Volume 16 Number 16 1988 Nucleic Acids Research

# Mutations within the decoding site of *Escherichia coli* 16S rRNA: growth rate impairment, lethality and intragenic suppression

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Received May 17, 1988; Revised and Accepted July 22, 1988

ABSTRACT

Several C⇒U transitions and small deletions were introduced into the conserved region centered on base C1400 in *Escherichia coli* 16S rRNA by *in vitro* mutagenesis. The mutations were placed within *rrn*B operons on multicopy plasmids under the transcriptional regulation of either the normal *rrn*B $P_1P_2$ promoters or the temperature-inducible $P_L$ promoter from bacteriophage lambda and introduced into *E. coli* hosts. When expressed from the $P_1P_2$ promoters, several of the mutant 16S rRNAs impaired cell growth while others, including one in which U replaced C at position 1400 within the ribosomal decoding site, had little or no effect on cell doubling time. However, C⇒U transitions at positions 1395 and 1407, as well as the deletion of C1400, appeared to render their hosts inviable. Cells in which these mutations were expressed from the $\lambda P_L$ promoter died within four generations after induction. Unexpectedly, the lethal phenotype was suppressed intragenically by replacement of G1505 with A, C or U. Suppression may alleviate a functional defect in 30S subunits containing the U1395, U1407 or ΔC1400 mutations.

## INTRODUCTION

Several lines of investigation indicate that the 3′ portion of prokaryotic 16S rRNA is in close contact with tRNA and mRNA during translation. Base pairing between the 3′ end of the 16S rRNA molecule and the complementary Shine-Dalgarno sequence in mRNA during initiation of protein synthesis has been well documented (1). In *Escherichia coli* 16S rRNA, additional evidence for the importance of the 3′ domain in ribosome function includes the observations that the target site of the antibiotic kasugamycin, which inhibits initiation, includes the methylated adenine residues at positions 1517 and 1518 (2), and that elongation of nascent polypeptide chains is halted by treatment of ribosomes with the bacteriocide, colicin E3, which cleaves 16S rRNA between nucleotides 1493 and 1494 (3). Furthermore, mutations that disrupt complementarity between nucleotides 1409 and 1491, which form the first base pair of the penultimate helix of 16S rRNA, induce resistance to paromomycin, an antibiotic which impairs codon recognition (4). Particular attention has centered on the region surrounding the cytosine at

position 1400 since $C_{1400}$ can be covalently cross-linked to the anticodon of $tRNA_1^{Val}$ when the tRNA is situated in the P site of *E. coli* ribosomes (5). More recently, it has been shown that tRNA bound to 70S ribosomes protects several nucleotides within this region from modification by a variety of structure-specific chemical probes (6, 7). The recognition that the $C_{1400}$ region constitutes at least a portion of the anticodon binding site lends added significance to the observation that the nucleotide sequence between positions 1390 and 1410 is conserved in nearly all small-ribosomal subunit RNAs (8).

We have initiated a detailed genetic study of the $C_{1400}$ region using site-directed mutagenesis *in vitro* to induce one or more mutations between positions 1388 and 1412 of the 16S rRNA gene. Although mutations in and around $C_{1400}$ have been reported previously, none has led to significant alterations in host-cell phenotype (9, 10). In part, this may have been due to selection against harmful mutations during the mutagenesis procedure. To avoid selective bias at that stage, we cloned the relevant restriction fragments from the *rrn*B rRNA operon into bacteriophage M13 vectors to construct phenotypically silent templates for mutagenesis. The altered restriction fragments were subsequently introduced into the *rrn*B operon of a multicopy plasmid for expression *in vivo* under the control of either the *E. coli rrn*B $P_1P_2$ promoters or the inducible $P_L$ promoter from bacteriophage lambda. Because the seven chromosomal *rrn* operons of the host cells were intact, the mutant rRNA comprised 50-60% of the cellular rRNA when transcribed from $P_1P_2$ (11), and less when the mutant operon was transcribed from $\lambda P_L$. To select those mutations most likely to impair ribosome function, the growth rate of *E. coli* host cells expressing the mutant rRNA genes was measured and compared with that of wild-type cells.

Despite the background of wild-type, host-encoded rRNA, we were able to identify several mutations that were harmful, or even lethal, to host cells. Surprisingly, alterations of $C_{1400}$, the base in close proximity to the tRNA anticodon, had less effect than anticipated: a C⇒U transition at position 1400 did not materially alter the growth rate, and even replacement of the $C_{1400}$-$G_{1401}$ dinucleotide with A only modestly impaired cell growth when the mutant genes were expressed from the $\lambda P_L$ promoter. Deletion of $C_{1400}$ did prove to be lethal, however, when the mutant rRNA was transcribed from either $P_1P_2$ or $\lambda P_L$ promoters. Unexpectedly, expression of mutant 16S rRNAs with a C⇒U transition at either position 1395 or 1407 was also lethal for host cells, in both the $P_1P_2$ and $P_L$ systems. It has been suggested that $C_{1395}$ may base

pair with the conserved nucleotide $G_{1505}$ to form part of a short tertiary helix (12). To investigate the possibility that disruption of this interaction was responsible for the lethal phenotype of the $C_{1395} \Rightarrow U_{1395}$ transition, oligonucleotide-directed mutagenesis was employed to induce point mutations at position 1505 which were then expressed in combination with either C or U at position 1395. While the results do not support a model in which base-pairing between $C_{1395}$ and $G_{1505}$ is an essential feature of ribosome structure or function, they do indicate that replacement of G at 1505 with any other base suppresses the lethal phenotype of cells expressing the $U_{1395}$ mutation. Moreover, the base changes at position 1505 also suppress the lethal $C_{1407} \Rightarrow U_{1407}$ transition and $C_{1400}$ deletion.

## MATERIALS AND METHODS

### Enzymes and Radioisotopes

Restriction endonucleases were obtained from New England Biolabs, International Biotechnologies, Inc. or Bethesda Research Labs and used according to the manufacturer's instructions. $T_4$ DNA ligase and *E. coli* DNA polymerase I (large fragment) were purchased from New England Biolabs, and $T_4$ polynucleotide kinase was obtained from Boehringer Mannheim. [$\alpha$-$^{32}$P]-dATP (800 Ci/mmol) and [$\gamma$-$^{32}$P]-ATP (7000 Ci/mmol) were products of New England Nuclear and ICN, respectively.

### Bacterial Strains

*E. coli* strains JM101 (13), CAG1574 (14), NO3203 (15), C600($\lambda$) (15) and XL1-B (16) have been described previously. Both NO3203 and C600($\lambda$) are defective lambda lysogens encoding a temperature-sensitive repressor of the $\lambda P_L$ promoter (cI857ts).

### Plasmids and Bacteriophages

Plasmid pKK3535 (17) and its derivative, pNO1301 (11), both carry an intact *rrn*B operon. pCT2 was derived from pKK3535 in three steps: first, the *Bam*HI to *Bgl*I restriction fragment spanning positions 1 to 2221 and containing the $P_1P_2$ promoters was removed; next, the *Nco*I site at position 4191 was destroyed by filling it in with *E. coli* DNA polymerase I (large fragment) in the presence of the appropriate deoxynucleotides; and, finally, a restriction fragment containing the *E. coli lac* operon promoter and operator as well as the *lac*Z and *lac*Y genes, was inserted between the *Sma*I and *Nco*I sites at positions 2801 and 2929, respectively. These manipulations resulted in a vector with three notable features: unique *Sma*I and *Nco*I restriction sites bracketing the $C_{1400}$ region of the 16S rRNA gene; a "silent" *rrn* operon, due

to the removal of the $P_1P_2$ promoter region; and, constitutive expression of *lac*Z from the *lac* promoter on the insert. pCT3 was derived from pNO1301 by replacement of the *Apa*I to *Xba*I restriction fragment of pNO1301, containing the 3' end of the 16S rRNA gene and a portion of the intergenic region, with a 1795-bp *Apa*I to *Xba*I DNA fragment designed to incorporate additional restriction sites to facilitate screening for prospective recombinant plasmids. Plasmid pPL215 (15), which contains the *rrn*B operon under the transcriptional control of the $\lambda P_L$ promoter, was kindly provided by Dr. Richard A. Gourse. Plasmid pPL*lac* was constructed by replacing the *Apa*I to *Xba*I restriction fragment from the *rrn*B operon of pPL215 with the *Apa*I to *Xba*I fragment from pCT2, thereby placing the *lac*Z gene within the inducible *rrn* operon. Bacteriophages M13mp9 and M13mp19 (New England Biolabs), were used to construct the vectors employed for mutagenesis and DNA sequencing of the $C_{1400}$ region. Plasmid Bluescript KS+ (Stratagene), which includes the M13 origin of replication for production of single-stranded DNA, was used for mutagenesis of position 1505 and the subsequent identification of base alterations by DNA sequencing.

Agarose Gel Electrophoresis

Analytical agarose gels were prepared in TBE buffer (100 mM Tris-HCl, 100 mM $H_3BO_4$, pH 8.3, and 2 mM EDTA). Sea-Plaque low melting-point agarose (FMC BioProducts) was used for the isolation of DNA restriction fragments: samples were applied to 0.7% agarose gels in 50 mM Tris-acetate buffer, pH 8.2, and were electrophoresed at 4°C; the desired bands were then excised, melted at 65°C, and used directly in ligation reactions (18).

Mutagenesis

Sodium bisulfite mutagenesis was performed according to Everett and Chambon (19) with the modifications described by Kalderon *et al.* (20). Oligonucleotide-directed mutagenesis was performed essentially according to Zoller and Smith (21). Details of the mutagenesis procedures used in this report are described in reference 14. The 18-nucleotide primer used to replace the $C_{1400}$-$G_{1401}$ dinucleotide with G, C or A hybridized to the 16S rRNA gene at positions 1392 through 1409. The 18-base mutagenic primer used to change nucleotide 1505 to A, C or U hybridized at positions 1497-1514 of the 16S rRNA gene. Because $G_{1505}$ falls within the unique recognition site of restriction endonuclease *Bst*EII, plasmids isolated from XL1-B cells that had been transformed with the mutagenesis mixture were digested with *Bst*EII in order to linearize those which retained a G at 1505. The mixture was then used to transform fresh XL1-B cells for isolation of plasmids containing

mutations at position 1505. Enrichment of mutants by alkaline sucrose gradient centrifugation and screening by differential hybridization, employed for the mutagenesis of $C_{1400}$-$G_{1401}$, were omitted in the case of $G_{1505}$. All of the mutations described in this report were verified by sequencing the appropriate segment of the reconstituted *rrn*B operons by the dideoxynucleotide technique (22).

## RESULTS

### Isolation of mutations in the $C_{1400}$ region

A 27-bp *Sma*I to *Nco*I restriction fragment spanning positions 1385 to 1412 of the *E. coli* 16S rRNA gene and encompassing the entire conserved $C_{1400}$ region was cloned into bacteriophage M13mp9 to provide the target for the induction of random C⇒U transitions by sodium bisulfite mutagenesis. The template for oligonucleotide-directed mutagenesis of the $C_{1400}$-$G_{1401}$ dinucleotide was constructed by inserting the 2151-bp *Eco*RI fragment from the *rrn*B operon, beginning at position 675 of the 16S rRNA gene, into bacteriophage M13mp9. Mutagenesis was performed using a primer designed to replace the dinucleotide with a single base. In all cases, mutations were identified by sequence analysis of the single-stranded M13 DNA derived from transformation of *E. coli* JM101 with the mutagenized material.

### Introduction of mutations into the intact *rrn*B operon of a multicopy plasmid

Both bisulfite- and oligonucleotide-induced mutations were introduced into a plasmid-borne *rrn*B operon via the two-step cloning procedure illustrated in Figure 1. First, the 27-bp *Sma*I/*Nco*I restriction fragment was removed from the mutagenized M13 vectors and cloned into plasmid pCT2, which contains a partial *rrn*B operon lacking both the 5′ portion of the 16S RNA gene and the $P_1P_2$ transcriptional promoters. Because the *lac*Z gene was located between the *Sma*I and *Nco*I sites of pCT2, recombinants containing the mutant 16S rRNA gene fragment in place of this insert were unable to synthesize β-galactosidase and therefore produced white colonies when grown on medium containing 40 μg/mL X-gal. The correct reconstruction of the plasmid was verified in all cases by restriction enzyme analysis. In the second step, the complete *rrn*B operon was assembled by replacing the *Apa*I/*Xba*I restriction fragment of pCT3 with that from the pCT2 recombinants, thereby placing the mutant rRNA gene under control of the $P_1P_2$ *rrn*B promoters. Recombinant plasmids were distinguished from pCT3 parents by restriction digest patterns, which differed markedly due to the 1795-bp fragment inserted between the *Apa*I and *Xba*I sites of pCT3. The presence of the mutations in the reconstructed

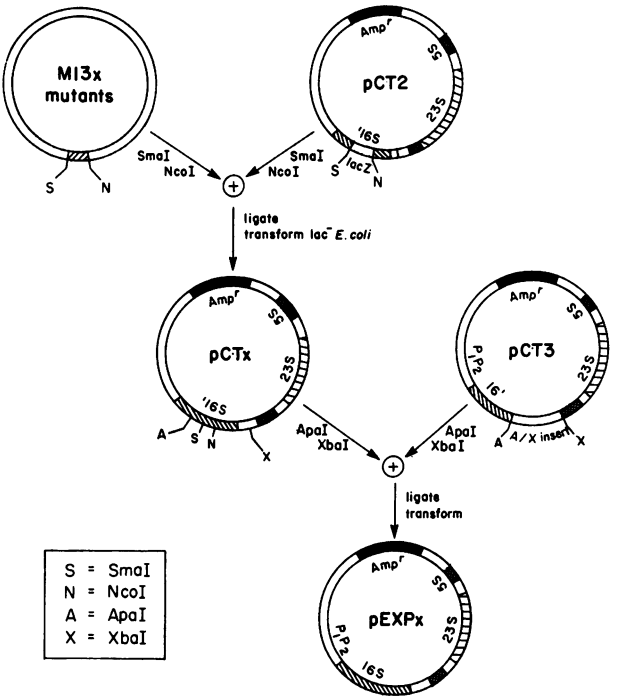


Figure 1. Strategy for placing mutations within the $C_{1400}$ region into a plasmid-borne *rrn*B operon under the control of the $P_1P_2$ promoters. *E. coli* CAG1574 (*lac*Z, *Rec*A) was used as the recipient in transformations with plasmids of the pCTx and pEXPx series. "x" denotes any of the various mutant alleles.

*rrn*B operons was verified by subcloning the relevant restriction fragments into M13 and sequencing the insert DNA.

The location and nature of the mutations described in this report are summarized in Figure 2. Under conditions in which the mutant 16S rRNA genes were expressed from the $P_1P_2$ promoters, only those plasmids containing single C⇒U transitions at positions 1389, 1397, 1399 or 1400, or double C⇒U transitions at positions 1388 and 1397 or 1397 and 1404, could be successfully introduced into *E. coli* hosts (Fig. 2a). The growth rates of the transformants are listed in Table 1. The presence of plasmids carrying the $U_{1389}$, $U_{1397}$, $U_{1400}$ and $U_{1388}/U_{1397}$ mutations had no apparent effect on host-cell growth. Cells transformed with plasmids containing C⇒U transitions at position 1399, or at both 1397 and 1404, exhibited doubling times 30-40% greater than that of cells harboring the wild-type *rrn*B plasmid. All of the

**a** *MUTATIONS WHICH ARE VIABLE WHEN EXPRESSED FROM THE $P_1P_2$ PROMOTERS*

| | 1390 | 1400 | 1410 |
|---|---|---|---|
| | G G G C C U U G U A C A C A C C G $^{m4}C_m$ C C G U $^{m5}$C A C A C C |
| 132 | ------- U ---------- |
| 136 | ---------- U ---------- |
| 125 | ---------- U ---------- |
| 145 | ---------- U ---------- |
| 185 | ----- U ---------- U ---------- |
| 212 | ---------- U ---------- U ---------- |
| AM8 | ---------- Δ A ---------- |

**b** *MUTATIONS NOT RECOVERED WHEN UNDER REGULATION OF THE $P_1P_2$ PROMOTERS*

| | | | | |
|---|---|---|---|---|
| | G G G C C U U G U A C A C A C C G $^{m4}C_m$ C C G U $^{m5}$C A C A C C |
| 111 | ---------- U ---------- |
| 201 | ------- U --------- U ---------- |
| 141 | ---------- U - U ---------- |
| 129 | ---------- U - U --- U ---------- |
| 168 | ------- U --------- U ---------- U --------- |
| 133 | ---------- U --------- |
| 130 | ---------- U ---------- U --------- |
| 196 | ---------- U ---------- U --------- |
| 119 | ---------- U - U ----- |
| 181 | ------- U ---------- U ---------- U --------- |
| 128 | ---------- U ---------- U - U ----- |
| 194 | ------- U ---------- U U ---------- U --------- |
| AM5 | ---------- Δ ---------- |
| AM6 | ---------- Δ ---------- |

Figure 2. Mutations obtained via site-directed mutagenesis of the $C_{1400}$ region of the 16S rRNA gene. (a) Bisulfite- or oligonucleotide-directed mutations that were successfully placed within the *rrn*B operon of plasmid pNO1301 under control of the $P_1P_2$ promoters. (b) Mutations that could not be cloned into plasmid pNO1301 despite persistent efforts and were suspected of being lethal under our experimental conditions.

Table 1. Growth rates of cells expressing mutations in the $C_{1400}$ region of 16S rRNA from the *rrn*B $P_1P_2$ promoters.

| Plasmid | Mutation(s) | Doubling time (min) |
|---|---|---|
| pNO1301 | Wild type | 42 |
| pEXP125 | $C \Rightarrow U_{1399}$ | 54 |
| pEXP132 | $C \Rightarrow U_{1389}$ | 42 |
| pEXP136 | $C \Rightarrow U_{1397}$ | 41 |
| pEXP145 | $C \Rightarrow U_{1400}$ | 42 |
| pEXP185 | $C \Rightarrow U_{1388}$<br>$C \Rightarrow U_{1397}$ | 42 |
| pEXP212 | $C \Rightarrow U_{1397}$<br>$C \Rightarrow U_{1404}$ | 58 |
| pEXPAM8 | $C_{1400}G_{1401} \Rightarrow A$ | Unstable |

*E. coli* CAG1574 (*rec*A) was transformed with plasmid pNO1301 or mutant derivatives of the pEXPx series (see Fig. 1) in which the *rrn*B operon was expressed from the $P_1P_2$ promoters. Cell were suspended at an initial $OD_{600}$ of approximately 0.04 in liquid medium containing 5g/L yeast extract, 10g/L tryptone, 10g/L NaCl and 200 mg/L ampicillin, and grown at 37°C for at least four doublings. pEXPAM8 was too unstable to permit determination of its growth rate (see text).

above phenotypes were stable over many generations in *rec*A strains of *E. coli*. Although transformants containing the $C_{1400}$-$G_{1401} \Rightarrow A$ replacement were isolated, their growth was severely impaired. It was not possible to measure the growth rate of this mutant as the culture rapidly converted to wild-type growth rate.

Despite numerous attempts, no transformants harboring plasmids from which either $C_{1400}$ or $G_{1401}$ had been deleted were recovered when the mutant *rrn*B operons were subject to regulation by the $P_1P_2$ promoters. As indicated in Figure 2b, the same observation held true for the remaining bisulfite-induced mutants. Significantly, all of the bisulfite mutants that failed to produce viable transformants were characterized by the presence of a C⇒U transition at position 1395 or 1407, either singly or in combination with other C⇒U transitions within the $C_{1400}$ region. Hence, we considered the C⇒U transitions at positions 1395 and 1407, as well as the deletion of $C_{1400}$ or $G_{1401}$, to be potentially lethal.

Placement of the mutant *rrn*B operons under control of the $\lambda P_L$ promoter

To investigate the possible lethality of the $U_{1395}$ and $U_{1407}$ transitions,

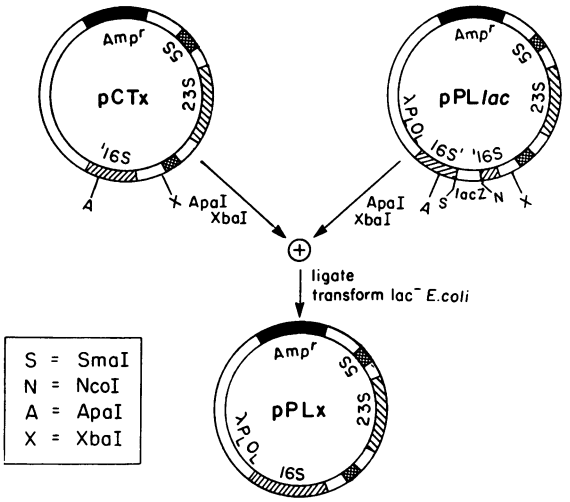


Figure 3. Construction of mutant, plasmid-borne *rrn*B operons regulated by the $\lambda P_L$ promoter. Plasmids were constructed in *E. coli* C600(λ),(Rec$^+$), then transferred to *E. coli* NO3203, a *rec*A strain, for experimentation and storage.

and of the $C_{1400}$ and $G_{1401}$ deletions, we placed the corresponding mutant operons under the control of an inducible promoter. For this purpose, we chose plasmid pPL215, in which an otherwise complete *rrn*B operon is regulated by the $P_L$ promoter of bacteriophage lambda, instead of the normal $P_1P_2$ promoters (15). Transformation of strains which encode a temperature-sensitive repressor of $P_L$ with mutant derivatives of pPL215 provided us with an easily inducible expression system by which to assess the physiological effects of the 16S rRNA mutants: at 30°C, transcription from the $P_L$ promoter was repressed, but at 42°C, the temperature-sensitive repressor was inactivated and the mutant rRNA operons were transcribed.

The method by which the mutations within the $C_{1400}$ region were transferred from plasmids of the pCTx series to pPL215 is described in Figure 3. The *ApaI/XbaI* restriction fragment of pCTx plasmids was cloned between the *ApaI* and *XbaI* sites of pPL*lac*, replacing the *lac* operon fragment and reconstituting a complete *rrn* operon. To avoid selection against deleterious mutations, transformations and incubations were performed at temperatures which ensured that transcription from $P_L$ remained repressed: host cells were grown at 28-30°C, heat shock was carried out at 35°C for five minutes, outgrowth for expression of ampicillin resistance was at 28-30°C for 60-90 minutes, and the transformation mixtures were incubated on solid medium at

Table 2. Growth rates of cells expressing mutations in the $C_{1400}$ region of 16S rRNA from the inducible $\lambda P_L$ promoter.

| Plasmid | Mutation(s) | Doubling time (min) |
|---|---|---|
| pPL215 | Wild type | 48 |
| pPL111 | $C \Rightarrow U_{1395}$ | Lethal |
| pPL125 | $C \Rightarrow U_{1399}$ | 46 |
| pPL133 | $C \Rightarrow U_{1407}$ | Lethal |
| pPL145 | $C \Rightarrow U_{1400}$ | 48 |
| pPL185 | $C \Rightarrow U_{1388}$<br>$C \Rightarrow U_{1397}$ | 46 |
| pPL212 | $C \Rightarrow U_{1397}$<br>$C \Rightarrow U_{1404}$ | 48 |
| pPLAM5 | $C_{1400}G_{1401} \Rightarrow G$ | Lethal |
| pPLAM6 | $C_{1400}G_{1401} \Rightarrow C$ | 60 |
| pPLAM8 | $C_{1400}G_{1401} \Rightarrow A$ | 58 |

*E. coli* NO3203 (*recA*) was transformed with plasmid pPL215 or mutant derivatives of the pPLx series in which the *rrnB* operon is expressed from the $\lambda P_L$ promoter. Cells were grown overnight at 30°C in liquid medium containing 5 g/L yeast extract, 8 g/L tryptone, 5 g/L NaCl, 0.1% glucose and 200 mg/L ampicillin. The overnight cultures were used to inoculate fresh cultures at a initial $OD_{600}$ of 0.007 or less, which were grown at 42°C for at least seven doublings or, in the case of pPL111, pPL133 and pPLAM5, for several hours after growth ceased.

30°C. Because the *lac* operon fragment of pPL*lac* includes the *lac* promoter, cells which contain intact pPL*lac* are blue on X-gal, even in the absence of transcription from the $P_L$ promoter. Transformation mixtures were therefore grown on plates containing X-gal, and potential recombinant plasmids were isolated from the white colonies. These plasmids were checked for the correct structure by restriction enzyme analysis and the presence of the appropriate mutations was verified by sequencing the double-stranded plasmid DNA by a modification of the dideoxynucleotide method.

The growth rates of the mutant pPL215 derivatives are shown in Table 2. As expected, those mutations which had no detectable effect on host-cell growth when expressed from $P_1P_2$ grew at the same rate as cells transformed

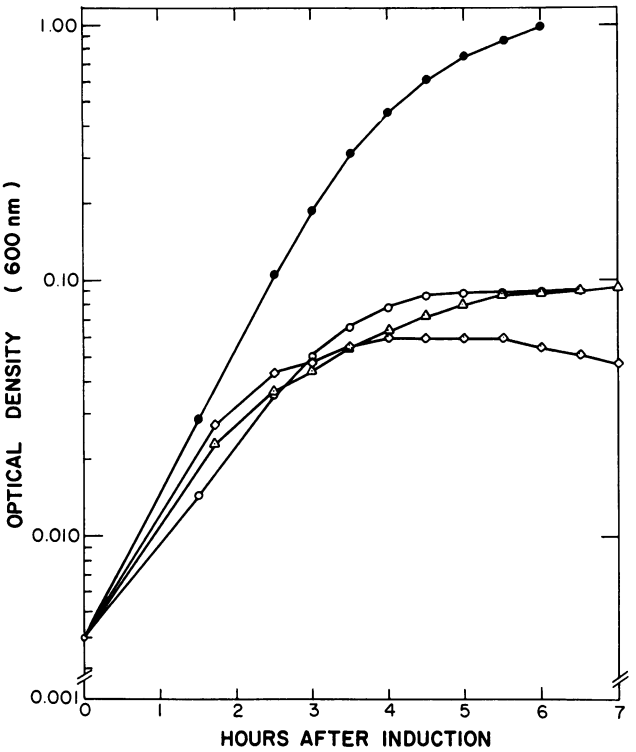


Figure 4. Representative growth curves for *E. coli* NO3203 transformed with mutant plasmids. Cells containing pPL215 (wild-type, ●), pPL111 ($C_{1395}$⇒U, ◊), pPL133 ($C_{1407}$⇒U, Δ) and pPLAM5 ($\Delta C_{1400}$, O) were grown at 42°C to induce transcription of the plasmid-borne *rrn* B operons. See Table 2 for conditions.

with the wild-type plasmid in the $P_L$ system as well. However, the $U_{1399}$ and $U_{1397}/U_{1404}$ mutants also grew at roughly the same rate as the wild-type control while the $G_{1401}$ deletion and the $C_{1400}G_{1401}$⇒A mutation conferred only a moderate increase in host-cell doubling time. In contrast, expression of the $U_{1395}$ and $U_{1407}$ mutations, as well as the $C_{1400}$ deletion, proved to be highly deleterious. Figure 4, which depicts growth curves for cells transformed with plasmids pPL111, pPL133 and pPLAM5, encoding the 1395 and 1407 transitions and the $C_{1400}$ deletion, respectively, shows that the cultures stopped growing within four generations after induction of the $P_L$ promoter. Cells removed from the cultures after the curves leveled off did not grow on solid medium and were presumed to be dead.

Isolation of mutations at position 1505

To investigate a proposed tertiary interaction between $C_{1395}$ and $G_{1505}$

Table 3. Growth rates of cells expressing a mutation at position 1505 of 16S rRNA.

| Plasmid | Mutation(s) | Doubling time (min) |
|---|---|---|
| pPL215 | wild type | 43 |
| pPL1301m2 | $G_{1505}\Rightarrow A$ | 40 |
| pPL1301m26 | $G_{1505}\Rightarrow U$ | 38 |
| pPL1301m38 | $G_{1505}\Rightarrow C$ | 42 |
| pPL111m5 | $C_{1395}\Rightarrow U$<br>$G_{1505}\Rightarrow A$ | 67 |
| pPL111m28 | $C_{1395}\Rightarrow U$<br>$G_{1505}\Rightarrow U$ | 67 |
| pPL111m4 | $C_{1395}\Rightarrow U$<br>$G_{1505}\Rightarrow C$ | 75 |
| pPL133m2 | $C_{1407}\Rightarrow U$<br>$G_{1505}\Rightarrow A$ | 70 |
| pPL133m26 | $C_{1407}\Rightarrow U$<br>$G_{1505}\Rightarrow U$ | 70 |
| pPL133m38 | $C_{1407}\Rightarrow U$<br>$G_{1505}\Rightarrow C$ | 66 |
| pPLAM5m2 | $\Delta C_{1400}$<br>$G_{1505}\Rightarrow A$ | 70 |
| pPLAM5m26 | $\Delta C_{1400}$<br>$G_{1505}\Rightarrow U$ | 76 |
| pPLAM5m38 | $\Delta C_{1400}$<br>$G_{1505}\Rightarrow C$ | 77 |

*E. coli* NO3203 (*rec*A) was transformed with plasmid pPL215 or the indicated mutant derivatives and grown as described in Table 2. The doubling time for cells containing pPL215 is less than that indicated in Table 2 owing to the use of different stocks of media in the two experiments.

(12), we substituted A, C or U for G at position 1505 and expressed these mutations in plasmids encoding either C or U at position 1395. The templates for oligonucleotide-directed mutagenesis of position 1505 were constructed by inserting the 795-bp *Apa*I/*Xba*I restriction fragment from pNO1301 and pPL111,

encoding $C_{1395}$ and $U_{1395}$, respectively, between the *Apa*I and *Xba*I sites of the plasmid Bluescript KS+. Mutagenesis was performed on the single-stranded DNA produced by the recombinant plasmids, using an 18-base mutagenic primer specifying the incorporation of A, C or U at position 1505. Altogether, six mutants were constructed in this manner, three containing a base change at position 1505 only, and three in which a mutation at position 1505 was combined with the lethal mutation, $U_{1395}$.

Expression of mutations at position 1505 from the $\lambda P_L$ promoter

The mutagenized *Apa*I/*Xba*I restriction fragments were transferred from the Bluescript plasmids to the wild-type *rrn*B operon of pPL215 for *in vivo* expression of the altered 16S rRNA genes. The mutant operons were reconstructed via pPL*lac* in a manner analogous to that described above for the $C_{1400}$ region (see Fig. 3). As a test of the specificity of the interaction between mutations at 1395 and 1505, each of the substitutions at position 1505 was also combined with the lethal $U_{1407}$ and $\Delta C_{1400}$ mutations by replacing the *Nco*I restriction fragment from the *rrn*B operon of pPL133 or pPLAM5--containing the 3′ portion of the 16S rRNA gene distal to $C_{1412}$--with the *Nco*I restriction fragment from the pPL215 derivatives encoding the mutations at position 1505.

Unexpectedly, the expression of 16S rRNA genes containing A, C or U at position 1505 together with *any* of the three lethal mutations, $U_{1395}$, $U_{1407}$ or $\Delta C_{1400}$, suppressed the lethal phenotype. Not only do cells harboring the double mutations remain viable after induction of the plasmid-borne operons, but their doubling times are only 40-70% greater than that of cells expressing the wild-type 16S rRNA from pPL215 (Table 3). Moreover, the data presented in Table 3 fail to provide evidence for an essential Watson-Crick base pair between positions 1395 and 1505. The replacement of $G_{1505}$ with A, C or U does not in itself impair host-cell growth when position 1395 is occupied by the wild-type C residue despite the fact that these nucleotide combinations cannot form stable, canonical base pairs.

## DISCUSSION

### Expression of 16S rRNA genes altered in the vicinity of nucleotide $C_{1400}$

To investigate the conserved 20-bp sequence surrounding nucleotide $C_{1400}$ of *E. coli* 16S rRNA, we have mutagenized the corresponding fragments of the 16S rRNA gene *in vitro*, placed them within plasmid-borne rRNA operons for expression *in vivo* and identified several deleterious mutations including three that are lethal to host cells. In an earlier study, a C⇒U transition at position 1402 was isolated following bisulfite mutagenesis of positions 1385 to 1505 of the 16S rRNA gene, but no other mutations within the $C_{1400}$ region

were recovered (9). It should be noted, however, that mutagenesis and screening were performed with plasmids carrying intact *rrn*B operons, an approach which may have precluded the recovery of most slow-growing mutants. Transcription of the $U_{1402}$ mutant 16S rRNA from the $P_1P_2$ promoters on a multicopy plasmid conferred only a moderate decrease in growth rate upon host cells. In another report, oligonucleotide-directed mutagenesis was used to induce the double mutations, $C_{1399}$ to $A_{1399}/G_{1401}$ to $C_{1401}$ and $C_{1399}$ to $A_{1399}/G_{1401}$ to $U_{1401}$ (10). When these mutations were cloned into a multicopy plasmid and expressed from the $P_1P_2$ promoters, the growth rates of the hosts corresponded to that of cells harboring the wild-type plasmid, and the mutant 16S rRNA was processed and incorporated into ribosomes capable of binding tRNA *in vitro*.

Our results indicate that several of the cytosines of the $C_{1400}$ region, including those at positions 1388, 1389, 1397 and 1400, can be replaced by uracil without adversely affecting host-cell growth, at least in our experimental system. However, our data also lead us to conclude that the highly conserved cytosines at positions 1395 and 1407 are of critical importance for proper rRNA function, and that $C_{1399}$ and $C_{1404}$ play a significant, although lesser, role. Moreover, the characteristics of the deletion mutations at positions 1400 and 1401 suggest that the length and/or geometry of the RNA chain within the $C_{1400}$ region are crucial to the biological activity of 16S rRNA.

At this point, we do not know why certain of the mutations reported here cause cells to grow slowly, or die. Cells expressing the $U_{1395}$, $U_{1407}$ or $\Delta C_{1400}$ mutations for two to three generations accumulate a large excess of subunits relative to 70S ribosomes. We have sequenced 16S rRNA extracted from free 30S subunits and 70S ribosomes of strains pPL111 and pPL133, and have determined that 16S rRNA containing $U_{1395}$ and $U_{1407}$ is assembled into 30S subunits; however, we did not detect the mutant rRNAs in 70S ribosomes (C.L.T. and R.A.Z., unpublished results). 30S subunits that contain the altered 16S rRNA could fail at several levels, including rRNA processing, formation of initiation complexes, association of 30S with 50S subunits or polypeptide elongation.

Structural and functional implications of mutations in the $C_{1400}$ region.

Because nucleotide $C_{1400}$ has been cross-linked to the anticodon of P-site bound $tRNA_1^{Val}$, it has been presumed to comprise part of the ribosomal decoding site (5). For this reason, we anticipated that mutations at, or in the immediate neighborhood of, this base might adversely affect ribosome function

and, therefore, cell growth. As cells which express 16S rRNA containing $U_{1400}$ grow at the wild-type rate, it is possible that the two pyrimidines are functionally equivalent at this location. There may of course be subtle differences in the efficiency of ribosomes containing $U_{1400}$ instead of $C_{1400}$ that cannot be detected by our growth-rate measurements.

One of the lethal mutations reported here results from the introduction of U at position 1407 in place of $m^5C$, which is one of only ten methylated bases in 16S rRNA (23). While the functional importance of such modifications has not yet been determined, a recently published tertiary-structure model of the *E. coli* 30S subunit suggests that all of the methylated bases are arranged in a ring around the "neck" that separates the head of the particle from its body (24), placing them in the vicinity of the decoding region. If methylation *per se* is crucial for proper rRNA function, replacement of $m^5C$ by U would be detrimental to ribosomes containing the altered 16S rRNA. It should be noted, however, that although the C residue at position 1402 is also methylated, substitution of U at this site did not seriously impede host-cell growth (9). Furthermore, recent work has shown that functional 30S subunits can be assembled from extracted ribosomal proteins and *in vitro* synthesized 16S rRNA lacking all ten modified bases (25).

With respect to the lethal mutation at position 1395, a possible structural role for this base has been advanced whereby $C_{1395}$ is paired with $G_{1505}$ to form part of a short Watson-Crick helix (12). We reasoned that if the proposed tertiary interaction were a critical feature of ribosome structure, replacement of the stable C-G base pair with U•G might be at least partially responsible for the lethal phenotype of the $C_{1395}$ to $U_{1395}$ transition. To test for a possible interaction between residues 1395 and 1505, we introduced mutations at position 1505 into the defective, $U_{1395}$-containing 16S rRNA. Clearly, the findings reported here do not support a model in which a Watson-Crick base pair between positions 1395 and 1505 is essential for ribosome structure or function. The ability of three different base substitutions at position 1505 to suppress the lethal $U_{1395}$ mutation was completely unexpected, however, prompting us to investigate the generality of this phenomenon. Accordingly, we combined two other lethal mutations, $U_{1407}$ and $\Delta C_{1400}$, with A, C or U at position 1505 and found that the 1407/1505 and $\Delta C_{1400}$/1505 double mutants were also viable, growing at roughly the same rates as cells expressing the 1395/1505 double mutations. Suppression of the lethal phenotype by base substitutions at 1505 is therefore not allele-specific.

Lethality, suppression and the regulation of rRNA synthesis

The extent to which several of the mutations in the $C_{1400}$ region impaired

cell growth depended on whether they were transcribed from the $P_1P_2$ *rrn*B or the $\lambda P_L$ promoters. The fact that host cells expressing the $C_{1400}$-$G_{1401}\Rightarrow A$ and $C_{1400}$-$G_{1401}\Rightarrow C$ mutations were either lethal or barely viable in the $P_1P_2$ system, but grew moderately well when the mutant 16S rRNA gene was transcribed from the $P_L$ promoter, suggests that plasmid-encoded transcripts represent a smaller fraction of rRNA in the inducible system than when expressed from the normal *rrn*B promoters. Hence, the fact that C⇒U transitions at positions 1395 and 1407, as well as the deletion of $C_{1400}$, were lethal even when they were transcribed from the $P_L$ promoter poses an intriguing question: how does the expression of these mutant rRNA molecules lead to cell death?

Our interpretation of the lethal effect follows from the observation that mutant 16S rRNAs are assembled into 30S subunits which are presumably defective at some stage of protein synthesis. For example, the mutant ribosomes may sequester mRNAs or diffusible translation factors in non-productive initiation complexes, or may block the progress of wild-type ribosomes during elongation. When the mutant rRNAs are expressed from a strong promoter such as $P_1P_2$, the functionally impaired ribosomes would be sufficiently numerous to halt protein synthesis and cause the cell to die. At the lower levels of transcription which result when the mutant rRNA genes are expressed from the $\lambda P_L$ promoter, the fraction of defective ribosomes is initially low and would not exceed a threshold needed for a fatal disruption of translation. Recent analyses of cells harboring pPL215 have shown that induction of the wild-type rRNA operon under $\lambda P_L$ control leads to feedback repression of chromosomal rRNA operons (15) at the *rrn* $P_1$ promoter (26). Although the effector has not been identified, it is believed that excess ribosomes or ribosomal subunits (15)--possibly only those that are initiation-competent (27, 28)--are the signal for feedback regulation. Since the $\lambda P_L$ promoter is not subject to this control, rRNA contributed by the plasmid-borne operons should accumulate at the expense of chromosomally-encoded rRNA (15). If 16S rRNA containing $U_{1395}$ or $U_{1407}$ is assembled into stable 30S subunits, these subunits would be expected to repress chromosomal rRNA operons, while transcription of plasmid-borne operons continued unabated. Consequently, production of wild-type ribosomes would decrease and mutant, functionally impaired ribosomes would comprise a steadily rising proportion of the total. This condition would lead to cell death when the number of ribosomes unable to carry out translation rose above the critical threshold.

In theory, substitutions at position 1505 could suppress the lethal $U_{1395}$ and $U_{1407}$ mutations by rendering the altered 16S rRNA unstable or otherwise

incompetent for assembly into 30S subunits. However, we have sequenced 16S rRNA extracted from strains expressing $U_{1505}$, alone or in combination with $U_{1395}$ or $U_{1407}$, and found that, within four generations, 16S rRNA containing $U_{1505}$ was not only present, but predominant, in the 30S, 70S, and polyribosome fractions of all three strains (C.L.T. and R.A.Z, unpublished results). We must therefore consider the possibility that alterations at position 1505 at least partially correct the putative functional defect in 30S subunits that contain the $U_{1395}$ or $U_{1407}$ mutations. Experiments are under way to determine which of the partial reactions of protein biosynthesis have been impaired by base changes in the $C_{1400}$ region, and in what manner these defects are suppressed by mutations at position 1505.

ACKNOWLEDGMENTS

We thank Drs. S. Brown, A. E. Dahlberg and T. L. Mason for valuable discussions during these experiments. This work was supported by National Institutes of Health grant GM22807.

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