Functional Effect of Deletion and Mutation of the *Escherichia coli* Ribosomal RNA and tRNA Pseudouridine Synthase RluA*

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The *Escherichia coli* gene *rluA*, coding for the pseudouridine synthase Rlu that forms 23 S rRNA pseudouridine 746 and tRNA pseudouridine 32, was deleted in strains MG1655 and BL21/DE3. The *rluA* deletion mutant failed to form either 23 S RNA pseudouridine 746 or tRNA pseudouridine 32. Replacement of *rluA* in trans on a rescue plasmid restored both pseudouridines. Therefore, RluA is the sole protein responsible for the *in vivo* formation of 23 S RNA pseudouridine 746 and tRNA pseudouridine 32. Plasmid rescue of both *rluA*- strains using an *rluA* gene carrying asparagine or threonine replacements for the highly conserved aspartate 64 demonstrated that neither mutant could form 23 S RNA pseudouridine 746 or tRNA pseudouridine 32 in *in vivo*, showing that this conserved aspartate is essential for enzyme-catalyzed formation of both pseudouridines. *In vitro* assays using overexpressed wild-type and mutant synthases confirmed that only the wild-type protein was active despite the overexpression of wild-type and mutant synthases in approximately equal amounts. There was no difference in exponential growth rate between wild-type and MG1655(*rluA*) either in rich or minimal medium at 24, 37, or 42 °C, but when both strains were grown together, a strong selection against the deletion strain was observed.

Ribosomal RNA, considered to be the functional heart of the ribosome (1), contains a variety of modified nucleosides of unknown function (2). The most common single modification is the conversion of uridine to pseudouridine (Ψ), the 5-ribosyl isomer of uridine (3). Ψ is formed by isomerization of specific uridines after the RNA chain is formed. The mechanism of the reaction, which involves breaking of the N7-glycosyl bond, rotation of the uracil ring, and formation of a C7-glycosyl bond is unknown but is thought to involve an active site carbonyl group of an aspartate residue (4). Ψ is found in the rRNA of all organisms so far examined (5), and in *Escherichia coli*, which has one Ψ in the 16 S RNA (6) and nine in the 23 S RNA (7, 8), it is the most prevalent of the modified nucleosides. The single Ψ in the 16 S RNA is found adjacent to the “530” loop, whose sequence has been almost completely conserved in all organisms and is known to be involved in the fidelity of codon recognition (reviewed in Refs. 9 and 10). In 23 S RNA, the nine Ψ residues are distributed among three distinct areas, which, despite their separation in two-dimensional secondary structure representations, are at or near the peptidyl transferase center when in the ribosome (11). However, despite the congruence of the Ψ residues with the two functional centers of the ribosome, namely decoding and peptidyl bond formation, there is so far no known role for Ψ in the process of protein synthesis.

To approach this problem, we have initiated a program to identify the genes for the specific synthases that make the 10 Ψ in *E. coli* rRNA on the assumption, subsequently shown to be correct, that distinct synthases are used to form Ψ at the different sites in the rRNA molecule. Once identified, gene inactivation will result in the loss of a specific synthase and should therefore cause the loss of specific Ψ residues for which the effect on cell physiology can then be assessed. Thus far, three Ψ synthase genes have been inactivated in this manner. One, *rluC*, codes for a synthase solely responsible for formation of Ψ residues 955, 2504, and 2580 in 23 S RNA (12) and a second, *rluD*, codes for the synthase that makes 23 S RNA Ψ1911, 1915, and 1917 (13). The third, *rsuA*, codes for the synthase that forms 16 S RNA Ψ516 (14). Inactivation of *rluC* and *rsuA* and the consequent loss of their respective Ψ had no physiological effect. However, disruption of *rluD* with the loss of its three Ψ severely inhibited cell growth.

Another synthase, RluA, was shown to form only Ψ746 when *in vitro* transcripts of 23 S RNA were the substrate. The synthase also specifically catalyzed the formation of Ψ32 in *E. coli* tRNA^Phe^ (15). The ability to be highly specific for a single site in more than one class of RNA, a property termed “dual specificity” (15), has since been reported for another Ψ synthase (16) as well as for a ribose methylating enzyme, although in the latter case the dual specificity resides in the guide RNA (17). A question left open by the work on RluA was whether it is the only synthase in *E. coli* capable of forming rRNA Ψ746 and Ψ32 and what the effect of its absence would be on the cell. This issue has now been addressed by deleting *rluA* and comparing the growth rate with wild type both separately and in a competition experiment. In addition, by mutating aspartate 64, which was predicted to be an essential residue by virtue of its location in a conserved sequence, HRLD (4), we have shown that RluA, as well as RsuA (14), RluD, and TruA (4), requires this residue for function.

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1 The abbreviations used are: Ψ, pseudouridine; PCR, polymerase chain reaction; M9+, medium, M9 (Ref. 22) plus 0.4% glucose and 1 mM MgSO4; CMC, N-cyclohexyl-N’-β-(4-methylmorpholinium)methylcarboxidimide.

2 S. Raychaudhuri and J. Ofengand, unpublished results.
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EXPERIMENTAL PROCEDURES

rluA and miaoA rluA Strains-The rluA gene was deleted by the method of Hamilton et al. (18). The insert, cloned into the XhoI and KpnI sites of pM4K705, was prepared by PCR as described by Nelson and colleagues (Fig. 2 in Ref. 19). It contained 818 bases 5’ to the AUG start and 785 bases 3’ to the UAA termination codon. Sixteen bases of the N-terminal portion of the gene and 52 bases of the C terminus were retained with the remainder being replaced by the kanamycin resistance gene, obtained by PCR amplification from pUC4K (Amersham Pharmacia Biotech, catalog no. 27-4958-01). The host strain for pM4K705 was the leucine auxotroph MC1061, as described by Hamilton et al. (18). The deleted rluA gene was moved into strains MG1655 (Ref. 20; the gift of Dr. Kenneth Rudd, this department) and BL21/DE3 (Novagen, Inc.) by bacteriophage P1 transduction (21). Selection was done on either rich (LB, Ref. 22) or minimal (M9) medium containing 0.1 mg/ml bovine serum albumin. The mixture was denatured at 95 °C for 60 s, and then subjected to the same amplification program again. The amplified product was purified by gel electrophoresis, digested with NcoI and XhoI and cloned into the MG1655 and MG1655(miaA) strains with selection on LB containing 34 mg/ml chloramphenicol in addition to kanamycin.

Wild-type Rescue Plasmids—The preparation of wild-type rescue plasmid pET15b/rluA has been described previously (15). Wild-type rescue plasmid pTrc99A/rluA was constructed by insertion into the NcoI and HindIII sites of pTrc99A (Amersham Pharmacia Biotech, catalog no. 27507-01) of a segment of DNA that was PCR-amplified from pET15b/rluA and consisting of the rluA gene starting from the initiator AUG and ending at the terminator UAA. The N-terminal primer had an NcoI site adjacent to the initiating AUG, whereas in the reverse (orientation) C-terminal primer incorporated a HindIII site after the terminator UAA.

Mutant Rescue Plasmids—These plasmids were prepared by the megaprimer PCR mutagenesis procedure (23). PCR reactions were performed using the pTrc99A/rluA rescue plasmid as template and three oligonucleotide primers, two outer primers, which were upstream and downstream of the mutagenesis site, and one mutagenic primer. The upstream primer was annealed to the restriction end of NcoI and HindIII, respectively, so that the product could be ligated directly into pTrc99A. Mutagenesis was carried out in three steps. The initial PCR reaction was performed with either mutagenic primer 5’-CATCG-TCTGACTATGCGACAC-3’ for the D64T mutation or 5’-CATCGTCT-GAAATGGTACCC-3’ for the D64N mutation (mutation sites shown in bold) and with the downstream primer 5’-GGGAAGCTTTTAAAAATTCGCGTGGCCG-3’ having a HindIII site (underlined). A 100-μl reaction contained 50 ng of template plasmid DNA, 15 pmol each of the mutagenic primer and downstream primer, 3 units of Pfu DNA polymerase (Promega), 0.2 mM dNTPs, 20 mM Tris, pH 8.75, 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgCl2, 0.1% Triton X-100, and 0.1 mg/ml bovine serum albumin. The mixture was denatured at 95 °C for 60 s, and then 10 cycles of amplification (55 °C, 47 °C, 60 °C, 72 °C, 70 °C) were performed, followed by a 5-min extension at 72 °C. Fifty pmol of the upstream primer was added (5’-GGGCGATCGGATGGGATAGGAAA-ACTAC-3’, NcoI site underlined), and the reaction mixture was subjected to the same amplification program. Finally, 50 pmol of downstream primer was added, and the sample was subjected to the same amplification program again. The amplified product was purified by gel electrophoresis, digested with NcoI and HindIII, and ligated with similarly digested and purified pTrc99A for 16 h at 16 °C. The ligation mixture was transformed into Novablue cells (Novagen, Inc.) by standard methods yielding 4 positive clones of 5 tested for D64T and 3 positive of 4 tested for D64N. DNA sequencing of the isolated plasmids verified that the expected mutation had been produced at the desired site. Transfer of the mutant rluA genes into pET15b was done by PCR amplification of the mutant rluA gene from pTrc99A. The 3′ terminal primer extended from 9 to 18, where the A of the initiating AUG of rluA is +1 with changes at −2 to −5, to create an XhoI site adjacent to the initiating AUG. The C-terminal primer, in the reverse orientation, extended from +643 to +669, where the last sense nucleotide is 657, and contained mismatches at +661 to +666 to create a BamHI site. The amplified product was digested, with XhoI and BamHI, and subcloned into identical sites of pET15b/rluA, yielding pET15b/rluA-D64T and pET15b/rluA-D64N. They were transferred into LB21/DE3(rluA) cells by standard methods. Overexpression and affinity purification were carried out as described previously (13). pET15b/rluA was processed in the same way.

rRNA and tRNA Isolation and Sequencing—5′[3H]Uridine-labeled transcripts of full-length 23 S RNA (specific activity 168 dpm/pmol uridine residues) were prepared as described previously (15). The 5′-[3H]Uridine-labeled transcript of tRNAAsp (199 dpm/pmol), tRNAHis (1269 dpm/pmol), and tRNALeu (454 dpm/pmol) were prepared as described for tRNAAsp (15) but using pTFTm-EC5 (the gift of Yu-Ming Hou, Thomas Jefferson University, Philadelphia, PA) as template for tRNAAsp and pUC19/tRNALeu(uaa) for tRNALeu, Ribosomal RNA for the sequence was prepared according to King and Schlessinger (24) with omission of the LiCl precipitation step. tRNA sequencing of RNA was done on either rich (LB, Ref. 22) or minimal (M9) medium containing 0.1 mg/ml kanamycin. The rluA and miaA genes were isolated from cells grown to an A500 of 1.0 in LB medium. tRNA sequencing of tRNAAsp was done exactly as for rRNA using a primer complementary to residues 61–76.

RESULTS

Identification of RluA as the Only Synthase for Formation of 7-methylguanine in E. coli RNA—Overexpressed and purified RluA converts U746 in E. coli 23 S RNA transcripts to U746 and U-32 in tRNAAsp transcripts to 32 (15). In vitro, the enzyme is highly specific for these two sites. Comparison of the sequence surrounding the sites of the two U residues selected for conversion to 7-methylguanine revealed that both possessed the same sequence immediately 3′ to the U in question, thus providing a rationale for the dual specificity exhibited by the enzyme (15). These experiments did not, however, show whether additional enzymes existed in the cell that were also capable of 7-methylguanine formation at these sites, nor did they show the effect of deletion of these enzymes existed in the cell that were also capable of 7-methylguanine formation at these sites, nor did they show the effect of deletion of these. Therefore, the gene was deleted by insertion of the kanamycin resistance gene (18) in strain MC1061. The deletion was confirmed by PCR amplification from total N and C termini of the rluA gene in the chromosomal DNA of the deletion mutant. The wild-type transcriptional start site was, as determined 670-base-pair band, whereas the mutant having a kan insertion was 1.4 kilobase pair bands, whereas no band was obtained from the wild type.

To assess the physiological effects of this gene deletion un-
complicated by the other mutant genetic loci present in MC1061 (18), the deletion was transferred by bacteriophage P1 transduction into MG1655 in which the sequenced genome (20) provided a well-defined background. Transductants were selected by resistance to kanamycin. PCR amplification confirmed the presence of the kanamycin insert, and $\Psi$ sequencing analysis of the ribosomal RNA from the mutant strain showed unequivocally that $\Psi 746$ was absent (data not shown).

To prove that the loss of $\Psi$ attendant on the deletion of $\textit{rluA}$ was a direct consequence of the deletion and not because of some downstream polarity or other indirect effect, the gene was replaced in trans by transformation of the deletion strain with a rescue plasmid that contained only the $\textit{rluA}$ gene inserted into pTrc99A. Wild-type and MG1655($\textit{rluA}$) were transformed with both the rescue plasmid and the control vector pTrc99A and selected on carbenicillin plates. Ribosomal RNA was isolated and sequenced for the presence of $\Psi$ (Fig. 1). Comparing the $\textit{rluA}^-$ lanes with the $\textit{rluA}^+$ lanes, it is clear that the stop at residue 746 in the + CMC lane of the $\textit{rluA}^+$ set is absent in the + CMC lane of the $\textit{rluA}^-$ pair. Recall that in this method of sequencing, reverse transcriptase halts one residue 3' to the CMC-$\Psi$ (7, 25). However, when the rescue plasmid was introduced into the $\textit{rluA}^-$ strain, $\Psi 746$ was again found. The stop seen in all lanes results from m$^1$G745, because all of the RNAs were isolated from cells. We conclude that the loss of $\Psi 746$ is the strain carrying the wild-type rescue plasmid. Neither plasmid carrying a mutant $\textit{rluA}$ was any more effective than the vector alone. Thus, in vivo, the single mutation D64T or D64N is sufficient to block synthesis of an active $\Psi 746$ synthase.

One might conclude from this experiment that Asp-64 is an amino acid that actively participates in catalysis, but it could also be that its role is in the maintenance of the correct conformation of the enzyme. In the latter event, the replacement of Asp-64 by another amino acid might make the protein susceptible to protease degradation.

To address this question, we turned to the BL21/DE3 strain and pET15b to obtain stable overexpression of the mutant proteins. The $\textit{rluA}^-$ gene was transferred into BL21/DE3 by P1 transduction from MC1061 with selection by kanamycin resistance. PCR amplification confirmed the presence of the kanamycin insert, and $\Psi$ sequencing analysis of the ribosomal RNA from the mutant strain showed the absence of $\Psi 746$ (data not shown, but see Fig. 4 for an equivalent result). Both the wild-type and mutant $\textit{rluA}$ constructs were subcloned into pET15b. DNA sequencing analysis (data not shown) confirmed that the desired mutants had been produced in pET15b. The BL21/ DE3($\textit{rluA}^-$) cells were then transformed with vector alone or with the $\textit{rluA}$ constructs in pET15b. Transformants were selected on carbenicillin plates. The BL21/DE3 cells carrying either the vector or the various $\textit{rluA}$ constructs were then induced. After a 3-h induction with isopropyl-1-thio-$\beta$-d-galactopyranoside, samples from each culture were taken out for protein analysis on SDS-polyacrylamide gels as well as for ribosomal RNA isolation and $\Psi$ sequencing analysis. Fig. 3 shows that a strongly overexpressed protein band at about 27 kDa, the expected size, was found in the cells carrying both wild-type and mutant $\textit{rluA}$ constructs, whereas there was no such overexpressed protein band in the cells carrying the vector only. Furthermore, induction was required to produce the band. The intensity of the 27-kDa band appeared the same in both wild-type and mutant constructs. $\Psi$ sequencing analysis of the rRNA showed that, as with the results obtained in Fig. 2, the mutant rescue plasmids were unable to form $\Psi 746$ (Fig. 4).

We conclude that the two mutant $\textit{rluA}$ constructs produced stable proteins that had, nevertheless, lost the capability to isomerize U746 to $\Psi$ as a result of the replacement of Asp-64 by Thr-64 or Asn-64.

Affinity purification of the overexpressed proteins shown in Fig. 3 and assay of $\textit{in vitro}$ activity using 23 S $[^3]$HrRNA transcripts as substrate gave the same result, namely that whereas unit stoichiometry of $\Psi$ formation could be obtained for the wild-type construct, as reported previously (15), both the D64T and D64N mutants were totally inactive (Fig. 5).

Identification of a Synthase Amino Acid Essential for $\Psi 746$ Formation—Recently, it has been shown that the replacement of Asp-60 in a conserved (G/H)(D/E/A)(L/A)(D) motif (lowercase identifies a rare event), by Ala, Asn, Glu, Lys, or Ser in the pseudouridine synthase $\textit{TruA}$ resulted in the loss of catalytic activity while retaining binding ability (4). There is an equivalent residue, Asp-64, in a similarly conserved motif, HRLD, in the $\textit{RluA}$ synthase, and it is the only Asp residue in such a motif in the molecule. To test the possibility that Asp-64 could be an essential residue of this enzyme, we mutated it to Thr and Asn. This was done by megaprimer mutagenesis (23). The two mutated $\textit{rluA}$ were cloned in pTrc99A and transformed into MG1655($\textit{rluA}^-$) cells to assess the function of these mutant enzymes in vivo. The wild-type rescue plasmid served as a control. Ribosomal RNA was isolated and sequenced for the presence of $\Psi$ (Fig. 2). It is clear that the only strain able to make $\Psi 746$ is the strain carrying the wild-type rescue plasmid.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Pseudouridine sequencing analysis of 23 S RNA from the wild-type and $\textit{rluA}$-deleted strains of \textit{E. coli} and \textit{plasmid}-containing derivatives. Preparation of the wild-type (\textit{rluA}+) and $\textit{rluA}$-disrupted (\textit{rluA}−) MG1655 strains and the plasmids pTrc99A (+) and pTrc99A carrying the \textit{rluA} gene (+\textit{rluA}), transformation of the \textit{rluA}− strain with the plasmids, RNA preparation, and $\Psi$ sequencing were done as described under “Experimental Procedures.” The single naturally occurring $\Psi 746$ in the region monitored in this figure is indicated by the arrow. RNA for A, C, U, and G sequencing lanes was a transcript of 23 S RNA (27).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Pseudouridine sequencing analysis of 23 S RNA from MG1655(\textit{rluA}+) containing various rescue plasmids. Preparation of the mutant constructs, transformation of the \textit{rluA}− strain with the plasmids, RNA preparation, and $\Psi$ sequencing were done as described under “Experimental Procedures.” Plasmids were pTrc99A (+), pTrc99A/\textit{rluA}-D64T (+/D64T), pTrc99A/\textit{rluA}-D64N (+/D64N), and pTrc99A/\textit{rluA} (+/D64D). $\Psi 746$ is indicated by the arrow. RNA for the A, C, U, and G sequencing lanes was a transcript of 23 S RNA (27).}
\end{figure}
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**FIG. 3.** Overexpression of the wild type and mutant rluA gene products in BL21/DE3(rluA-) cells. Cells grown at 37 °C were transformed with the various pET15b/rluA constructs, harvested either before (-) or after (+) induction with 1 mM isopropyl-1-thio-β-D-galacto-pyranoside, lysed by boiling in SDS, and analyzed on SDS-polyacrylamide gels. Lanes: A, pET15b; B, pET15b/rluA-D64T; C, pET15b/rluA-D64N; D, pET15b/rluA-D64D; S, molecular mass standards of the indicated sizes.

![Image 65x423 to 281x535](image)

**FIG. 4.** Pseudouridine sequencing analysis of 23 S RNA from strain BL21/DE3(rluA-) containing mutated rescue plasmids. pET15b (+), pET15b/rluA-D64T (+/D64T), pET15b/rluA-D64N (+/ D64N), and pET15b/rluA (+/D64D). Constructs were prepared, the rluA- strain was transformed with the plasmids, RNA was isolated, and sequencing was done as described under “Experimental Procedures.” ▶ indicates the position of each modified nucleoside.

![Image 308x483 to 554x729](image)

**FIG. 5.** Pseudouridine formation in 23 S RNA transcripts by wild type and mutant RluA synthases. ◦, H release from a 5-[^3]H]uridine-labeled 23 S RNA in vitro transcript was assayed with 0.15 μg of affinity-purified recombinant synthase at 10 mM Mg2⁺ and 100 mM substrate. ◦, Asp-64 (wild type); ◦, Thr-64; ▲, Asn-64.

![Image 335x198 to 527x425](image)

**FIG. 6.** Kinetics of pseudouridine formation in tRNA transcripts by RluA. 5[^3]H]uridine-labeled in vitro transcripts of tRNA⁸ψ (●), tRNA⁸ψ (○), and tRNA⁸ψ (▲) were reacted with recombinant RluA as described in Ref. 15 at 400 nM RNA and 1–5 mM EDTA in the absence of Mg2⁺. Assay was by the H release procedure.

![Image 93x618 to 253x729](image)

**FIG. 7.** Note that this method obviated the need to purify tRNA⁸ψ from the total RNA preparation. In the mia-rluA- strain, CMC-dependent stops were found at positions 33, 40, and 56 corresponding to the ▶, ▼, and ▲ residues known to be in this tRNA. No stop corresponding to ▶ was found in the mia-rluA- strain transformed with the pTrc99A vector only, although both ▼ and ▲ were present. However, when plasmid carrying the rluA gene was used, ▶ reappeared. Clearly, RluA is the only protein able to form ▶ in *E. coli*. Furthermore, when the two rluA genes mutant at

**RLuA Is the Only Protein Capable of ▶ Formation in tRNA-Ψ32 is found in four tRNAs of *E. coli*, tRNA⁸ψ, tRNA⁸ψ, tRNA⁸ψ(UUA), and tRNA⁸ψ(CAA) (28, 29). We previously showed that RluA formed ▶ on a transcript of tRNA⁸ψ in an *in vitro* reaction (15), a result that led to the concept of dual specificity for this enzyme. All five of these RNAs, the 23 S RNA and the four tRNAs, share a common sequence surrounding the ▶ residue, namely (A/G)ΨUN(A/G/CAA). Therefore, it seemed reasonable that these other tRNAs could also serve as a substrate for RluA. To test this hypothesis, tRNA⁸ψ and tRNA⁸ψ transcripts were assayed for their ability to react with RluA (Fig. 6). Both transcripts were active. The rate and yield with tRNA⁸ψ was virtually identical to that with tRNA⁸ψ, whereas tRNA⁸ψ was somewhat less reactive for unknown reasons.

To determine whether RluA is the only protein in *E. coli* capable of ▶ formation, ▶ from the rluA- strain was analyzed. However, before the reverse transcription assay could be used, obstacles created by the presence of other modified nucleosides in the tRNAs that block reverse transcriptase had to be overcome. ms⁸A37, present in all three tRNAs, is a strong inhibitor of reverse transcriptase. Moreover, tRNA⁸ψ also has aps⁹U47, another strong blocker, and tRNA⁸ψ has cmms³Um34 (29) only two residues away from ▶. To replace ms⁸A37 by A37, a deletion of miaA, the gene responsible for the enzyme that forms A37 (30, 31), was transduced into MG1655(rluA-) by bacteriophage P1. To avoid the other modified nucleosides, tRNA⁸ψ was chosen for analysis. ▶ sequencing of tRNA⁸ψ by the reverse transcription procedure is shown in Fig. 7. Note that this method obviated the need to purify tRNA⁸ψ from the total RNA preparation. In the mia-rluA- strain, CMC-dependent stops were found at positions 33, 40, and 56 corresponding to the ▶, ▼, and ▲ residues known to be in this tRNA. No stop corresponding to ▶ was found in the mia-rluA- strain transformed with the pTrc99A vector only, although both ▼ and ▲ were present. However, when plasmid carrying the rluA gene was used, ▶ reappeared. Clearly, RluA is the only protein able to form ▶ in *E. coli*. Furthermore, when the two rluA genes mutant at
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Aasp-64 were used, no \(\Psi32\) was formed, showing that both \(\Psi746\) and \(\Psi32\) formation requires the same essential Asp residue.

Effect of the rluA Deletion on Growth—Cells with ribosomes lacking 23 S RNA \(\Psi746\) and tRNA \(\Psi32\) were viable and appeared to grow normally. To better detect small metabolic defects, growth rates were measured at different temperatures in both rich and minimal glucose media. The growth experiments were done in the MG1655 genetic background after transduction of the \(\text{rlu}^-\) gene from strain MC1061. Both wild type and MG1655(\(\text{rlu}\)) were transformed with both the rescue plasmid and its control, and exponential growth rates were measured for all four strains (Table I). Although both rich and minimal media were tested over a temperature range from 24 to 42 °C, no significant difference in growth rate between the wild-type and \(\text{rlu}^-\) strain was observed.

The above experiment did not, however, test the effect of the \(\text{rlu}^-\) mutation under more natural conditions, namely in competition with non-mutant cells. Moreover, only the exponential phase of growth was examined. To test these additional conditions, the following experiment was performed. Exponential phase wild-type and \(\text{rlu}^-\) cells were mixed and grown for 24 h to stationary phase at 37 °C in LB medium with aeration. A sample was taken for analysis of viable cells, and the cultures were diluted either \(1:10^3\) or \(1:1.6 \times 10^3\) and grown for an additional 24 h. In this way, the wild-type and mutant cells compete for nutrients through the entire cycle of growth, starting from stationary phase at the start of the experiment, then by dilution into lag phase and exponential phase, and again into stationary phase. Aliquots were diluted into fresh medium, and the process was repeated for a total of five or six cycles. The results are shown in Fig. 8 together with control experiments in which \(\text{rsu}^-\) and \(\text{rlu}^-\) cells were also competed against wild-type cells. These strains, in which gene deletions also carry a kanamycin insert like the \(\text{rlu}^-\) cells, control for an effect due to the presence of the kanamycin insert.

Fig. 8A shows the relative percentage of loss of \(\text{rlu}^-\) cells as a function of the number of cycles of growth to saturation followed by dilution into fresh medium. Compared with the slow decrease in the \(\text{rsu}^-\) and \(\text{rlu}^-\) cells in mixed culture, the \(\text{rlu}^-\) cells in an equivalent mixed population decreased sharply. Thus, when the \(\text{rsu}^-\) strain had decreased to 56% of its original value, the \(\text{rlu}^-\) strain had gone down to 0.4% in one case and 0.5% in the other, which is a 124-fold decrease relative to the control \(\text{rsu}^-\) strain. Clearly \(\text{rlu}^-\) cells are at a marked disadvantage when growing in competition with wild-type cells. Moreover, there was no effect of a 1600-fold difference in the extent of dilution between cycles. If selection against \(\text{rlu}^-\) cells had occurred during the exponential phase of growth, a large effect would have been expected, because to reach the same cell density after a \(1.6 \times 10^8\) dilution requires 20.6 doublings, whereas a \(10^3\) dilution only requires 9.97 doublings. Twice as many doublings should have had twice the effect if selection had occurred in exponential phase. This effect is illustrated in Fig. 8B, where the same data are plotted versus the calculated number of cell doublings of the mixed culture. The discrepancy in the rate of decrease of the two dilutions of \(\text{rlu}^-\) cells shows that the selection against \(\text{rlu}^-\) cells does not occur during exponential phase but must take place elsewhere in the growth cycle.

**DISCUSSION**

Specificity—In a previous report, we showed that RluA in *vitro* was able to form \(\Psi746\) in transcripts of *E. coli* 23 S RNA (15) but did not form any of the other known \(\Psi\) residues. In the present work, we have demonstrated that RluA is the only gene product in *E. coli* that can carry out this reaction, because deletion of the gene caused only the loss of \(\Psi746\) and replacement of this gene on a plasmid restored it. Nevertheless, it is possible that RluA in *vivo* might share the ability with another \(\Psi\) synthase for formation of one or more of the other \(\Psi\) sites in 23 S RNA. Only synthases for two such \(\Psi\) are still possible candidates, namely \(\Psi2457\) and \(\Psi2605\), because we have shown that deletion of \(\text{rlu}^C\) results in the loss of \(\Psi\) residues 955, 2504, and 2580 (12) and that deletion of \(\text{rlu}^D\) results in the loss of \(\Psi\) residues 1911, 1915, and 1917 (13). Deletion experiments on candidate genes for these last two synthases are in progress.

RluA was also shown capable of forming \(\Psi32\) in tRNA\(^{\text{pre}}\) *in vitro* (15), and in this work the reaction was extended to tRNA\(^{\text{pre}}\) and tRNA\(^{\text{pre}}\) as well (Fig. 6). All three tRNAs are known to have \(\Psi\) at position 32 when isolated from cells (28). The question then arose whether the deletion in \(\text{rlu}^-\) also caused the loss of \(\Psi32\) from tRNA or whether a second synthase exists in *E. coli* that is able to catalyze this reaction. By sequencing tRNA\(^{\text{pre}}\) from the \(\text{rlu}^-\) strain, we showed that \(\Psi32\), but not \(\Psi39\) or \(\Psi55\), was absent. Therefore, RluA is the only protein in the cell able to carry out both reactions. Nevertheless, it is not clear whether only one of the \(\Psi\) is the desired one, and the other is a benign by-product, or whether both are desired by the cell, and one protein has been co-opted to perform both functions.

**Relationship of \(\Psi746\) to \(m^3G745\) and \(m^3U747\)**—This segment of *E. coli* 23 S RNA is notable for its concentration of three modified nucleosides, \(m^3G745\), \(\Psi746\), and \(m^3U747\) adjacent to each other in a small stem-loop structure. Because we previously showed that \(\Psi746\) could be formed *in vitro* on rRNA transcripts, it is clear that \(\Psi\) formation does not require the presence of either \(m^3G745\) or \(m^3U747\) (15). The present results, which show the existence of \(m^3G745\) in the deletion strains lacking \(\Psi746\) (Figs. 1, 2, and 4), demonstrate that \(m^3G745\) synthesis is independent of \(\Psi746\) formation. The existence of \(m^3U747\) in the deletion strains was not examined.

**Reaction Mechanism**—The minimum reaction required for the isomerization of uridine to pseudouridine involves cleavage of the uracil N1-ribosyl C1 bond, rotation of the uracil ring either 180° about the N2-C8 axis in the ring plane or 120° about an axis perpendicular to the ring plane, and formation of a uracil C2'-ribosyl C1 bond. Recently, a reaction mechanism was proposed for this type of isomerization that involves the pres-
and C

Moreover, recent studies have shown that the aspartate in the

sequence motif in RsuA, the synthase responsible for

mation in 16 S RNA, has also been shown to be essential.

In other work (14), the conserved aspartate in the same se-

quence motif in RluD is needed to make

formation because replacement of this aspartate by threonine

were not tested. Thus, the essential nature of the con-

served aspartate has now been shown for both TruA and RluA.

In other work (14), the conserved aspartate in the same se-

sequence motif in RluA, the synthase responsible for \( \Psi_{516} \)

formation in 16 S RNA, has also been shown to be essential.

Moreover, recent studies have shown that the aspartate in the

same sequence motif in RluD is needed to make \( \Psi_{1911} \), \( \Psi_{1915} \),

and \( \Psi_{1917} \) in 23 S RNA in vitro. 3

Function of \( \Psi - \) There was no difference in exponential phase
growth rates when cells lacking 23 S RNA \( \Psi_{746} \) and tRNA \( \Psi_{32} \)

were grown in separate cultures, even when both the medium

and the temperature were varied (Table I). However, when the

rluA\(^{-}\) strain was grown in competition with wild-type cells, a

marked selection against the mutant cells was observed (Fig.

8). Compared with the rsuA\(^{-}\) strain, which also carries the

same kanamycin resistance cassette, rluA\(^{-}\) cells were more

than 100 times more likely to die. Moreover, it is possible that

there is little or no effect of the presence of the kanamycin

resistance gene on survival. The slow decay observed for rsuA\(^{-}\)

cells may be the result of an intrinsic decrease in fitness be-

cause of the absence of the RsuA protein. This view is sup-

ported by the results with the rluC\(^{-}\) strain, which, while also a

much better survivor than the rluA\(^{-}\) strain, was itself less fit

than the rsuA\(^{-}\) strain. Thus, the true lack of survival of rluA\(^{-}\)
cells may be even greater than indicated by the comparison

with rsuA\(^{-}\) cells.

At what stage of growth does the discrimination occur? The

fact that a more than 1000-fold difference in dilution for each

cycle in Fig. 8 did not change the decay rate argues strongly

against the effect occurring in exponential phase. Other possi-

bilities are the approach to stationary phase, stationary phase

itself, or the lag phase before reinitiation of growth. Further

experimentation will be required to answer this question. It is

also not known whether the growth defect manifest in the

absence of RluA is because of its inability to form \( \Psi_{746} \) in 23 S

RNA and \( \Psi_{32} \) in four tRNAs or to some other unknown func-

tion. Such a situation was found for the

RUMT enzyme, which catalyzes the m\(^5\)U\(^{54} \) formation in tRNA.

Deletion and Mutation of Pseudouridine Synthase RluA

TABLE I

Growth rate of rluA deletion and rescue strains

| Strain                  | Medium  | Doubling time\(^{a}\) |
|-------------------------|---------|----------------------|
| MG1655/pTrc99A          | Rich    | 72 (3)               |
| MG1655/pTrc99A(rluA)    | Rich\(^{b}\) | 66 (3)               |
| MG1655/rluA/\( \Psi \)Trc99A | Rich\(^{c}\) | 65 (3)               |
| MG1655/rluA/\( \Psi \)Trc99A(rluA) | Rich\(^{c}\) | 66 (3)               |
| MG1655/pTrc99A          | Minimal | 230 (3)              |
| MG1655/pTrc99A(rluA)    | Minimal | 228 (3)              |
| MG1655/rluA/\( \Psi \)Trc99A | Minimal | 244 (3)              |
| MG1655/rluA/\( \Psi \)Trc99A(rluA) | Minimal | 244 (3)              |

\(^{a}\) Values in parentheses are the number of exponential phase doublings over which the doubling time was measured.

\(^{b}\) LB broth (22).

\(^{c}\) M9 (22) plus 0.4% glucose, 1 mM MgSO\(_{4}\), and 0.1 mg/ml carbenicillin.

FIG. 8. Growth competition between wild-type and mutant MG1655 strains. The competition experiment is described in the text. 20-ml cultures in LB medium were shaken at 37 °C in 250-ml flasks. Aliquots were sampled and viable cells determined as described under "Experimental Procedures." The percentage of cells that were kanamycin-resistant at each cycle was normalized by dividing the values by the initial percentage of kanamycin-resistant cells. Initial percentages were 49 (rluA\(^{-}\), ○), 39 (rluA\(^{+}\), ●), 36 (rsuA\(^{-}\), △), and 46% (rluC\(^{-}\), ▲). The values were plotted as the number of cycles of growth to saturation and dilution into fresh medium (panel A) and as the number of cell doublings calculated from the dilution factor (panel B). ○, dilution factor of 1:10\(^3\) between cycles; ●, △, and ▲, dilution factor of 1.6 \( \times \) 10\(^{3}\).
Deletion and Mutation of Pseudouridine Synthase RluA

scribed in this work should provide a means to test this possibility because even though normal amounts of mutant RluA were produced, the mutants had no Ψ synthase activity.

This work adds RluA to the category of Ψ synthases that cannot be deleted without a serious effect on cell growth. Previously, we showed that disruption of the rluD gene, which codes for the synthase that makes Ψ’1911, Ψ’1915, and Ψ’1917, severely inhibits the growth of *E. coli* (13). On the other hand, the lack of RluC, which makes Ψ’955, Ψ’2504, and Ψ’2580, has much less of an effect on growth. Although no effect was observed when individual exponential growth rates were compared with wild type (12), Fig. 8 shows that in competition with wild type there is an effect, albeit much less than for rluA− cells. The 5-fold drop observed after only six cycles would still result in the eventual loss of the rluC− cell from the culture. There is an even smaller effect in the case of rslA− cells, which also grew exponentially at the same rate as wild type (14). In competition, only a 2-fold drop was observed after six cycles (Fig. 8), some or all of which could be attributed to the presence of the kanamycin resistance protein and not to the absence of RsuA. Thus, there already appears to be a gradient of effects of depriving the cell of the Ψ synthases. The most severe effect was found by blocking RluD formation, which resulted in tiny colonies on plates and a readily detectable decrease in exponential growth rate (13). A strong RluA effect was found only in competition studies; the RluC effect was much less and was found only in competition, and the effect of the loss of RsuA was either very small or nonexistent.

In eukaryotes, which use a guide RNA system to specify those uridines in ribosomal RNA to be converted into Ψ, deletion of a number of guide RNAs, and thus the absence of those Ψ residues, also had no apparent effect on cell growth or metabolism (reviewed in Ref. 5). However, so far no competition studies have been performed. It seems unlikely that two distinct systems for forming Ψ in ribosomal RNA should have evolved without the driving force of a significant role in the survival of the cell. The discovery of strong effects in two cases in *E. coli*, RluD (13) and RluA (this work), marks the first two times any kind of role for Ψ in cellular metabolism has been established. The exact nature of that role remains to be deciphered.

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