., 1995b
		tRNA	32				
RluC (YceC)	24.66	23S rRNA	955 2504 2580	III	36.0	P23851	Conrad et al., 1998
RluD (SfhB)	58.93	23S rRNA	1911 1915 1917	II	37.1	P33643	this work
YciL	28.56	23S rRNA	[c]	[c]	32.7	P37765	Ofengand et al., 1997
YjbC	91.14	23S rRNA	[c]	[c]	32.5	P32684	Ofengand et al., 1997
YqcB	62.98	23S rRNA	[c]	[c]	29.7	Q46918	Ofengand et al., 1997
YmfC	25.74	23S rRNA	[c]	[c]	24.9, 23.7[b]	P75966	C. Alabiad & J. Ofengand, unpubl. results

[a]Ofengand and Fournier (1998); Conrad et al. (1998).
[b]Value using next downstream AUG as initiation codon. The true start site is unknown.
[c]Unknown.

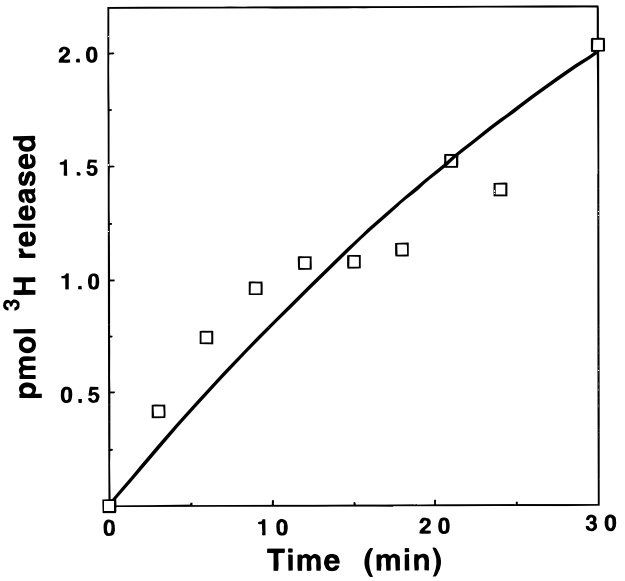

FIGURE 1. Pseudouridine formation in 23S RNA transcripts by the *rluD* gene product. 5-[^{3}H]-uridine-labelled 23S rRNA in vitro transcript was incubated with affinity-purified RluD. Eighteen μl samples were taken at the indicated times for analysis. An enzyme blank taken at 12 min has been subtracted.

ence the specificity of the synthase reaction in vitro, especially when comparing a partially purified native enzyme with its affinity purified, N-terminal tag-containing recombinant counterpart. We chose, therefore, to bypass this issue by determining the specificity of the natural synthase in its native cellular milieu.

In vivo specificity

To assess the specificity of RluD under in vivo conditions, an *E. coli* strain with an inactivated *rluD* gene was needed. Serendipitously, one of us (B.H.), for other reasons, had created such a strain, MH040, by miniTn10(cam) insertion (Hall, 1998). The location of the insert with respect to the gene sequence is shown in Figure 3A. The wild-type *rluD*$^+$ is 326 amino acids in length. Overlapping the terminator UGA is another ORF, yfiH, of unknown function, that is 243 amino acids long. The insertion occurs between A589 and C590 of the *rluD* gene, and results in a change in amino acid sequence after Thr 197 with termination at His208. Therefore, the protein presumed to be made in the disrupted strain consists of the N-terminal 197 residues (60%) of RluD fused to a C-terminal segment of 11 amino acids. Ψ sequencing analysis of ribosomal RNA from strain MH040 showed that Ψ1911, Ψ1915, and Ψ1917 were the only Ψ missing in this mutant (data not shown), and therefore that this synthase must be solely responsible for their biosynthesis.

To better correlate potential physiological effects of this gene disruption with the loss of Ψ, the mutation was moved by P1 transduction from MH040 into strain MG1655 whose gene sequence is completely known (Blattner et al., 1997). Transductants were selected by resistance to chloramphenicol and further characterized by Ψ sequencing of the ribosomal RNA from the mutant strain which showed unequivocally that all three Ψ, 1911, 1915, and 1917, were absent (see Fig. 4).

To ensure that the loss of Ψ was directly associated with *rluD* and not with some other effect such as a downstream perturbation of the yfiH ORF, a rescue plasmid was constructed containing only the *rluD* gene plus 31 additional nt (thick arrows in Fig. 3A) inserted into pTrc99A as shown in Figure 3B. Wild-type and mutant MG1655 were transformed with both the rescue plasmid and the vector pTrc99A with selection on carbenicillin plates. Wild-type and mutant MG1655 were grown in LB broth, as were the transformed strains except that the latter were grown in the presence of carbenicillin to retain the plasmids in their carbenicillin-sensitive host strains. Ribosomal RNA was isolated and sequenced for the presence of Ψ in 23S RNA (Fig. 4).

In the wild-type *rluD*$^+$ (SfhB$^+$), strong bands could be seen corresponding to Ψ one base 3′ to positions 1911, 1915, and 1917 (Bakin & Ofengand, 1993). The Ψ sequencing analysis procedure results in a band one base 3′ to the Ψ residue, which is absent in the minus CMC lanes because the effect depends on adduct formation of CMC with Ψ (Ho & Gilham, 1971). The exception, position 1915, showed a strong stop to reverse transcriptase both in the presence and absence of CMC because it is N_3-methyl pseudouridine (Kowalak et al., 1996). The presence of the methyl group at N_3 effectively blocked reverse transcription independent of reaction with CMC. Parenthetically, it probably does not even allow CMC adduct formation with Ψ because the end product of the normal reaction is a CMC adduct on N_3. However, it might be that the initial adduct at N_1 (Ho & Gilham, 1971) is resistant to the alkaline treatment if the N_3 is methyl-

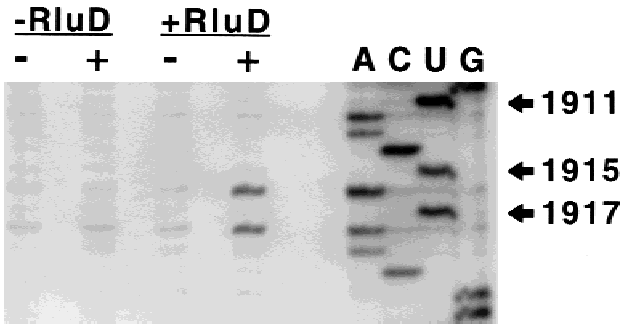

FIGURE 2. Ψ sequencing analysis of 23S RNA after in vitro reaction with recombinant RluD synthase. Reaction was as in Figure 1 for 30 min in the absence (−RluD) or presence (+RluD) of the synthase. −, +; absence or presence, respectively, of CMC treatment. A, C, U, G, sequencing lanes. Arrows show the positions of residues 1911, 1915, and 1917.

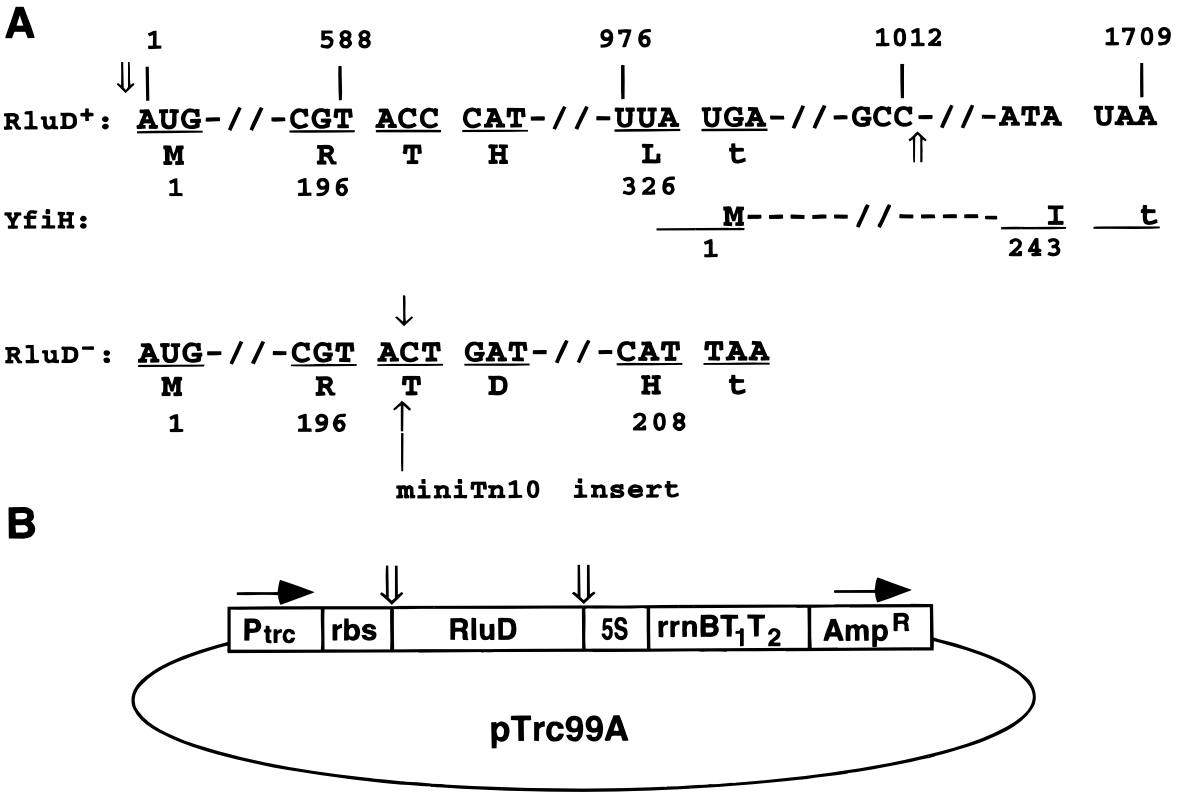

FIGURE 3. Schematic gene and protein sequence of *rluD*, YfiH, the *rluD* mutant, and the rescue plasmid. **A**: Nucleotide and amino acid sequence for the relevant parts of the *rluD*, yfiH, and miniTn10 inserted *rluD* genes. Numbers above the lines are for the nucleotide sequence, those below the line are for the amino acids. Codons are underlined. The site of insertion is shown. Thick arrows show the segment inserted into the rescue plasmid. **B**: Diagram of the plasmid used showing the site of insertion of the *rluD* gene between the ribosome binding site (rbs) and the 5S RNA coding region.

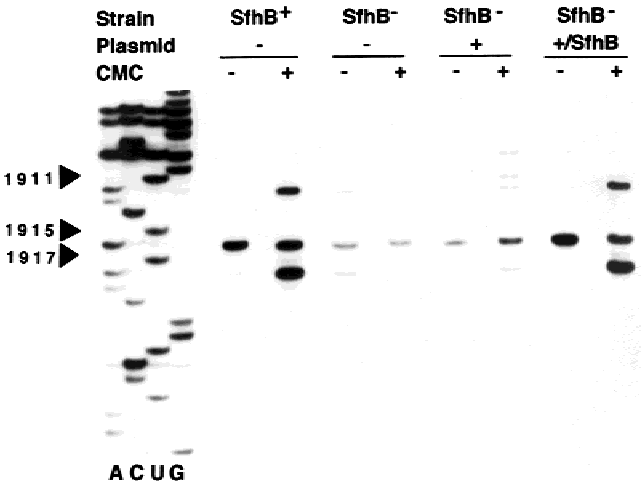

FIGURE 4. Pseudouridine sequencing analysis of the wild-type and *rluD* (*sfhB*)-disrupted strains and plasmid-containing derivatives. The wild-type (SfhB$^+$) and *rluD*-disrupted (SfhB$^-$) strains of MG1655 and the plasmids pTrc99A (+) and pTrc99A carrying the *rluD*$^+$ gene (+/SfhB) were prepared as described in Materials and Methods. Transformation of the *rluD*$^-$ strain with the plasmids, RNA preparation, and Ψ sequencing were done as described. The three naturally occurring Ψ sites monitored in this figure are indicated by arrows. RNA for ACUG sequencing lanes was from a transcript of 23S RNA (Weitzmann et al., 1990).

ated. This remains to be clarified. In the mutant *rluD*$^-$ (SfhB$^-$), the bands at 1911 and 1917 disappeared and the 1915 band was markedly diminished. Essentially the same results were obtained when *rluD*$^-$ (SfhB$^-$) was transformed with the vector, pTrc99A (+). In the latter case, the band in the + CMC lane appeared stronger than the others because more sample was applied (deduced by the fact that the background bands were proportionately darker compared to SfhB$^-$ in longer exposure autoradiograms, not shown). In contrast, in *rluD*$^-$ (SfhB$^-$) transformed with the rescue plasmid (+/SfhB), both Ψ1911 and Ψ1917 were evident, and the 1915 lanes were much stronger, like the wild type. The weak band corresponding to U1915 in the nonrescued mutant strains is most likely due to a weak methylation at N_3 of the unmodified uridine because, unlike the situation with Ψ which does not impede reverse transcription in the absence of CMC derivatization, a band of equal intensity was found both with and without CMC treatment. The band was weak compared with the wild-type and rescue strains in the absence of CMC indicating that this methyltransferase strongly prefers Ψ1915 rather than U1915 as a substrate. Therefore, in the normal cell, Ψ1915 formation should precede methylation. We con-

clude that RluD is the sole synthase capable of forming the 3 grouped Ψ, 1911, 1915, and 1917 in *E. coli*.

The location of the three sites recognized by RluD is shown in Figure 5 in the context of the secondary structure of 23S RNA and the location of the other six Ψ. Note that these three are the only U residues in this stem-loop until U1923 at the base of the stem, suggesting that once the stem-loop is recognized by the synthase, all U residues within "range" are isomerized to Ψ.

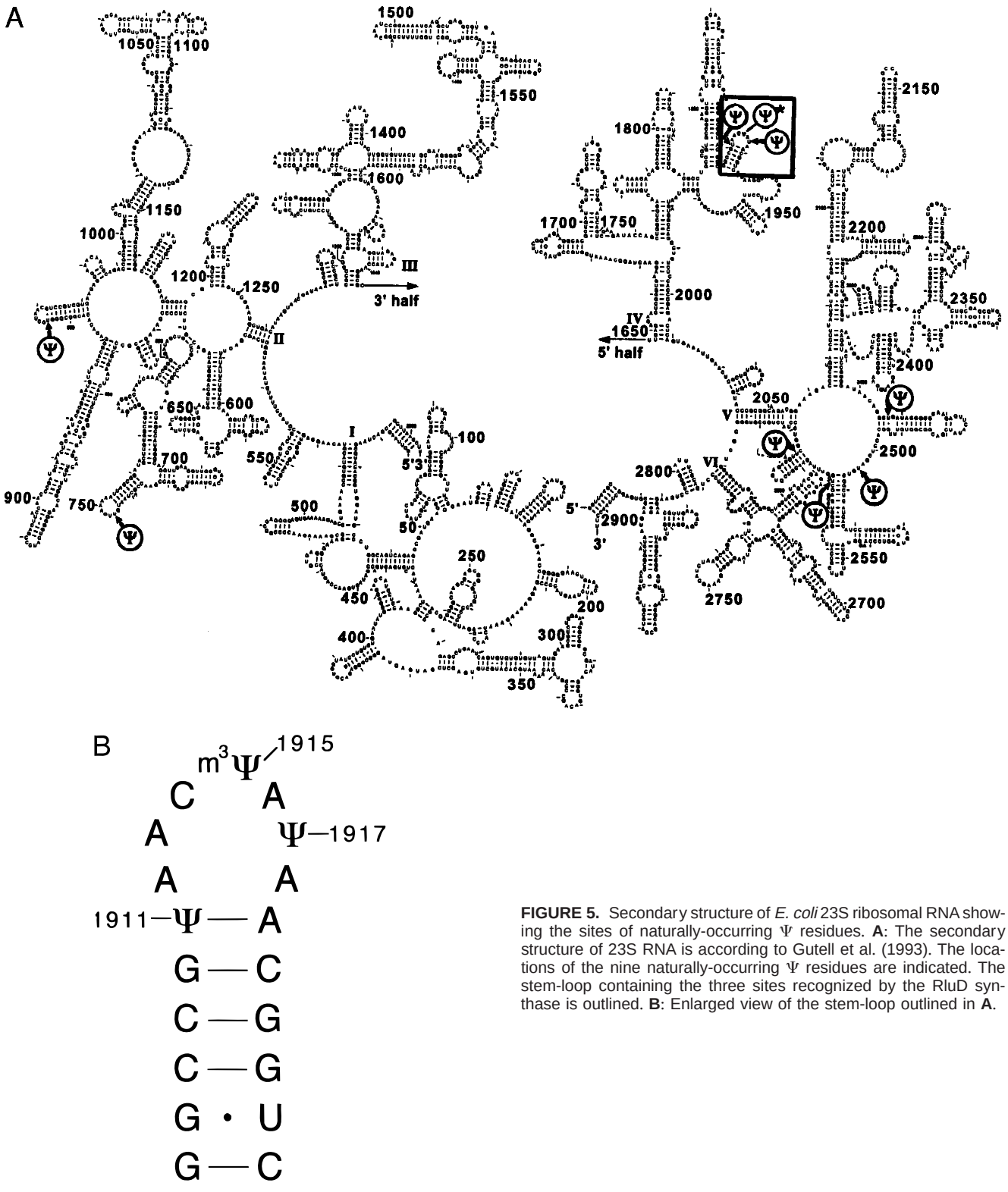

FIGURE 5. Secondary structure of *E. coli* 23S ribosomal RNA showing the sites of naturally-occurring Ψ residues. **A**: The secondary structure of 23S RNA is according to Gutell et al. (1993). The locations of the nine naturally-occurring Ψ residues are indicated. The stem-loop containing the three sites recognized by the RluD synthase is outlined. **B**: Enlarged view of the stem-loop outlined in **A**.

Growth of the *rluD*-disrupted strain

For growth comparison on plates, the four transformed strains shown in Figure 6 were used. Transformation of the wild-type strain with both the vector pTrc99A and the rescue plasmid yielded normal-sized colonies. The mutant strain transformed with the vector pTrc99A yielded only pinpoint colonies, but the mutant transformed with the rescue plasmid pTrc99A(rluD$^+$) produced normal-sized colonies. Clearly, tiny colony size was a direct result of *rluD* disruption.

To quantitate this growth defect, growth rates were determined in liquid culture. For these experiments, wild-type and *rluD*$^-$ mutant MG1655 transformed with both the rescue plasmid and its vector were used. Therefore, all four strains carried the Amp^R gene and could be grown in carbenicillin-containing LB broth. Stock cultures were plated on LB plus carbenicillin. As before, the MG1655(*rluD*$^-$)/pTrc99A strain produced only tiny colonies whereas the other three, including MG1655(*rluD*$^-$)/pTrc99A(*rluD*$^+$), gave normal-sized colonies. Inocula were grown for 14 h at 37 °C in LB plus carbenicillin. For the two *rluD*$^-$ strains, chloramphenicol was also present. The mutant [MG1655(*rluD*$^-$)/pTrc99A] strain A_{600} was 0.70, whereas the mutant-rescue [MG1655(*rluD*$^-$)/pTrc99A(*rluD*$^+$)] and both wild-type strains' A_{600} values ranged from 4.6–5.2. The cultures were diluted to an A_{600} of 0.015 and grown for 3 h at 37 °C in LB plus carbenicillin but without chloramphenicol. The culture was again diluted to a calculated A_{600} of 0.0032 for the wild-type and mutant-rescue strains and 0.0016 for the mutant strain, and growth continued in the same medium at 37 °C. The results are shown in Figure 7. It is clear that the wild-type and mutant-rescue strains grow at one rate whose generation time is 28–30 min, while the *rluD*-disrupted strain grows at approximately half that rate with a generation time of 58 min. From these generation times, a cell number ratio of 817,000:1 can be calculated for the 19 h of growth on the plates shown in Figure 6, assuming the same growth rates on the plates as in liquid culture for purposes of illustration. Thus, it is understandable how a twofold difference in growth rate can translate into the large difference in colony size shown in Figure 6.

Second site suppressor mutations were readily detected. When a sample from the mutant culture shown in Figure 7 was taken at 540 min and plated on LB containing carbenicillin and chloramphenicol, some wild-

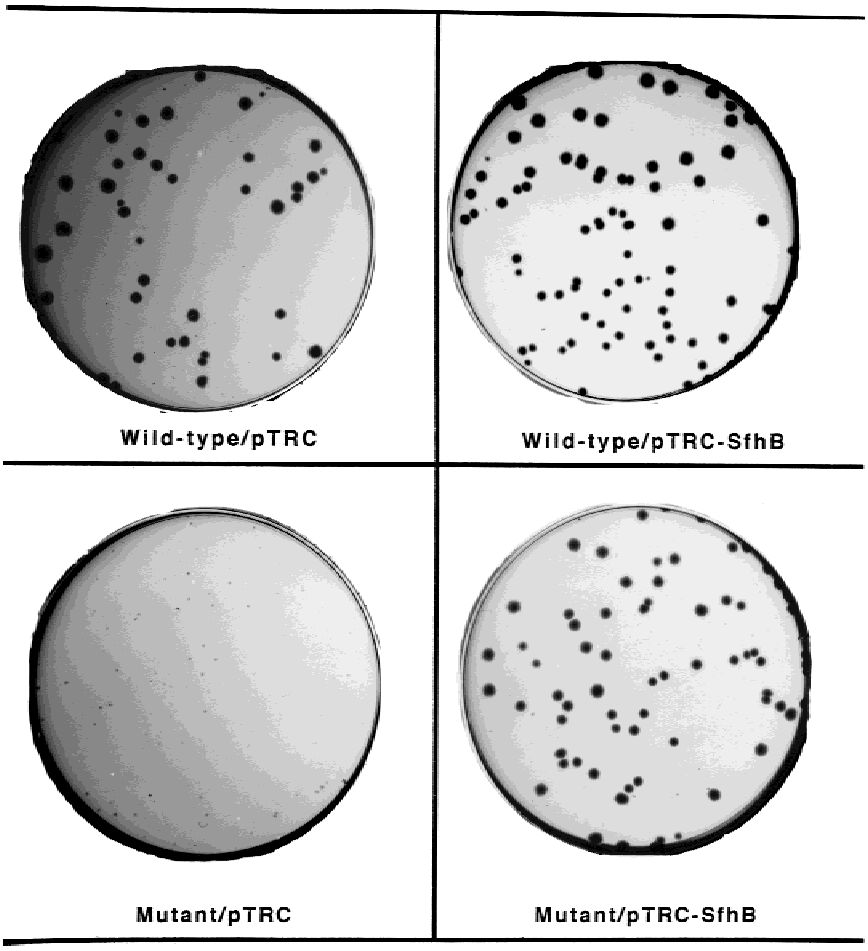

FIGURE 6. Colony morphology of *rluD* wild-type, mutant, and mutant-rescue strains of *E. coli*. Wild, MG1655(rluD$^+$); mutant, MG1655(rluD$^-$). Strains were transformed with plasmids pTrc99A [pTRC] and pTrc99A(rluD$^+$) [pTRC-SfhB] and stock cultures prepared as in Materials and Methods. Aliquots were spread on LB plus carbenicillin plates and incubated for 19 h at 37 °C.

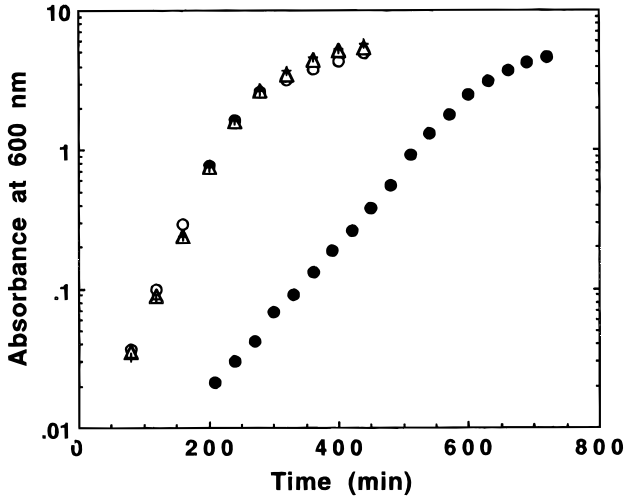

FIGURE 7. Exponential growth rate of *rluD*-disrupted and control strains of *E. coli*. Strains and inocula were prepared as described in the text. △ MG1655(rluD$^+$)/pTrc99A; +: MG1655(rluD$^+$)/pTrc99A(rluD$^+$); ●: MG1655(rluD$^-$)/pTrc99A; ○ MG1655(rluD$^-$)/pTrc99A(rluD$^+$).

type sized colonies were found after an overnight growth. Six colonies were selected and their exponential growth rates measured in the same way as described above. All six grew with generation times (31 min) like the wild type. Ribosomal RNA from three of the six "revertant" cultures was analyzed for the presence of Ψ at 1911, 1915, and 1917. No Ψ was present at any of these sites, proving that the *rluD* gene was in fact still disrupted by the chloramphenicol-carrying miniTn10 (Fig. 8). The marked decrease in band intensity at position 1915 compared to the controls provides further evidence that RluD is the synthase for Ψ1915 as well as for Ψ1911 and Ψ1917. When miniTn10(cam) from each of the six pseudorevertants was independently transduced into wild-type MG1655 by P1 transduction, and selected for chloramphenicol resistance, all of the transductants produced from each of the original pseudorevertants displayed a tiny colony morphology like that shown in Figure 6 (data not shown). Growth rate measurements on a single transductant colony from each of the pseudorevertants confirmed the mutant phenotype. After overnight growth in LB plus chloramphenicol, the culture was diluted 1:100 in the absence of chloramphenicol for growth rate measurements. All of the growth rates were mutant-like, confirming that a mutant phenotype had been re-obtained by transduction of the pseudorevertants (data not shown).

We conclude from these experiments that *rluD* disruption and consequent failure to form Ψ1911, Ψ1915, and Ψ1917 results in a severe growth inhibition of *E. coli* cells. We postulate that the cells respond by rapidly mutating at a second site so as to restore the growth rate. This suppressor mutation appears to be readily segregated out by P1 transduction and antibiotic selection, since the tiny colony and slow growth phenotype of the mutant were restored by this procedure.

Although strain MG1655 was chosen for the growth experiments because of its dearth of known mutations, the strain does contain a mutation in the *rph* gene. This

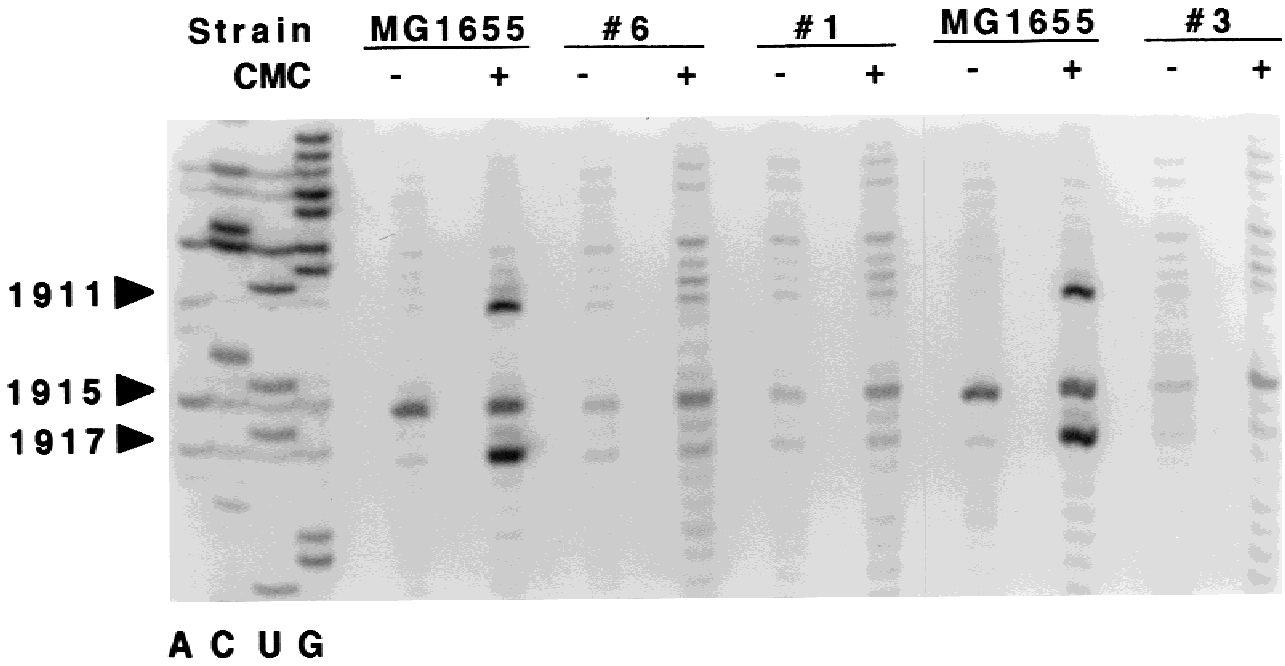

FIGURE 8. Pseudouridine sequencing analysis of three of the suppressed strain isolates. RNA from isolates 1, 3, and 6 were analyzed along with RNA from MG1655 as a positive control. Conditions were as in Figure 4.

mutation reduces the activity of RNase PH, the gene product, to very low levels, and also reduces the level of orotate phosphoribosyltransferase, encoded by *pyrE*, because of close coupling between the two genes (Jensen, 1993). This fact is relevant to our studies because Tsui et al. (1991) showed that disruption of the *truA* gene, which makes a Ψ synthase for tRNA, causes a 3–4-fold reduction in growth rate when present in strain MG1655 and related strains. Inhibition of growth was observed in minimal medium but virtually disappeared when uracil was added (Tsui et al., 1991). Moreover, there was no inhibition of growth in rich medium (Tsui et al., 1991; M. Winkler, pers. comm.). Jensen (1993) suggested that the growth defect is due to the *rph* mutation in MG1655 that in turn affects pyrimidine synthesis by down-regulation of the *pyrE* gene. Despite the fact that this effect was only seen in minimal medium and was absent in rich (LB) medium, whereas our experiments were done entirely in the same rich medium where no effect should be anticipated, we have directly examined the effect of the *rph* gene by the following gene transfer experiment.

P1 transduction from MH040 was repeated using as recipient strain CA244, which is known to be *rph*$^+$ (Reuven & Deutscher, 1993), and strain VH1000, which is MG1655 in which the *rph* mutation has been repaired. MG1655 was included as a recipient to serve as a tiny colony control. In all three cases, chloramphenicol-resistant, poorly growing colonies were found. When replated and grown for 18 h at 37 °C, only tiny colonies were obtained, like Mutant/pTRC in Figure 6. As a growth control, the rescued strain MG1655(*rluD*$^-$)/pTrc99A(*rluD*$^+$), which grows normally in the presence of chloramphenicol, was used, and in the same time interval yielded the same normal colony pattern shown in Figure 6.

DISCUSSION

Specificity

In this work, we have shown that the *yfiI*, later named *sfhB*, gene product is a Ψ synthase which forms Ψ at positions 1911, 1915, and 1917 in *E. coli* 23S RNA. In view of this specificity, the gene has been renamed *rluD*. Moreover, it is the only synthase in *E. coli* capable of this reaction in vivo since disruption of the gene caused the loss of these three Ψ residues, and addition of the gene on a plasmid restored them (Fig. 4). However, RluD might be able to recognize other Ψ sites which are also recognized by still-unidentified synthases. Deletion experiments would not reveal this until all the other relevant synthases had been deleted. There can be only two such additional sites, however, because in vivo deletion of the *rsuA* gene causes the loss of Ψ516 in 16S RNA (L. Niu & J. Ofengand, unpubl. results), deletion of the *rluC* gene (formerly called yceC) results in the absence of Ψ residues 955, 2504, and 2580 (Conrad et al., 1998), and deletion of *rluA* (Wrzesinski et al., 1995b) causes the loss of Ψ746 (S. Raychaudhuri, L. Niu, & J. Ofengand, unpubl. results). Thus, only Ψ2457 and Ψ2605 are potential candidate sites for dual enzyme action.

Synthases for 8 of the 10 Ψ residues in *E. coli* ribosomes have now been identified (Table 1). So far, each synthase appears to be associated with a specific subset of Ψ sites with no overlap. In addition to other pertinent data, Table 1 shows the classification of the Ψ synthases according to specificity class. Briefly, class I specificity is for a single site in a single class of RNA, class II is for a cluster of nearby sites in a single class of RNA, class III is for a set of separated sites in a single class of RNA, and class IV is for a single site in more than one class of RNA. RluD falls in class II while the recently discovered RluC, which also makes 3 Ψ in 23S RNA, is in class III. Table 1 also includes the remaining ORFs in *E. coli* with known homology to Ψ synthases (Koonin, 1996; Gustafsson et al., 1996; K. Rudd, pers. comm.). We have cloned and overexpressed the YciL, YjbC, YqcB, and YmfC proteins, showed that they each possess Ψ synthase activity (Ofengand et al., 1997; C. Alabiad & J. Ofengand, unpubl. results), and specificity analyses are underway. Since only two Ψ sites remain to be identified, namely 2457 and 2605, at most two of the ORFs should be involved in rRNA Ψ synthesis. It is likely that the remaining two ORFs code for synthases involved in tRNA pseudouridylation since there are five distinct sites in tRNA for Ψ (Sprinzl et al., 1998), three of which have already been connected to specific synthases (Kammen et al., 1988; Nurse et al., 1995; Wrzesinski et al., 1995b). Of course, it is also possible that one or more of these proteins form Ψ in some other class of RNA which is not yet known to be pseudouridylated, and there could be rRNA Ψ synthase ORFs with as yet unrecognizable sequence motifs. The possibility that RluD might also show class IV specificity, that is, for a site in another class of RNA like RluA (Wrzesinski et al., 1995b), has not yet been tested.

RNA recognition mechanism

Because Ψ1911, 1915, and 1917 are within 7 nt of each other in the loop region of a small stem-loop structure (Fig. 5B), the stem-loop may be the site of recognition by the synthase and any U within a defined distance of the active site may then become a target for isomerization. Implicit in this view is the concept that the active site is floppy so it can accommodate different parts of the loop. In this regard, it is noteworthy that the isomerized U residues are the only ones in the stem-loop except for U1923 near the base of the stem where it exists as a G-U base pair (Fig. 5B). The only other obvious common structural element of the three Ψ sites is the sequence ΨA. U1923 is not followed by A, and

the next closest U followed by an A is U1898 and U1926, on the 5′ and 3′ sides, respectively, of the stem and well removed from the site of action. Thus, exclusion of U1923 could be a result of a requirement by the synthase for a UA sequence in addition to the distance constraint. Testing of this proposal by studies with mutant RNAs is underway.

In eukaryotes, guide RNAs determine the sites of Ψ formation (reviewed in Ofengand & Fournier, 1998). Are guide RNAs also used in *E. coli* as specificity determinants? As yet, there is no evidence to support this hypothesis. Four distinct Ψ synthases have been identified in *E. coli* and together account for 8 of the 10 Ψ found in the ribosomes of this organism. Although gene inactivation experiments do not rule out the existence of guide RNAs as an adjunct to synthase proteins (e.g., they could determine the specificity for each of the sites in specificity classes II and III), in vitro experiments with overexpressed and affinity-purified synthases RsuA, RluA, RluC, and RluD and in vitro transcribed 16S and 23S RNA do not show any requirement for an added cofactor. Thus, even though the LSU RNA Ψ residues cluster similarly around the peptidyl transferase center in both prokaryotes and eukaryotes, each class of cells appears to use a distinctly different system for site recognition. This subject is discussed further in Ofengand & Fournier (1998).

Function

Two of the three Ψ residues made by RluD, Ψ1915 and Ψ1917, are universally conserved according to available data (Ofengand & Bakin, 1997), although in most organisms, the equivalent to N_3-methyl Ψ1915 in *E. coli* is an ummodified Ψ. Even in the mitochondria of *Trypanosoma brucei*, a relative of *Leishmania*, where no secondary structure of the LSU RNA has been defined, the two Ψ residues likely to correspond to 1915 and 1917, 579 and 581 (Ofengand & Bakin, 1997), are found in a sequence identical to the loop in Figure 5B except that the C residue in *E. coli* is changed to A. In agreement with this universal occurrence, genes exist in a wide variety of organisms, including *Leishmania*, with homology to *rluD* (Myler et al., 1994). What do these Ψ do? In *E. coli*, although these residues are in the 50S subunit, they are juxtaposed to the decoding center in the 30S subunit as shown by crosslinking of the 23S RNA segment 1912–1920 to residues 1408–1411 of the 16S RNA (Mitchell et al., 1992). They are functionally related as well since the fidelity of codon recognition is perturbed upon mutation of C1914 or A1916 (O'Connor & Dahlberg, 1995).

It is tempting, therefore, to assign the severe growth defect found here to the lack of one or more of the three Ψ lost upon disruption of *rluD*. However, what has actually been shown is that the lack of RluD results in growth inhibition which can be reversed by addition of the *rluD* gene on a plasmid. In addition to the proposal that the growth defect is directly related to the loss of one or more of the Ψ residues 1911, 1915, and 1917, there are at least two other possibilities. The RluD protein may perform some other essential function unrelated to its synthase activity. For example, the *E. coli* RUMT enzyme which catalyzes m^5U54 formation in tRNA is essential yet its methylation activity is dispensable (Persson et al., 1992). Another alternative is that RluD is in reality a class IV synthase, which makes an essential Ψ in an essential but unidentified RNA, and that its activity on rRNA is not related to the growth defect. With regard to a role in the ribosome, our bias is toward an effect on ribosome function and in particular on decoding, but it is also possible that Ψ residues are necessary for ribosome biosynthesis, for example by assisting in the proper folding of the RNA. In any event, it is clear that the growth defect attendant on the loss of the RluD synthase is specific, and not related to the loss of a synthase or Ψ residues in general. RluC (see Table 1), like RluD, is responsible for the biosynthesis of three different Ψ in 23S RNA, and grows indistinguishably from wild type (Conrad et al., 1998). Clearly, simply the lack of three Ψ in 23S RNA or the lack of a synthase does not inhibit growth. To our knowledge, this is the first time that deletion of a Ψ synthase or of a Ψ residue has had such a marked physiological effect.

Pseudouridines in rRNA are also likely to be important in eukaryotes in view of the elaborate guide RNA system which directs their precise placement in rRNA, despite the fact that depletion of various guide RNAs singly or in combination have so far failed to show an effect (reviewed in Ofengand & Fournier, 1998). For example, a recent report shows that the rare bone-marrow disorder dyskeratosis congenita is due to mutation in a gene which contains Ψ synthase motifs (Heiss et al., 1998), leading to speculation that rRNA Ψ are somehow involved in this genetic disease (Luzzatto & Karadimitris, 1998).

MATERIALS AND METHODS

RNA

The rRNA transcript of full-length 23S RNA was prepared by linearization of pCW1 (Weitzmann et al., 1990) and transcription in 40 mM HEPES pH 8.0, 20 mM $MgCl_2$, 25 mM NaCl, 2 mM spermidine, 10 mM DTT, 2.5 mM each of ATP, CTP, UTP, GTP, 2 units/ml inorganic pyrophosphatase (Sigma, cat. #1891), 1,000 units/ml RNasin (Promega), 22 nM linearized plasmid, 5,000 units/ml T7 RNA polymerase (Ambion, Inc.), and 300 μCi/ml of [5-^{3}H]UTP (Amersham) at 37 °C for 2–4 h. rRNA was purified by phenol extraction, gel filtration, and ethanol precipitation. The final specific activity was 197 dpm/pmol of uridine residues. Ribosomal RNA for Ψ sequencing was prepared according to King & Schlessinger (1983) with omission of the LiCl precipitation step.

Cloning and overexpression

The *rluD* ORF was amplified and prepared for insertion into pET-28a by PCR. The N-terminal primer had an *Nhe* I site adjacent to the initiating AUG, while in the reverse orientation, the C-terminal primer incorporated an *Xho* I site 31 nt after the terminator UGA (see Fig. 3A). The amplified product was purified by gel electrophoresis, digested with *Nhe* I and *Xho* I, and ligated with a similarly digested and purified pET-28a vector for 16 h at 16 °C. Transformation of Novablue cells was carried out by standard methods and yielded 5 positive clones out of 10 tested. Plasmids from 2 positive clones were transformed into BL21/DE3 cells. For overexpression of the N-terminal His-tagged protein, the transformed BL21/DE3 cells were grown in LB at 37 °C to an A_{600} of 0.6. IPTG (1 mM) was added and cells grown at 37 °C for 4 h. Cells were resuspended in 0.1 times the original culture volume of 50 mM Tris, pH 7.9, lysozyme was added to a final concentration of 100 μg/ml, and the mixture incubated at 30 °C for 30 min. After disruption by sonication and centrifugation at 15,000 $\times$ *g*, the S15 supernatant (3 ml), was applied to a 1 ml column of His-Bind resin (Novagen, Inc.). By gel analysis, approximately 60% of the overexpressed protein was in the supernatant. Conditions of preparation and operation of the column were as described in the pET System Manual, 7th edition (Novagen, Inc.). After elution of the His-tagged protein, pooled fractions were dialysed versus 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl for 2 h, and then against 20 mM HEPES, pH 8.0, 250 mM NH_4Cl, 5 mM mercaptoethanol, 0.1 mM EDTA, and 10% glycerol for 3 h. Under these conditions, the protein stayed soluble. The protein solution was diluted with an equal volume of glycerol and stored at −20 °C.

Gene disruption

The *rluD* (*sfhB)* gene was disrupted by a miniTn10(cam) insertion in strain SJ134 as part of a search for genes whose disruption affects adaptive mutagenesis (Hall, 1998). The mutant designation is MH040. Insertion of the 1471 nucleotide miniTN10 occurred between nt A589 and C590 of the *sfhB* coding region (GenBank Accession Number U50134) (Fig. 3) as determined by direct sequencing (Hall, 1998). The disrupted gene was moved into strains MG1655, CA244, and VH1000 by P1 transduction (Miller, 1992) using chloramphenicol selection. Isolated colonies were grown in LB plus 34 μg/ml chloramphenicol. Glycerol was added to 20%, and the cultures stored at −60 °C.

Rescue plasmid

Plasmid pTrc99A(rluD$^+$) was constructed by insertion into the *Nco* I and *Hin*d III sites of pTrc99A (Pharmacia, cat. #27-5007-01) of a PCR-amplified segment of DNA consisting of the *rluD* gene starting from the initiator AUG and terminating 31 nt after the terminator UGA (see Fig. 3A). The *trc* promoter is inducible with IPTG but considerable expression occurs even without induction, as detected by SDS-PAGE of cell lysates.

Polyacrylamide gel electrophoresis

SDS gels were 10% acrylamide and contained 0.345 M Tris-HCl, pH 8.8, and 0.1% SDS. The 3.8% stacking gel contained 0.13 M Tris-HCl, pH 6.8, 0.1% SDS. Samples were heated at 95 °C for 5 min in 50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.1% bromphenol blue and then quenched on ice before loading. Gels were stained with Coomassie Blue.

Other methods and materials

Transformants of wild-type and *rluD*-disrupted MG1655 with pTrc99A and pTrc99A(rluD$^+$) were selected on LB (Zyskind & Bernstein, 1992) plates containing 100 μg/ml carbenicillin. A colony was picked, grown in LB broth plus carbenicillin, and sterile glycerol was added to 20%. Stock cultures were stored at −60 °C. All growth media for the transformants contained 100 μg/ml carbenicillin to retain the plasmid in the carbenicillin-sensitive host cells. Ψ sequencing was performed as previously described (Bakin & Ofengand, 1993, 1998). In vitro assays of Ψ synthase activity were done as described previously (Wrzesinski et al., 1995b) at 10 mM Mg^{2+} and 100 nM RNA. Growth experiments were performed as described in Results with cell density being monitored at 600 nm. Restriction enzymes, T4 DNA ligase, and calf intestinal phosphatase were from New England Biolabs. T7 RNA polymerase was from Ambion, Inc. Deoxyoligonucleotide primers were purchased from Gibco-BRL. *E. coli* strain MH040 was obtained as described (Hall, 1998). MG1655 (Blattner et al., 1997) and CA244 (Bachmann, 1996) were the gifts of K. Rudd and M. Deutscher, respectively, of the University of Miami. VH1000 (MG1655[lacI$^-$lacZ$^-$rph$^+$], V.J. Hernandez, pers. comm.) was obtained from R.L. Gourse, University of Wisconsin.

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