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Identification and site of action of the remaining four putative pseudouridine synthases in *Escherichia coli*

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ABSTRACT

There are 10 known putative pseudouridine synthase genes in *Escherichia coli*. The products of six have been previously assigned, one to formation of the single pseudouridine in 16S RNA, three to the formation of seven pseudouridines in 23S RNA, and three to the formation of three pseudouridines in tRNA (one synthase makes pseudouridine in 23S RNA and tRNA). Here we show that the remaining four putative synthase genes make bona fide pseudouridine synthases and identify which pseudouridines they make. RluB (formerly YciL) and RluE (formerly YmfC) make pseudouridine2605 and pseudouridine2457, respectively, in 23S RNA. RluF (formerly YjbC) makes the newly discovered pseudouridine2604 in 23S RNA, and TruC (formerly YqcB) makes pseudouridine65 in $tRNA^{Ile1}$ and $tRNA^{Asp}$. Deletion of each of these synthase genes individually had no effect on exponential growth in rich media at 25 °C, 37 °C, or 42 °C. A strain lacking RluB and RluF also showed no growth defect under these conditions. Mutation of a conserved aspartate in a common sequence motif, previously shown to be essential for the other six *E. coli* pseudouridine synthases and several yeast pseudouridine synthases, also caused a loss of in vivo activity in all four of the synthases studied in this work.

Keywords: rRNA Ψ2457, 2604, 2605; tRNA Ψ65; *yciL* is *rluB*; *yjbC* is *rluF*; *ymfC* is *rluE*; *yqcB* is *truC*

INTRODUCTION

Pseudouridine (Ψ), the 5-ribosyl isomer of uridine, is the most common modified nucleoside in RNA molecules. It is found in ribosomal RNA (rRNA), transfer RNA (tRNA), and, in eukaryotes, it is also found in small nuclear and nucleolar RNAs (Maden, 1990; Massenet et al., 1998; Ofengand & Fournier, 1998; Sprinzl et al., 1999). It is noteworthy that Ψ appears to be confined to those RNAs whose tertiary structure is important to their function. The localization of Ψ residues within those RNA molecules to elements known or likely to be involved in either intra- or intermolecular RNA–RNA interactions further supports this notion. Ψ is made by enzyme-catalyzed isomerization of specific U residues after the polynucleotide chain is formed (Johnson & Söll, 1970; Ciampi et al., 1977). Site specificity for Ψ in rRNA and tRNA of bacteria and in tRNA of eukaryotes is achieved by the existence of a set of Ψ synthases, each specific for one or at most three sites (see Figs. 1 and 2). For rRNA of eukaryotes, site specificity is determined by guide RNAs acting in conjunction with a (probably) single Ψ synthase protein plus additional auxiliary proteins (Ofengand & Fournier, 1998; Bachellerie et al., 2000). These two different ways to achieve specificity in Ψ formation in the rRNA of bacteria and eukaryotes are either a cause or a consequence of the disparate number of Ψ in their respective rRNAs, eukaryotes having up to 10 times the number found in bacteria (Ofengand & Fournier, 1998). Although the number of Ψ in Archaea is so far like that in bacteria (Massenet et al., 1999; Ofengand & Rudd, 2000), analysis of the 10 archaeal genomes whose sequence is publicly available failed to identify a set of potential Ψ synthase open reading frames (ORFs). Rather, a single potential rRNA Ψ synthase was identified (J. Ofengand, unpubl. results) that had homology to the one identified in *Saccharomyces cerevisiae* as being part of the guide RNA system (Zebarjadian et al., 1999; Bachellerie et al., 2000). It seems likely, therefore, that despite the so far small number of Ψ in archaeal rRNAs, guide RNAs are used for site identification in the process of rRNA Ψ formation in these organisms.

In *Escherichia coli*, there are 11 Ψ in the ribosome, 1 at position 516 in the small subunit rRNA made by Ψ synthase RsuA (Wrzesinski et al., 1995a, Conrad et al., 1999), and 10 in the large subunit rRNA made by the enzymes indicated in Figure 1. RluA makes Ψ746; RluC

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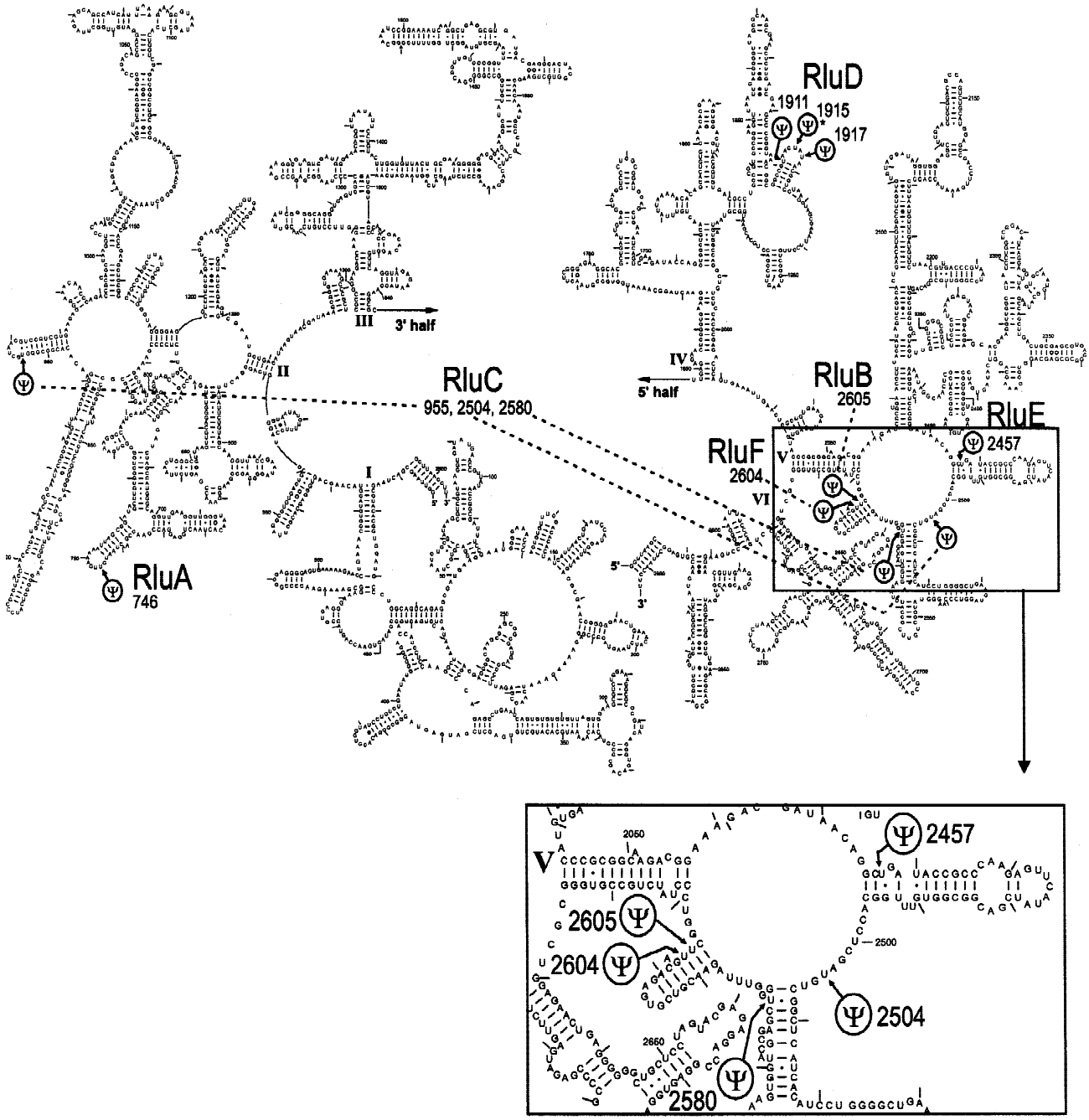

FIGURE 1. Secondary structure of *E. coli* 23S RNA (Gutell et al., 1993) showing the location of the 10 Ψ in this RNA along with the identification of the six synthases that form them. RluA (Wrzesinski et al., 1995b; Raychaudhuri et al., 1999), RluB (this work), RluC (Conrad et al., 1998; Huang et al., 1998a), RluD (Huang et al., 1998a; Raychaudhuri et al., 1998), RluE (this work), RluF (this work).

makes Ψ955, Ψ2504, and Ψ2580; and RluD makes Ψ1911, Ψ1915, and Ψ1917. The 2 Ψ at positions 2457 and 2605 were known previously but did not have identified synthases associated with them until this work. The remaining Ψ, at position 2604, was only discovered in this work during the course of identification of RluB.

Ψ in the tRNAs of *E. coli* can be found at five sites (Fig. 2). In addition to Ψ55 in all tRNAs made by TruB, TruA makes Ψ at positions 38–40 in the tRNAs indicated in the figure. RluA, which also makes Ψ746 in 23S RNA (Fig. 1), is the synthase responsible for Ψ32. Newly identified in this work is TruC, the synthase that makes Ψ65 in the two tRNAs indicated. Still unidentified is the synthase responsible for Ψ13 in $tRNA^{Glu}$.

In this work, we provide the evidence that the *E. coli* ORF *yciL* is *rluB*, *ymfC* is *rluE*, *yjbC* is *rluF*, and *yqcB* is *truC*. We show that deletion of each gene results in

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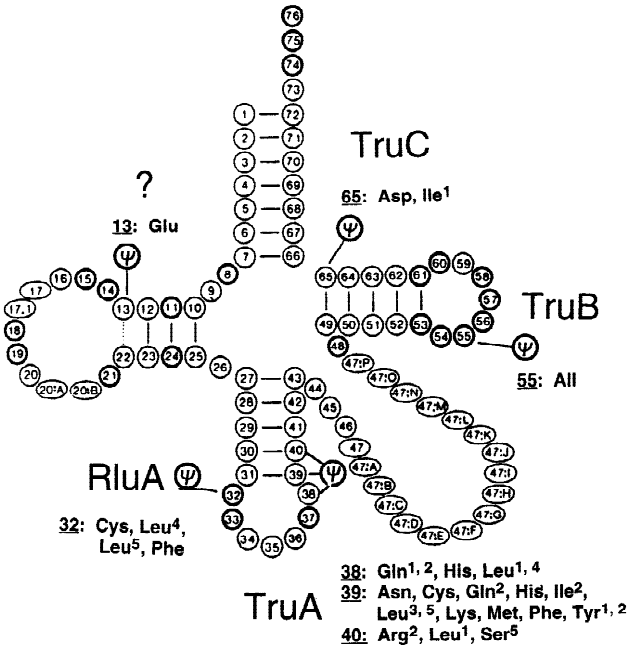

FIGURE 2. Assignment of synthases to the sites of Ψ formation in *E. coli* tRNAs. The location of Ψ residues and the tRNAs in which they occur are from Sprinzl et al. (1999). Isoacceptors are numbered according to the tDNA listings because of discrepancies between the listed isoacceptor tRNAs versus their tDNAs. This numbering is different from that conventionally used for tRNA isoacceptors. Reprinted in modified form with permission from Gutgsell et al. (2000). Synthase identification was as follows: TruA (Arps et al., 1985; Marvel et al., 1985; Kammen et al., 1988), TruB (Nurse et al., 1995; Gutgsell et al., 2000), RluA (Wrzesinski et al., 1995b; Raychaudhuri et al., 1999), TruC (this work).

the loss only of the respective Ψ, that the Ψ reappears in vivo upon transformation of the deletion strain with a plasmid carrying only the relevant synthase structural gene, and that mutation of a conserved aspartate residue essential for function in RsuA (Conrad et al., 1999), RluA (Ramamurthy et al., 1999; Raychaudhuri et al., 1999), RluC (S. Jean-Charles & J. Ofengand, unpubl. results), RluD (Gutgsell et al., 2001), TruA (Huang et al., 1998b), and TruB (Ramamurthy et al., 1999; Gutgsell et al., 2000) also blocks the function of all four of these synthases. Functional characterization by growth rate measurements did not reveal the need for any of these Ψ when singly deleted. Double deletion of *rluB* and *rluF* also had no effect.

RESULTS

Identification of RluE as the synthase for Ψ2457

Previous sequencing studies identified 10 Ψ residues in *E. coli* rRNA. Ψ516 was the only Ψ found in 16S rRNA (Bakin et al., 1994a) and the Ψ residues shown in Figure 1 (less Ψ2604) were identified in 23S rRNA (Bakin & Ofengand, 1993; Bakin et al., 1994b). As indicated in Figure 1, the synthase and associated Ψ are known for RluA, RluC, and RluD and account for 7 Ψ in 23S rRNA. The synthase(s) that formed Ψ2457 and Ψ2605 were not known nor was the existence of Ψ2604 suspected. RluA, RluC, and RluD were not involved in Ψ2457 or Ψ2605 synthesis, as inactivation of their genes did not alter the presence of these two Ψ in 23S rRNA.

From the synthase standpoint, identification of *yejD*, *yabO*, *yhbA*, and *hisT* as *rsuA*, *rluA*, *truB*, and *truA*, respectively, (references cited in Ofengand & Rudd, 2000) allowed Koonin (1996) to identify five additional putative Ψ synthase genes by amino acid sequence homology, namely *yceC*, *yfiI*, *yqcB*, *yciL*, and *yjbC*. Completion of the *E. coli* genome sequence revealed one more homolog, *ymfC* (K. Rudd, pers. comm. cited in Ofengand & Fournier, 1998). *yceC* and *yfiI* were identified as *rluC* and *rluD*, respectively (Fig. 1). Thus four unassigned synthase genes were available for the two 23S rRNA Ψ residues.

Overexpression of YmfC and reaction with a transcript of 23S rRNA yielded Ψ at U2457 but not at U2605 (J. Conrad, C. Alabiad, & J. Ofengand, unpubl. results). To prove this assignment unequivocally, the *ymfC* gene was deleted and the deletion strain complemented with a plasmid-borne wild-type or mutant version of the *ymfC* structural gene. Ψ sequencing (Fig. 3) established that Ψ2457 was absent in the deletion strain (compare the first two lanes with the last two) but reappeared when the deletion strain was transformed by a rescue plasmid carrying the *ymfC* structural gene. In this sequencing method, a band is produced one residue 3′ to the Ψ (U in the RNA sequencing lanes) and only upon CMC treatment. As noted previously (Ofengand & Fournier, 1998), there is some uncertainty about the true initiation site of this gene. In these constructs, the longer (by 10 amino acids) sequence was used. When Asp79 was mutated to Thr or Asn, rescue did not occur. Two controls show that the absence of rescue is not an artifact. First, because Ψ2504 was detected in all samples, the sequencing procedure worked. Second, mutant protein was present in the cell in about the same amount as wild-type. The RNA samples assayed for Ψ in Figure 3 were extracted from IPTG-induced transformed cells whose protein profiles, taken at the same time, are shown in Figure 4. Clearly, approximately the same band intensity was observed in all cases whether 1 × (Fig. 4A) or 3.3 × (Fig. 4B) amount of extract was applied. We conclude that *ymfC* is the gene for a synthase able to make Ψ2457 in vivo and that it has an essential aspartate at position 79. Therefore, *ymfC* has been renamed *rluE*.

Identification of RluB as the synthase for Ψ2605

A previous study (Niu et al., 1999) had identified a Ψ synthase gene, *ypuL,* in *Bacillus subtilis* that, by over-

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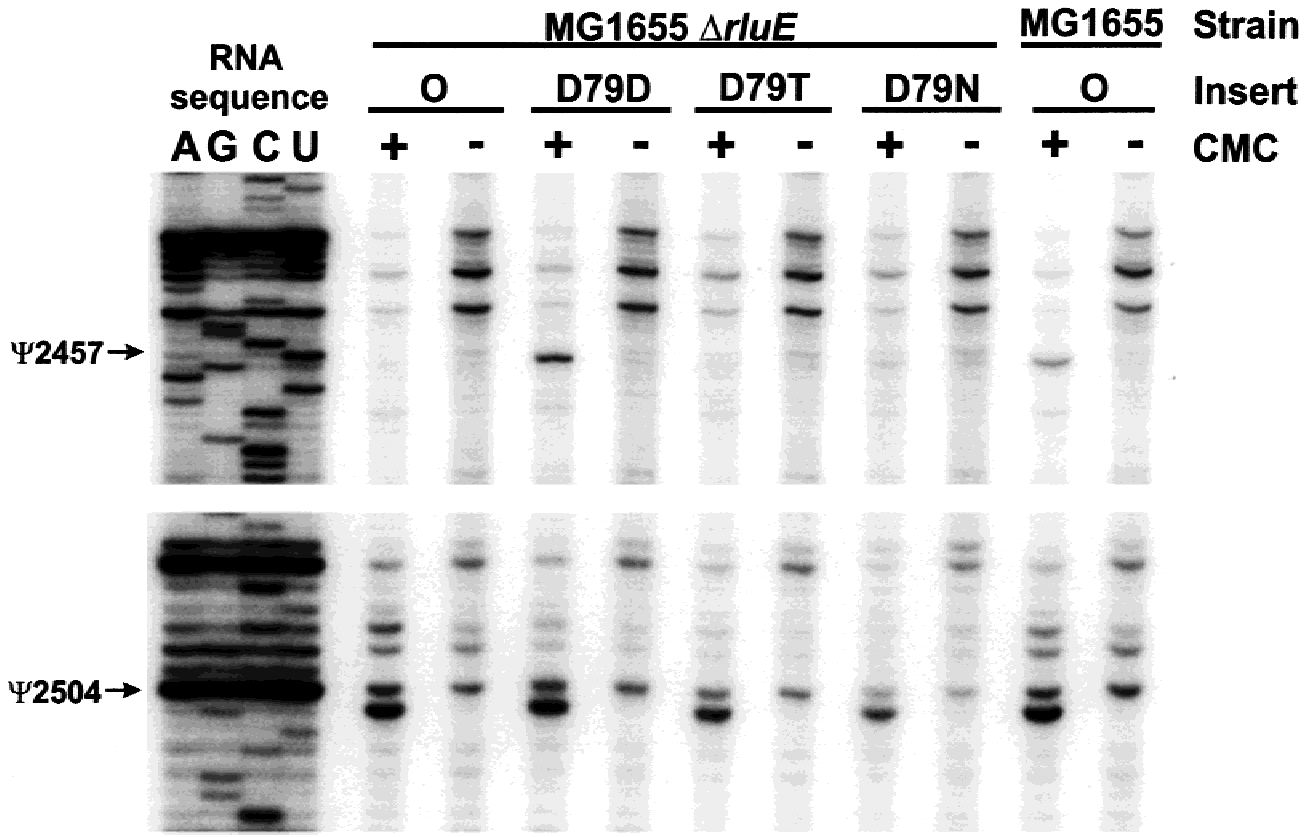

FIGURE 3. Ψ sequence analysis of the *rluE* deletion strain (Δ*rluE*). Deletion of the *ymfC* (renamed *rluE*) gene, construction of wild-type and mutant *rluE*-containing pTrc99A, transformation of the Δ*rluE* strain, growth and induction of the transformed cells, isolation of RNA, and Ψ sequencing were all performed as described in Materials and Methods. The primer was complementary to 23S RNA residues 2549–2569. Part of the gel between Ψ2457 and Ψ2504 was removed to save space; both segments shown come from the same gel. The Δ*rluE* strain was transformed with pTrc99A with no insert (○), with the wild-type *rluE* structural gene (D79D), and with two mutants (D79T, D79N). RNA was reacted with (+) or without (−) CMC following the standard sequencing protocol. A, G, C, U: RNA sequencing lanes.

expression and in vitro reaction, was found to primarily make Ψ2633 (2605 in *E. coli* numbering). The closest homolog in *E. coli* to that gene was *yciL* (expect value of $7e^{-29}$ in BLAST) compared to the next highest value of $5e^{-25}$ for *yjbC*. On that basis, *yciL* was deleted. Ψ sequencing revealed that *yciL* was indeed the gene for the Ψ2605 synthase. This Ψ disappeared from the deletion strain, and was restored by the plasmid-borne wild-type but not the mutant *yciL* structural gene (Fig. 5). The internal control in this case is Ψ2580, which was clearly present in all strains. Figure 4 shows that both mutant and wild-type RluB synthase were present in good amount at the time of RNA isolation for sequencing and that the amount of wild type was clearly less than even twice that of either mutant (seen in both Fig. 4A and B). We conclude that *yciL* is the gene for the synthase able to make Ψ2605 in vivo and that it has an essential aspartate at position 110. Therefore, *yciL* has been renamed *rluB*.

Identification of an additional Ψ at position 2604 in 23S rRNA and assignment of its synthase gene as yjbC

Ψ2605 has always yielded a double stop on sequencing gels, a behavior that has also been noted for certain other Ψ sites (Bakin & Ofengand, 1993, 1998; Bakin et al., 1994b). This effect introduces a complication in Ψ sequencing when the 5′ residue is a U, because then it is difficult to distinguish one Ψ with stuttering from two adjacent Ψ. The hydrazine-aniline cleavage reaction should, in principle, resolve this issue (Bakin & Ofengand, 1993; Bakin et al., 1994b) and on that basis it was concluded that only U2605 was converted to Ψ. However, a reexamination of this issue in a strain lacking RluB and thus lacking Ψ2605 (Fig. 5) shows the presence of a Ψ residue at U2604. Because only two unassigned putative Ψ synthase genes remained, *yjbC* and *yqcB*, and the function of *yqcB* was known

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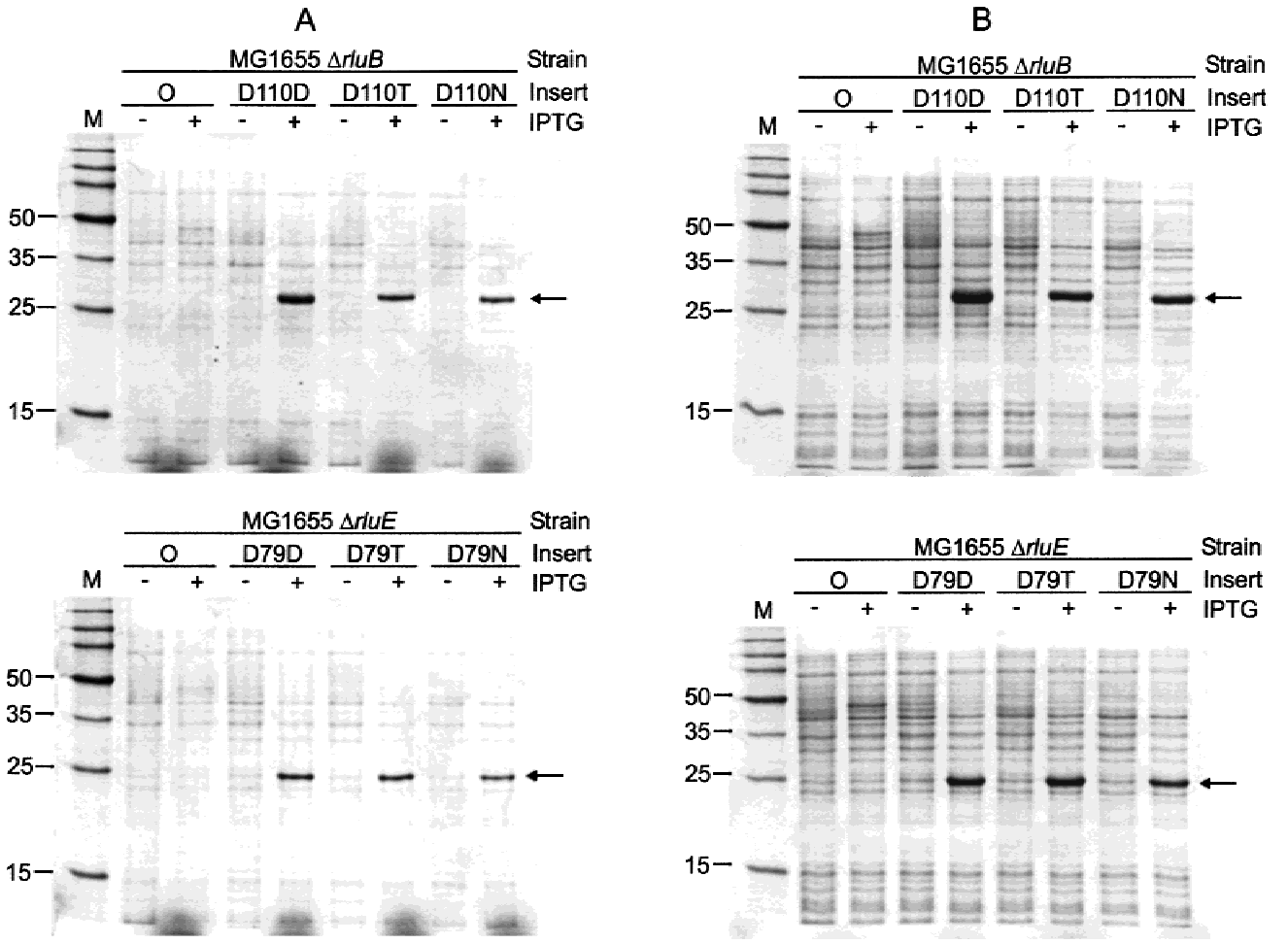

FIGURE 4. Overexpression of wild-type and mutant RluE and RluB upon induction of transformed Δ*rluB* and Δ*rluE* cells. Construction and designation of strains is as in the legends of Figures 3 and 5. Cells at an A_{600} of 0.6 were induced with 1 mM IPTG for 1 h at 37 °C, harvested, and protein isolated and electrophoresed as described (Raychaudhuri et al., 1998). Extracts from equal amounts of cells were applied to each lane. +: induced; −: uninduced; M: marker proteins with sizes in kilodaltons as indicated. 3.3 times as much sample was applied in **B** as in **A**. Arrow marks the overexpressed protein.

(see below), we tentatively assigned YjbC as the synthase for Ψ2604.

Proof of the assignment as well as of the separate and independent existence of Ψ2604 is shown in Figure 6. Deletion of *yjbC* (*rluF* in the figure) and transformation with a plasmid with no insert caused the upper of the two bands in the MG1655 control to disappear. It reappeared when the plasmid contained the *yjbC* (*rluF*) gene. By contrast, deletion of *rluB* in Figure 5 caused the lower band to disappear. To assess the specificity of each of the synthases, a double deletion mutant, Δ*rluB*Δ*rluF*, was constructed. Both bands disappeared in this strain, and only the lower band reappeared when RluB was present. There was no Ψ2604 band. When the same experiment was done with *yjbC* (*rluF*), a Ψ2604 band was found. In this case, a faint band at Ψ2605 was also seen. It appears that YjbC is not completely specific for U2604 but can, to a small extent, also react with U2605. Aspartate107 mutants of YjbC(RluF) were also tested and found to be unable to make Ψ2604. This was not due to a problem in overexpression, as both wild-type and mutant YjbC(RluF) were produced in approximately the same amount (Fig. 8A). Therefore, *yjbC* has been renamed *rluF*. It too has an essential aspartate residue, at position 107.

Identification of TruC as the synthase for Ψ65 in RNA^{Ile1}

All of the rRNA Ψ had by now assigned synthases and because they could all be accounted for by deletion of one or another of the synthase genes, it was clear that only one synthase made a specific Ψ. Nevertheless, one putative synthase, *yqcB*, remained unassigned. However, as shown in Figure 2, two sites of Ψ formation on tRNA had no assigned synthase, namely Ψ13

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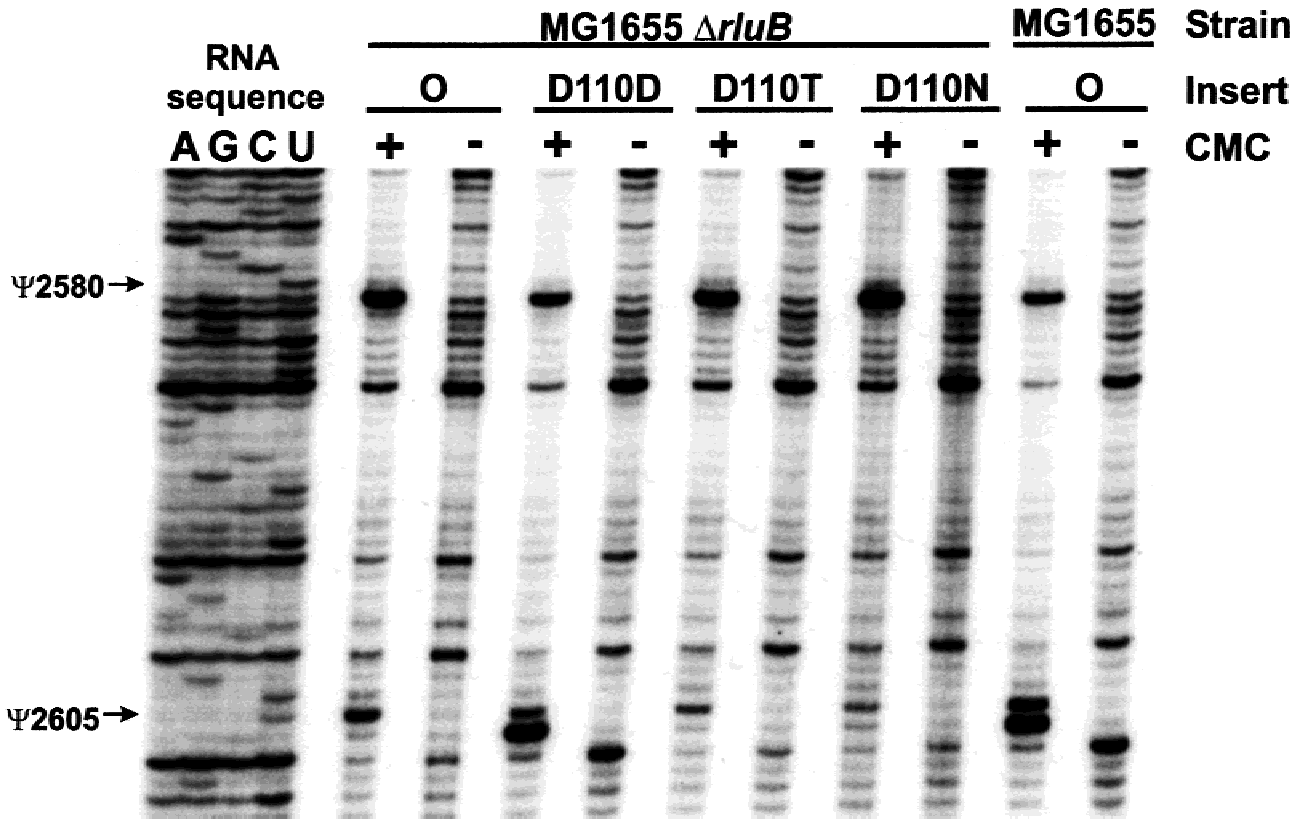

FIGURE 5. Ψ sequence analysis of the *rluB* deletion strain (Δ*rluB*). Deletion of the *yciL* (renamed *rluB*) gene, construction of wild-type and mutant *rluB*-containing pTrc99A, transformation of the Δ*rluB* strain, growth and induction of the transformed cells, isolation of RNA, and Ψ sequencing were all performed as described in Materials and Methods. The primer was complementary to 23S RNA residues 2647–2665. The Δ*rluB* strain was transformed with pTrc99A with no insert (○), with the wild-type *rluB* structural gene (D110D), and with two mutants (D110T, D110N). RNA was reacted with (+) or without (−) CMC following the standard sequencing protocol. A, G, C, U: RNA sequencing lanes.

in $tRNA^{Glu}$ and Ψ65 in $tRNA^{Asp}$ and $tRNA^{Ile1}$. Ψ sequencing of the relevant tRNAs isolated from a strain in which *yqcB* was deleted showed that YqcB (renamed TruC) was the synthase specific for Ψ65 in both tRNAs. The sequencing analysis of $tRNA^{Ile1}$ is shown in Figure 7. Except for two A residues that could not be read, the $tRNA^{Ile1}$ sequence was confirmed. The wild-type control had a strong band corresponding to Ψ65, and,

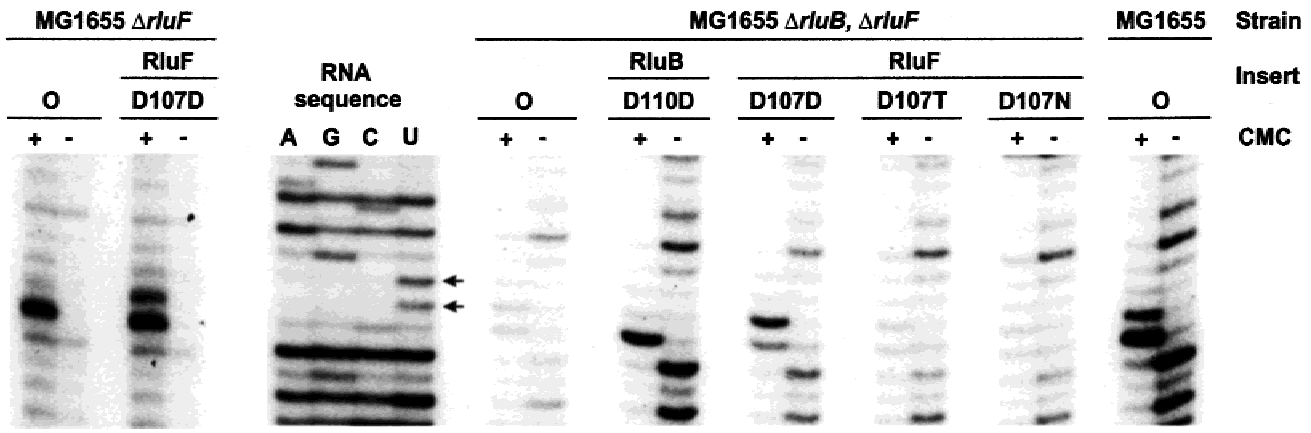

FIGURE 6. Ψ sequence analysis of the *rluF* deletion strain (Δ*rluF*). Deletion of the *rluB* and *yjbC* (renamed *rluF*) genes and all subsequent procedures were as in the legend to Figure 5 except that D107 was mutated in RluF and the host strains were Δ*rluF* and the double mutant Δ*rluB*Δ*rluF* as indicated. Arrow heads mark Ψ2604 and Ψ2605.

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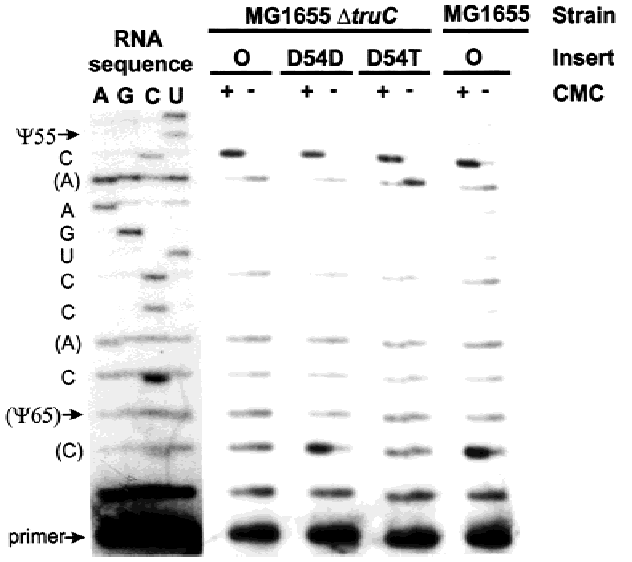

FIGURE 7. Ψ sequence analysis of the *truC* deletion strain (Δ*truC*). Deletion of the *yqcB* (renamed *truC*) gene, construction of wild-type and mutant *truC*-containing pTrc99A, transformation of the Δ*truC* strain, growth and induction of the transformed cells, isolation of RNA, and Ψ sequencing were all performed as described in Materials and Methods. The primer was complementary to $tRNA^{Ile1}$ residues 68–76. The Δ*truC* strain was transformed with pTrc99A with no insert (○), with the wild-type *truC* structural gene (D54D), and with the mutant D54T. RNA was reacted with (+) or without (−) CMC following the standard sequencing protocol. A, G, C, U: RNA sequencing lanes. The sequence is shown. (A) denotes residues that could not be experimentally confirmed.

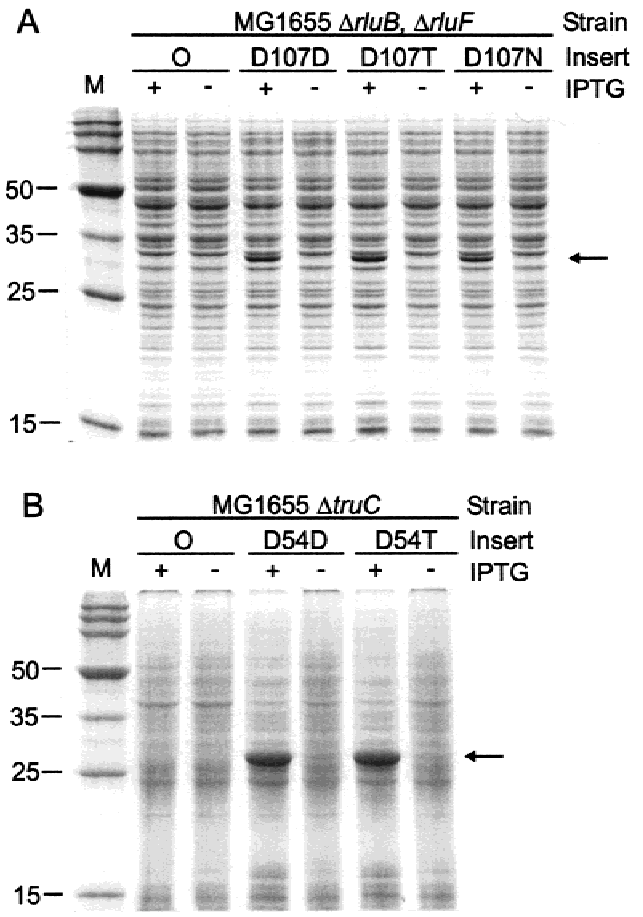

FIGURE 8. Overexpression of wild-type and mutant *rluF* and *truC* gene products upon induction of transformed Δ*rluB*Δ*rluF* and Δ*truC* cells. **A:** Δ*rluB*Δ*rluF* cells transformed with pTrc99A containing no insert (○), the wild-type *rluF* gene (D107D), or two mutant *rluF* genes (D107T, D107N). **B:** Δ*truC* cells transformed with pTrc99A containing no insert (○), the wild-type *truC* gene (D54D), or a mutant *truC* gene (D54T). Construction and designation of strains was as in the legends of Figures 6 and 7 and the procedure was as described in the legend of Figure 4. +: induced; −: uninduced; M: marker proteins with sizes in kilodaltons as indicated. Arrow marks the overexpressed protein.

as expected, only in the +CMC lane. This band was absent in the Δ*truC* lanes transformed with plasmid with no insert, but was restored when the wild-type gene (D54D) was added back via a plasmid. Therefore, YqcB is the only synthase able to make Ψ65 in *E. coli*. A mutant (D54T) gene did not restore Ψ65 forming ability. The control was Ψ55, which was made in all samples. Analysis of the protein content of the cells at the time the RNA was taken for analysis showed approximately equal amounts of wild-type and mutant TruC synthases (Fig. 8B). We conclude that *yqcB* is the gene for the synthase able to make tRNA Ψ65 in vivo and that it has an essential aspartate at position 54. The results for $tRNA^{Asp}$ were essentially the same (data not shown).

Effect of the absence of specific Ψ and their associated Ψ synthase on growth

Each of the Ψ synthase deletion constructs resulted in the loss of a single Ψ, either from 23S RNA or from tRNA, except for the double deletion of *rluB* and *rluF*, which caused the absence of both Ψ2604 and Ψ2605. The effect of these single (or double) Ψ synthase deletions and consequent Ψ absence on exponential growth rate in rich media was determined over a range of temperatures. The results (Table 1) show that none of the deletions had any effect.

DISCUSSION

Identification of Ψ synthase genes

In this work, we have shown that the genes *yciL*, *ymfC*, *yjbC*, and *yqcB* encode the Ψ synthases RluB, RluE, RluF, and TruC, respectively. With the identification of these four ORFs as bona fide Ψ synthases, the last of the five predicted by Koonin (1996) and subsequently by Gustafsson et al. (1996) plus the one, *ymfC*, predicted by Rudd (cited in Ofengand & Fournier, 1998) have been confirmed. These results once again illustrate the power of amino acid sequence homology analysis for identifying the function of unknown genes.

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TABLE 1. Exponential growth rate of the Ψ synthase deletion mutants.

Expt.	Strain	Doubling time in minutes at the indicated temperature		
		25 °C	37 °C	42 °C
I	MG1655 (WT)	72 ± 2	26 ± 1	18 ± 1
	Deletion *rluB*	74 ± 2	26 ± 1	18 ± 1
	Deletion *rluE*	70 ± 1	25 ± 1	17 ± 1
II	MG1655 (WT)	76 ± 1	26 ± 0	20 ± 0
	Deletion *rluF*	78 ± 1	25 ± 0	18 ± 1
	Deletion *truC*	77 ± 1	26 ± 1	20 ± 1
	Deletion *rluB*, *rluF*	76 ± 1	25 ± 1	20 ± 1

Cells were grown with aeration by shaking in LB medium at 37 °C and assayed by absorption at 600 nm over 3–6 doublings. Doubling time was determined from a semi-logarithmic plot of A_{600} versus time. Each plot consisted of 4–8 time points. Doubling times are the average of two determinations.

The 10 pseudouridine synthase ORFs can be grouped into four families on the basis of their amino acid sequence correspondence (Koonin, 1996; Ofengand & Rudd, 2000), coincidentally defined by the first four Ψ synthases to be characterized, namely RsuA, RluA, TruA, and TruB (Table 2). Although these initial four synthases used three different RNA substrates, identification of the substrates for all 10 synthases now shows that the family classification is, in fact, not substrate oriented. Thus TruC, which acts on tRNA, is a member of the RluA family whose first member uses 23S rRNA. Moreover, RluB, RluE, and RluF belong to the RsuA family, whose archetype member acts on 16S rRNA, whereas they act on 23S rRNA. At least in *E. coli*, it is more accurate to consider that the Ψ synthases, except for TruB and TruA, which are so far the single members of their respective families, fall, perhaps arbitrarily, into either the RsuA or RluA families.

TABLE 2. Properties of *E. coli* pseudouridine synthases.

Name	Previous name	No. of amino acids	MW (kDa)	RNA substrate	Ψ site	SWISS-PROT access. no.	Essential aspartate and mutants	Aspartate mutant activity (% of WT)		References
								In vivo	In vitro	
RsuA family										
RsuA	YejD	231	25.9	16S RNA	516	P33918	D102T/N	<1	n.t.[a]	Wrzesinski et al., 1995a Conrad et al., 1999
RluB	YciL	291	32.7	23S RNA	2605	P37765	D110T/N	<1	n.t.	This work
RluE	YmfC	217[b]	24.9[b]	23S RNA	2457	P75966	D69T/N	<1	n.t.	This work
RluF	YjbC	290	32.5	23S RNA	2604	P32684	D107T/N	<1	n.t.	This work
RluA family										
RluA	YabO	219	24.9	23S RNA tRNA	746 32	P39219	D64T/N/A/C	<1	<2	Wrzesinski et al., 1995b Raychaudhuri et al., 1999 Ramamurthy et al., 1999
RluC	YceC	319	36.0	23S RNA	955 2504 2580	P23851	D144T/N	<1	n.t.	Conrad et al., 1998 Huang et al., 1998a Jean-Charles & Ofengand, unpubl.
RluD	YfiI	326	37.1	23S RNA	1911 1915 1917	P33643	D139T/N	n.t.	<5	Raychaudhuri et al., 1998 Huang et al., 1998a Gutgsell et al., 2001
TruC	YqcB	260	29.7	tRNA	65	Q46918	D54T	<5	n.t.	This work
TruB family										
TruB	YhbA	314	35.1	tRNA	55	P09171	D48C/A	<1	<0.1	Nurse et al., 1995 Ramamurthy et al., 1999 Gutgsell et al., 2000
TruA family										
TruA	HisT	270	30.4	tRNA	38–40	P07649	D60X[c]	n.t.	<0.01	Arps et al., 1985 Kammen et al., 1988 Huang et al., 1998b

[a] n.t.: not tested.
[b] Best estimate of true initiation site. Next downstream AUG yields 207 amino acids and 23.7 kDa.
[c] X = A, E, K, N, S.

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Essentiality of the conserved aspartate

The aspartate residues found to be essential for in vivo activity in this work are all conserved residues embedded in a similarly conserved motif II common to all known and putative Ψ synthases (Koonin, 1996; Ofengand & Rudd, 2000). This aspartate was first shown to be essential for the in vitro activity of *E. coli* TruA (Huang et al., 1998b). It has subsequently been shown to be essential for the in vivo and/or in vitro activity of an additional five *E. coli* synthases (Gutgsell et al., 2001, and references therein) and three yeast Ψ synthases (Ansmant et al., 2000, 2001; Zebarjadian et al., 1999). RluB, RluE, RluF, and TruC can now be added to this list. With a total of 13 examples now known, it is safe to predict that this aspartate will be essential in all Ψ synthases. Nevertheless, two (as of July, 1999) examples of a putative Ψ synthase with glycine in place of aspartate have been noted (Ofengand & Rudd, 2000). These could be sequencing errors, although one of the examples would require two base changes. It is also possible that these are two examples of Ψ synthases that no longer need to make Ψ but are retained because they perform another valuable function. Recently, Gutgsell et al. (2001) showed that the growth defect induced by the absence of Ψ synthase RluD could be rescued by an aspartate mutant of the synthase. Thus, it is possible that an enzyme that was once capable of Ψ formation lost that activity (by mutation of its catalytic aspartate) but retained its second, more important, function.

The Ψ13 synthase

Deletion of each of the 10 synthases caused the disappearance of specific Ψ, and formation of all of the known Ψ could be blocked except for Ψ13 in $tRNA^{Glu}$ (Figs. 1 and 2). Thus, each enzyme has its own defined site of action and, in vivo, there is no overlapping specificity. No synthase has yet been identified that makes Ψ13, and as $tRNA^{Glu}$ Ψ13 is present in all 10 of the known synthase deletion/disruption strains (Y. Kaya & J. Ofengand, unpubl. results), none of these synthases also catalyze Ψ13 formation in a "dual specificity" manner like that of RluA. It is possible that Ψ13 is the one case where two (or more) synthases are able to make a Ψ. In that event, it will be necessary to delete at least two synthases to block Ψ13 formation. Another possibility is that the Ψ13 synthase is sufficiently different in its amino acid sequence so as to have escaped identification. An enzymatic activity able to make Ψ13 in yeast tRNA, but not Ψ32 or Ψ55, was previously partly purified (Samuelsson & Olsson, 1990) but so far none of the known Ψ synthase open reading frames in yeast have been identified with this activity (Ansmant et al., 2001). Possibly, both in yeast and *E. coli*, this enzyme is a representative of a hitherto undetected Ψ synthase family.

Specificity of RluB and RluF

The fact that RluB and RluF recognize adjacent U residues raises interesting questions about the mechanism of this specificity. Both proteins carry out the same catalytic reaction, and both must, in some manner, recognize the same segment of 23S rRNA. Nevertheless, one protein is largely specific for U2604 and the other completely so for U2605. How this is achieved is, so far, a mystery. A gapped BLAST comparison of their respective amino acid sequences (Fig. 9) shows considerable homology and of course the same amino acid sequence (underlined) around the essential aspartate residue but no obvious clue to either their common or specific substrate specificity. However, they are the two most related *E. coli* synthases in the RsuA family (Expect values of $7–9e^{-27}$). A BLAST search of *rluB* and *rluF* against the *E. coli* genome only identified with reasonable Expect values the other two members of the RsuA family, RsuA and RluE, and both numbers were considerably lower, $2–7e^{-19}$ and $1–3e^{-16}$, respectively. Current attempts to crystallize both proteins as well as to study their enzyme–substrate complexes both biochemically and by structure analysis may unravel the mechanism of this striking reciprocal specificity.

Function

None of the genes discussed in this work are essential, or even detectably affect exponential growth in rich media (Table 1). The double mutant Δ*RluB* and Δ*RluF* was likewise unaffected in its growth rate. Growth in minimal media, competition experiments, or effects of chemical or thermal stress have not yet been tested. Nevertheless, three of the Ψ, Ψ2457, Ψ2604, and Ψ2605, are to be found at a functionally very important site of the ribosome, namely the peptidyl transferase center, and thus merit consideration as important for the process of protein synthesis in some as yet unspecified way. Ψ2457 is only six residues away from A2451, the putative catalytic residue for peptide bond formation (Muth et al., 2000; Nissen et al., 2000), and Ψ2604Ψ2605 is within a few residues of sites involved in various aspects of peptide bond formation (reviewed in Ofengand & Bakin, 1997). In both cases, the same or an adjacent residue is Ψ in yeast, fruit flies, mice, and humans (Ofengand & Bakin, 1997). The geographic localization and persistence in widely diverse species strongly implies some important role for these Ψ in protein synthesis, but such a role has still to be demonstrated.

MATERIALS AND METHODS

Deletion strains

The *rluB*, *rluE*, *rluF*, and *truC* deletion strains were constructed by a modification (N. Hus & K. Rudd, pers. comm.) of

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RluB: 2    SEKLQKVLARAGHGSRREIESIIEAGRVSVDGKIAKLGDRVEVTPGLKIRIDGHLISVRE 61
          S +L K ++ +G  SRRE +  IE G V ++GK A +GD+V+  PG  ++++G LI  RE
RluF: 6    SVRLNKYISESGICSRREADRYIEQGNVFLNGKRATIGDQVK--PGDVVKVNGQLIEPRE 63

RluB: 62   SAEQICRVLAYYKPEGELCTRNDPEGRPTVFDRLPKLRGARWIAVGRLDVNTCGLLLFTT 121
          + + +  ++A  KP G + T  D E R  + D +   +  R   +GRLD ++ GL+  T
RluF: 64   AEDLV--LIALNKPVGIVSTTEDGE-RDNIVDFVNHSK--RVFPIGRLDKDSQGLIFLTN 118

RluB: 122  DGELANRLMHPSREVEREYAVRVFGQVDDAKLRDLSRGVQLEDGPAAFKTIKFSGGEGIN 181
           G+L N+++    + E+EY V V   + +  +R +S GV +        T K    +
RluF: 119  HGDLVNKILRAGNDHEKEYLVTVDKPITEEFIRGMSAGVPI----LGTVTKKCKVKKEAP 174

RluB: 182  QWYNVTLTEGRNREVRRLWEAVGVQVSRLIRVRYGDIPLPKGLPRGGWTELDLAQTNYLR 241
            + +TL +G NR++RR+ E  G +V +L R R  ++ L  G+P G W +L   +   L
RluF: 175  FVFRITLVQGLNRQIRRMCEHFGYEVKKLERTRIMNVSL-SGIPLGEWRDLTDDELIDLF 233

RluB: 242  ELVELPPETSSKVAVEKDRRRMKANQIRRAV 272
          +L+    E SS     K + + K   I+R V
RluF: 234  KLI----ENSSSEVKPKAKAKPKTAGIKRPV 260
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FIGURE 9. Amino acid sequence comparison of RluB and RluF. The gapped BLAST program available on the National Center for Biotechnology Information web site (Tatusova & Madden, 1999) was used. Identities and similarities are shown by the letter and +, respectively, between the two sequences.

the pKO3 gene replacement method (Link et al., 1997). In this method, deletion alleles are constructed by three PCR amplifications. The first one generates a product consisting of 400–600 bp upstream of the sequence to be deleted followed by a short sequence from the N-terminus of the structural gene and ending with a 32-bp restriction site insert (RSI) containing the five restriction sites *Avr*II, *Nde*I, *Sac*I, *Pst*I, and *Nru*I. For deletion of *rluB*, 0.15 pg of chromosomal DNA prepared as described (Wizard Genomic DNA Purification kit, Promega) were amplified with 15 pmol of the upstream outer primer, 5′-*GCTCGCggatcc*GTTATCTCACTGGCATGGGG-3′ (italics: nongene sequence; lower case: *Bam*HI site; normal upper case: sequence of residues −599 to −580 where the A of the initiating AUG is +1) and 15 pmol of the upstream inner primer, 5′-TCGCGACTGCAGGAGCTCCATATGCCTAGGTTCACTTTCTGTAGCTTTTCGCTC-3′ (underlined: RSI; normal: complement of residues +24 to +3). The second PCR product contained the same RSI followed by part of the C-terminal region of the structural gene and continuing for ~400 bp downstream. Fifteen picomoles of the downstream inner primer, 5′-AACCTAGGCATATGGAGCTCCTGCAGTCGCGAGTTCGTTACGGTGATATCCC-3′ (underlined: RSI; normal: sequence of residues +637 to +656) and 15 pmol of the downstream outer primer 5′-*CGGCAC*ggatcc*C*TAATGGCGAACGCAATCTG-3′ (italics: nongene sequence; lower case: *Bam*HI site; normal upper case: complementary to residues +1298 to +1279) were used with 0.15 pg of DNA. For the third PCR, 0.05 pmol each of the two agarose gel-purified PCR products were annealed via the common RSI (95 °C, 5 min; 65 °C, 5 min; 68 °C, 2 min) followed by addition of 15 pmol of each of the outer primers and amplification. The *rluB* deletion allele so generated was flanked by *Bam*HI sites and contained the following from 5′ to 3′: 599 bp upstream of the AUG start codon, 24 bp of N-terminal *rluB*, 32 bp of RSI, 240 bp of C-terminal *rluB*, and 422 bp downstream of the TAA stop codon. After cloning the allele into the *Bam*HI site of pKO3, the wild-type gene was replaced as described (Link et al., 1997). In this construct, there is a reading frame shift where the RSI joins the C-terminal codons. This results in a 62 amino acid mutant protein that, however, contains only eight wild-type N-terminal residues.

The *rluE* deletion allele, constructed similarly, was flanked by *Bam*HI sites and contained the following from 5′ to 3′: 584 bp upstream of the AUG start codon, 88 bp of N-terminal *rluE*, 32 bp of RSI, 17 bp of C-terminal *rluE*, and 664 bp downstream of the TAA stop codon. Sequences of the primers used were as follows: upstream outer, 5′-*CGCGACT ggatcc*GGAGTGCCCTGATAGTAACG-3′; upstream inner, 5′-TCGCGACTGCAGGAGCTCCATATGCCTAGGTTAGAACGTTGCGAGCTGAATC-3′; downstream inner, 5′-AACCTAGGCATATGGAGCTCCTGCAGTCGCGAGAGAAGTGACAGATTAAGGATGC-3′; downstream outer, 5′-*CGCCTGggatcc*TCCTCACCGTCGTCTTCTTC-3′. This construct also has a reading frame shift where the RSI joins the C-terminal codons, resulting in a 50 amino acid mutant protein that contains 29 wild-type N-terminal residues.

The *rluF* deletion allele was constructed in the same way and consisted of 816 bp upstream of the AUG start codon, 24 bp of N-terminal *rluF*, 32 bp of RSI, 138 bp of C-terminal *rluF*, and 587 bp downstream of the TAA stop codon. Sequences of the primers used were as follows: upstream outer, 5′-*CACCACACT*gcggccgcTGACCTTTACTCACTGTGATCCAG-3′ (italics: nongene sequence; lower case: *Not*I site; normal upper case: sequence of residues −816 to −792); upstream inner, 5′-TCGCGACTGCAGGAGCTCCATATGCCTAGGTTACGGACTGATGACTCGGGC-3′ (underlined: RSI; normal: complement of residues +24 to +6); downstream inner, 5′-AACCTAGGCATATGGAGCTCCTGCAGTCGCGACGAAGG

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CCAAACCGAAAACAGC-3′ (underlined: RSI; normal: sequence of residues +740 to +761); downstream outer, 5′-*GTGGTGAGA*gcggcc**gc**CTGTACTCTGTTAACCAGCG-3′. (Italics: non-gene sequence; lower case: *Not*I site; normal upper case: complement of residues +1457 to +1438; bold lower case: part of *Not*I recognition sequence and complement of residues +1459 to +1458.) This construct should result in synthesis of a 33 amino acid mutant protein, which, however, contains only eight wild-type N-terminal residues. The double deletion of *rluB* and *rluF* was constructed by transformation of this pKO3::Δ*rluF* construct into the MG1655::Δ*rluB* strain described above and following the replacement procedure of Link et al. (1997).

Deletion of *truC* proceeded similarly. There was 707 bp upstream of the AUG start codon, 30 bp of N-terminal *truC*, 32 bp of RSI, 95 bp of C-terminal *truC*, and 647 bp downstream of the TAA stop codon. Sequences of the primers used were as follows: upstream outer, 5′-*CCGCC*ggatccAG GATGATGACACTTCTGCAG-3′(italics: nongene sequence; lower case: *Bam*HI site; normal upper case: sequence of residues −707 to −687); upstream inner, 5′-TCGCGACTG CAGGAGCTCCATATGCCTAGGTTCCATTCATCCTGATAG AGTATTTC-3′ (underlined: RSI; normal: complement of residues +30 to +6); downstream inner, 5′-AACCTAGGCA TATGGAGCTCCTGCAGTCGCGACGTTATCACAATTTGGC TGGC-3′ (underlined: RSI; normal: sequence of residues +689 to +709); downstream outer, 5′-*GGCGCG*ggatccGAA AACAAGAACAAGAAAGGAAGGG-3′ (italics: nongene sequence; lower case: *Bam*HI site; normal upper case: complement of residues +1430 to +1406). This construct should yield a 32 amino acid mutant protein containing 10 wild-type N-terminal residues. All of the above deletions were confirmed by PCR and subsequent DNA sequencing.

Plasmids

Wild-type and mutant versions of the four genes were constructed in plasmid pTrc99A. Wild-type *rluB, rluE, rluF,* and *truC* were PCR amplified from MG1655 genomic DNA and cloned into the *Nco*I and *Hin*dIII sites of the pTrc99A expression vector. For *rluB*, the N-terminal primer (5′-CAGCTA*GAAGAC*TTCATGAGCGAAAAGCTACAGAAAG-3′) incorporated a *Bbs*I site (italics) 3 nt preceding the AUG start codon (underlined) and the C-terminal primer (5′-GTCAGC*AAGCTT*CGTCCGGCATACTTTGATTAAC-3′) incorporated a *Hin*dIII site (italics) 17 nt following the complement of the TAA stop codon (underlined). For *rluE*, the N-terminal primer (5′-CAGCTA*GAAGAC*ATCATGCGGCAAT TCATAATCTCTG-3′) incorporated a *Bbs*I site (italics) 3 nt preceding the AUG start codon (underlined) and the C-terminal primer (5′-GTCTGC*AAGCTT*CTTAATCTGTCACTTCTCG CC-3′) incorporated a *Hin*dIII site (italics) 1 nt following the complement of the TAA stop codon (underlined). For *rluF*, the N-terminal primer (5′-GCACTAAA*CCATGG*GAATGCTGCC GGACTCATCAGTC-3′) incorporated a *Nco*I site (italics) 2 nt preceding the AUG start codon (underlined) so as to introduce an upstream AUG start codon and GGA codon for glycine. The C-terminal primer (5′-CTAG*TCTAGA*CTCGAGGG CCGTTTTCATCAGTCTGAGG-3′) incorporated a *Xba*I site (italics) 34 nt following the complement of the TGA stop codon. For *truC*, the N-terminal primer (5′-GCACTAAA*CCATGG* GAATGCTGCCGGACTCATCAGTC-3′) incorporated a *Nco*I site (italics) 2 nt preceding the AUG start codon (underlined), introducing a prior AUG start codon and GGA codon for glycine. The C-terminal primer (5′-CTAAA*TCTAGA*CTCGAGC CCGTACATGGTGCCGAC-3′) incorporated a *Xba*I site (italics) 62 nt following the complement of the TAA stop codon.

Aspartate mutants of *rluB* and *rluE* were constructed by the megaprimer method (Colosimo et al., 1999). Briefly, a portion of each gene containing the desired mutations was created by PCR amplification from either pTrc99A::*rluB* or pTrc99A::*rluE* using the corresponding N-terminal primer (above) and a mutagenic primer in the reverse orientation. This product was diluted 50-fold and used in a second PCR amplification adding both corresponding N- and C-terminal primers and no additional template. The resulting PCR products were cloned into the *Nco*I and *Hin*dIII sites of the pTrc99A expression vector. For *rluB* D110T and D110N, the mutagenic primers were 5′-CAGACCACAGGT*GTTAAC*GGTCAA GCGACCCACG-3′ and 5′-GGTATT*AACGTT*CAAGCGACC CAC-3′, respectively. For *rluE* D79T and D79N, the mutagenic primers were 5′-CCCTTCGCTAT*CCCGGGT*AAGGCGACC TG-3′ and 5′-CGCTA*TCGCGA*TTAAGGCGACCTG-3′, respectively. Each mutagenic primer incorporated base changes (underlined in primer sequence) necessary to mutate the desired aspartate residue and, at the same time, incorporated amino acid-silent base changes to introduce a *Hpa*I (D110T) or *Psp*1406I (D110N) restriction site (*rluB*) or a *Sma*I (D79T) or *Nru*I (D79N) restriction site (*rluE*), italicized in the primer sequence, for ease in screening clones. Aspartate mutants of *rluF* and *truC* were constructed using the Quick-change XL site-directed mutagenesis kit (Stratagene, catalog #200516) according to its instruction manual. For *rluF* D107T and D107N, the mutagenic primers were 5′-CGCGTGTTCCCGAT*CGGCCG*CCTGACTAAAGACCTCCC AGGGGC-3′ and 5′-CGCGTGTTCCCGAT*CGGCCG*CCTG AATAAAGACCTCCCAGGGGC-3′, respectively. For *truC* D54T, the mutagenic primer was 5′-CTGCTCATCGTCTG ACT*CGACCG*ACTTCTGGTGTGTTGTTGATGG-3′. Each mutagenic primer incorporated base changes (underlined in the primer sequence) necessary to mutate the desired aspartate residue and, at the same time, incorporated amino acid-silent base changes to introduce a *Eag*I restriction site (*rluF*) or a *Bsi*EI restriction site (*truC*), italicized in the primer sequence, for ease in screening clones. All of the mutant rescue plasmids were verified by DNA sequencing.

RNA isolation

Total cellular RNA was isolated from 16 mL of cells that had been induced (at A_{600} of 0.5–0.7) for 1 h with 1 mM IPTG. Cells were pelleted by centrifugation, the supernatant decanted, and the pellet frozen on dry ice/ethanol. The pellet was washed in 1 mL of 50 mM Tris-acetate, pH 5.5, and 1 mM $MgCl_2$, centrifuged, and the pellet resuspended in 0.3 mL of lysis buffer (200 mM Tris-acetate, pH 5.5, 10 mM EDTA, 1% SDS). After addition of an equal volume of 50% phenol (pH 7.5–7.8, Gibco BRL):50% chloroform, the sample was vortexed for 5 min and then incubated at 65 °C for 5 min. The sample was then transferred to a 2-mL phase lock gel tube (Eppendorf), and centrifuged at 14,000 rpm for 5 min to separate the phases. The sample was extracted again in the same tube by addition of another equal volume of 50% phenol:50% chloroform to the aqueous layer, mixing by multiple

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inversions, centrifugation, addition of an equal volume of 24:1 chloroform:isoamyl alcohol, and centrifugation. The RNA in the final aqueous layer was ethanol precipitated using pellet paint (Novagen), resuspended in 0.1 to 0.2 mL of RNase-free water, quantitated by A_{260}, and stored at −20 °C. Unfractionated tRNA was isolated as previously described (Deutscher & Hilderman, 1974) from cells grown to an A_{600} of 0.8–1.0 in LB except that the tRNA was fractionated using isopropanol concentrations of 40 and 65%.

Ψ sequencing

Ribosomal RNA Ψ sequencing was performed as described previously (Bakin & Ofengand, 1993, 1998) with the following modifications. The amount of RNA used for CMC modification was between 15 and 25 μg. RNA was incubated with CMC at 37 °C for 20 min and with Na_2CO_3 for 4 h at 37 °C. All precipitations were done with addition of pellet paint (Novagen) at room temperature; in addition, effective precipitation of RNA after CMC treatment required 0.9 M (final) sodium acetate and 3.5 vol of ethanol. The mixture for labeling cDNA contained 5 μCi [α-^{32}P]dATP and 4 U of AMV reverse transcriptase (Promega). The final cDNA pellet was resuspended in 6 μL of DNA loading buffer. The primers used for reverse transcription are listed in the figure legends. tRNA Ψ sequencing was done after adding a tail of polyA to the 3′ end of tRNA in order to facilitate primer extension, as the expected Ψ65 was otherwise too close to the A76 natural terminus. The tRNA, obtained as described above, was incubated at 30 °C for 20 min in a reaction mixture composed of 20 mM Tris-HCl, pH 7.0, 50 mM KCl, 0.7 mM $MnCl_2$, 0.2 mM EDTA, 0.1 mg/mL acetylated bovine serum albumin, 10% glycerol, 100 pmol of ATP, and 1 μL [500 U] of polyA polymerase (USB) in a final volume of 10 μL. After incubation, the mixture was extracted twice with phenol/chloroform (1:1) and twice with chloroform. The tRNA was precipitated from 1 M ammonium acetate, pH 6, with 3 vol of ethanol, washed with 80% ethanol, vacuum dried, and resuspended in water. 5′-labeling of the primer and tRNA sequencing were performed as described previously (Gutgsell et al., 2000). For $tRNA^{Asp}$, the primer was 5′-TTTTTTGGCGGAAC-3′; for $tRNA^{Ile1}$, the primer was 5′-TTTTTTGGTAGGCC-3′.

Other methods and materials

Transformants of wild-type and deletion strains with pTrc99A with and without inserts were selected on LB (1% Bacto Tryptone, 0.5% yeast extract, 1% NaCl) plates containing 0.1 mg/mL carbenicillin. All subsequent growth media for the transformants also contained 0.1 mg/mL carbenicillin to retain the plasmid in the carbenicillin-sensitive host cells. Primers were obtained from Gibco-BRL and used without further purification. All other materials were obtained, and SDS polyacrylamide gel electrophoresis performed, as described previously (Raychaudhuri et al., 1998).

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