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Mutations in the *Escherichia coli* 23S rRNA Increase the Rate of Peptidyl-tRNA Dissociation from the Ribosome

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Abstract—We have studied *in vivo* the phenotypes of 23S rRNA mutations G2582A, G2582U, G2583C, and U2584C, which are located at the A site of *Escherichia coli* 50S ribosomal subunit. All mutant rRNAs incorporated into 50S ribosomal subunits. Upon sucrose gradient fractionation of cell lysates, 23S rRNAs mutated at G2582 to A and G2583 to C accumulated in the 50S and 70S fractions and were underrepresented in the polyosome fraction. Induction of 23S rRNAs mutated at G2582 and G2583 lead to a drastic reduction in cell growth. In addition, mutations G2582A and G2583C reduced to one-third the total protein synthesis but not the RNA synthesis. Finally, we show that 23S rRNA mutations G2582A, G2582U, and G2583C cause a significant increase in peptidyl-tRNA drop-off from ribosomes, thereby reducing translational processivity. The results clearly show that tRNA–23S rRNA interaction has an essential role in maintaining the processivity of translation.

Key words: peptidyl-tRNA, 23S rRNA, peptidyl-tRNA hydrolase, translation, *Escherichia coli*

INTRODUCTION

Studies on the structure and functional role of ribosomal RNA (rRNA) started with the pioneering works of A.S. Spirin in the 1960s; rRNA was accepted as the main functional component of the ribosome 30 years later. The atomic structure of ribosomal subunits has recently been determined by X-ray diffraction [1–3]. Notably, the crystallographic analysis of ribosomes was also initiated in Spirin's lab [4]. The availability of atomic models of ribosomal structure has made it possible to analyze the functional importance of different atoms in rRNA. Ribosome interacts with many different molecules during translation. Ribosomal RNA is involved in the binding of all ribosomal ligands [5]. In spite of the solid body of biochemical data, unraveling the molecular mechanisms of translation of genetic message is still an important task for ribosome research.

The frequency of amino acid substitution errors in proteins ranges from 10^{-3} to 10^{-4} per codon [6]. All errors that prevent completion of the full-length protein after its initiation are termed processivity errors. Translational processivity can be influenced by mRNA polarity, spontaneous frameshifts, termination at sense codons, and peptidyl-tRNA dissociation (drop-off) from ribosomes. Peptidyl-tRNA drop-off, occurring at a frequency of $4 \cdot 10^{-4}$ per codon [7], is the

dominant factor of translational processivity errors [8]. Peptidyl-tRNA dissociation is enhanced by antibiotics streptomycin, neomycin [9, 10], erythromycin, carbomycin, spiramycin [11], and starvation for amino acids [12], and suppressed by chloramphenicol [7]. Peptidyl-tRNA is degraded by a ubiquitous and essential enzyme, peptidyl-tRNA hydrolase (Pth) [7]. Accumulation of peptidyl-tRNA in the absence of functional Pth leads to arrest of protein synthesis and cell growth [13]. This effect was attributed to the sequestering of tRNA^{Lys} [14]. Mutations G2582 to A, G2583 to C, and U2584 to C in the 23S rRNA have been shown to cause loss of peptidyl transferase activity by the mutant ribosomes and to inhibit bacterial growth in combination with second site mutations A2058G [15, 16] or A1067U [17, 18]. In addition, the G2583C mutation confers increased translational accuracy upon mutant ribosomes [17, 19]. In this study, we have analyzed the single point mutations G2582A, G2582U, G2583C, and U2584C in *E. coli* 23S rRNA in respect to cell growth, translational activity, and peptidyl-tRNA accumulation *in vivo*. The mutations that have a strong effect on the peptide bond formation *in vitro* were found to transdominantly inhibit translation *in vivo* and to induce peptidyl-tRNA drop-off from ribosomes.

EXPERIMENTAL

Plasmids and strains. Plasmid ptBsB contains the *E. coli* 23S rRNA structural gene under control of the

Abbreviations: DTT, dithiothreitol; IPTG, isopropyl- β -D-thiogalactopyranoside; Pth, peptidyl-tRNA hydrolase.

tac promoter [20]. *E. coli* strain KH-1 [Δ (gpt-lac)⁵, supE44 = glnV44, gal t22, λ^- , dcm-6, dam3, thi-1/Fr, proAB-lac, lacI^q, lac2 Δ M15, pth^{ts}, tet^r] contains a temperature-sensitive allele of peptidyl-tRNA hydrolase, strain GM 1674 which contains the wild-type *pth* is isogenic to KH-1.

Sucrose gradient analysis of plasmid-borne 23S rRNA. Cultures of KH-1 cells transformed with ptBsB or its mutated derivatives were grown at 30°C in LB medium containing ampicillin (25 μ g/ml). Mutant rRNA expression was induced with 1 mM IPTG, polysomal gradients were prepared and analyzed for mutant rRNA content as described in [21].

Growth kinetics of bacteria. KH-1 cells were grown overnight at 30°C in 2 ml LB medium containing ampicillin (25 μ g/ml). Then 50 ml of LB was inoculated to OD₆₀₀ = 0.02. At OD = 0.1 the mutant rRNA expression was induced with IPTG (1 mM). Incubation was continued for additional 12 h with cell density measured every 60 min.

Incorporation of labeled amino acid into protein. Protein synthesis activity of KH-1 cells was determined essentially as described in [22]. Cells growing in 15 ml of M9 glucose medium containing 10 μ g/ml thiamine, 1mg/ml casein lysate, 20 μ g/ml uridine were induced with 1 mM IPTG at A₆₀₀ = 0.05–0.08. After growth for 2 h at 30°C the culture was divided into four 2-ml samples. For RNA labeling, two of the samples were supplemented with [³H]uridine (0.5 μ Ci/ml), for protein labeling the remaining two were supplemented with [¹⁴C]amino acids (0.5 μ Ci/ml). After 10, 30, and 60 min 200 μ l samples were collected and stopped with 200 μ l 20% TCA. Ice-cold TCA was used for [³H]uridine samples, whereas [¹⁴C]amino acid samples were heated at 95°C for 15 min to hydrolyze any charged tRNAs. The samples were filtered through Whatman GF/C filters, washed with cold 5% TCA, and counted in a liquid scintillation counter. The basal signal was determined by inhibiting translation with chloramphenicol (34 μ g/ml) and transcription with rifampicin (100 μ g/ml). Incorporation of ¹⁴C amino acids was corrected separately for transcription rates [³H]uridine incorporation and cell densities. Both corrections gave similar results.

Peptidyl-tRNA accumulation was assayed by viability of KH-1 (*pth*^{ts}) and by direct measurement of peptidyl-tRNA concentration. KH-1 and GM 1674 cells harboring mutant 23S rRNAs were shifted to elevated temperature (42°C), plated, and analyzed for viability as described in [11]. Isolation of total cell tRNA and measurements of the fraction of peptidyl-tRNA were conducted as described in [7]. Peptidyl-tRNA hydrolase-unmaskable leucine-accepting activity was measured as described in [7].

RESULTS AND DISCUSSION

Mutated 23S rRNA genes were expressed from the plasmid ptBsB under the control of IPTG-inducible *tac* promoter. To determine the fraction of mutant 23S rRNA in ribosomes, the single mutations were combined with the second site mutation A1067U. This expression system was earlier shown to encode functional 23S rRNA [17, 20]. In all other experiments, 23S rRNA genes with single-site mutations were used. Three *E. coli* strains were used for mutant rRNA expression. Strain KH-1 is temperature sensitive for peptidyl-tRNA hydrolase (Pth), the isogenic strain GM 1674 contains a wild-type allele of *pth*. XL-1 Blue which was used to verify the growth phenotype of 23S rRNA mutants is a standard laboratory strain with the wild-type *pth* allele.

Effect of Mutations on Cell Growth

Mutant 23S rRNA expression in KH-1 was induced with IPTG in the early exponential growth phase (A₆₀₀ = 0.03–0.05), and cell density was measured every hour. The effects of the mutations on the cell growth were tested by growing the cells in liquid media at 30°C where Pth is active. Plasmid ptBsB encoding the wild-type 23S rRNA gene was used as a control. The expression of 23S rRNA genes containing single mutations G2582A and G2583C completely blocked the growth of bacteria after 3–4 h of induction with IPTG (Fig. 1). The expression of the mutant gene G2582U lead to severe growth inhibition, while the expression of the mutation U2584C produced no effect on growth. Similar results were obtained using *E. coli* strain XL-1 Blue as the host for the expression of mutant 23S rRNAs at 37°C (data not shown).

Incorporation of Mutant 23S rRNAs into Ribosomes

Escherichia coli contains seven chromosomal rRNA operons. Expression of plasmid-borne mutant 23S rRNA, therefore, results in a mixed population of rRNAs. In order to differentiate between plasmid-encoded mutant and chromosomally encoded wild-type ribosomes, the marker mutation A1067U was used. KH-1 cells were grown at permissive temperature (30°C) and the expression of plasmid-borne 23S rRNA was induced with IPTG. Polysomes were fractionated by sucrose gradient centrifugation. 23S rRNA from the 50S ribosomal subunits, 70S ribosomes, and polysomes was prepared by phenol extraction. The A1067U marker mutation was determined by RNA sequencing using the primer extension procedure [23]. 23S rRNAs with the single marker A1067U constituted 38% of the total 23S rRNA in the 50S subunits and about 32% thereof in the 70S ribosomes and the polysomes (table). These fractions are in agree-

ment with the previous results [21]. The overall level of the 23S rRNA variant 2582U/1067U in the ribosomal fractions was the same as the level of control 23S rRNA with 1067U marker mutation, except that it was overrepresented in the 50S fraction. The 23S rRNA variants mutated at 2582A/1067U and 2583C/1067U were overrepresented in the 50S subunits and 70S ribosomes but underrepresented in trisomes (table). The fact that the 50S mutants 2582A and 2583C exhibit a fractional decline in the order 50S > 70S > disomes > trisomes and accumulate in the 50S and 70S gradient fractions indicates that these 23S rRNA mutations impair the translational capability of the mutant ribosomes.

Translational Activity of Bacteria Containing Mutant Ribosomes

The 23S rRNA mutations G2582A and G2583C lead to growth retardation and underrepresentation of mutant ribosomes in polysome fractions, indicating that the mutant 23S rRNA affects translation. The cell translational and transcriptional activities were assayed in the presence and absence of the mutant ribosomes in strain KH-1. The translational activity was assayed by measuring the incorporation of [¹⁴C] amino acids into protein and the activity of transcription by measuring the incorporation of [³H]uridine into total cell RNA. The total transcriptional activity of the bacteria was not affected by the expression of either mutants or wild-type plasmid-derived rRNA (data not shown). The level of ¹⁴C incorporation into proteins was normalized to the level of [³H]uridine incorporation into total cell RNA, which in its turn was normalized to the cell density (data not shown). There was no difference in the incorporation of [¹⁴C] amino acids or [³H]uridine between KH-1 cells harboring different plasmid constructs when grown without the inducer (data not shown). When the transcription of plasmid-encoded rRNA genes was induced, mutations G2582A and G2583C caused a 60% decrease in the level of incorporation of [¹⁴C] amino acid into peptides (Fig. 2). Therefore, these mutations cause a threefold reduction in total cell protein synthesis. Mutation U2584C had no effect on the total translational level *in vivo*. Since about 70% of the cell ribosomes are wild-type (table), mutations G2582A and G2583C must exert their influence on translation at least partly through inhibiting translation by wild-type ribosomes, i.e., by a transdominant effect.

The expression of 23S rRNA carrying the single point mutations G2582A or G2583C stopped cell growth completely. However, the mutant rRNA expression did not kill the cells as judged by the colony-forming ability (shown in Fig. 3). Only about one-third of the ribosomes present in the induced cells contained the mutant 23S rRNA. Therefore, the

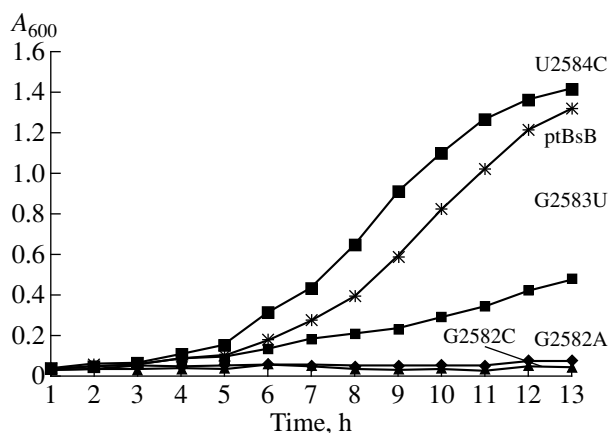


Fig. 1. Growth of KH-1 cells expressing mutant 23S rRNA at 30°C. The expression of the mutant rRNA was induced at $A_{600} = 0.05$ (at 1 h time point). Optical densities of the cultures at different time points are shown. 23S rRNA mutations are indicated at right margin. ptBsB denotes plasmid-encoded wild-type 23S rRNA.

mutant ribosomes have a dominant bacteriostatic phenotype.

Effect of Mutations on Peptidyl-tRNA Dissociation from Ribosomes

The strain KH-1 carries a temperature-sensitive *pth* allele. At 42°C the Pth protein is inactivated and peptidyl-tRNA released by translating ribosomes accumulates, resulting in cell death. The cell death rate reflects the rate of peptidyl-tRNA accumulation *in vivo* [24]. Therefore it is possible to infer peptidyl-tRNA accumulation from the colony-forming ability of KH-1 cells. Strain GM 1674, which is isogenic to

Mutant 23S rRNA incorporation into *E. coli* polysomes, 70S ribosomes, and 50S ribosomal subunits

Fraction	1067U	2582U/1067U	2582A/1067U	2583C/1067U
50S	38.2 ± 2.4	46.4 ± 4.5	48.0 ± 4.0	50.8 ± 1.9
70S	32.2 ± 1.9	33.8 ± 2.2	38.5 ± 2.75	40.0 ± 2.4
Disome	31.2 ± 3.1	31.0 ± 2.4	28.0 ± 3.0	27.5 ± 1.5
Trisome	32.2 ± 3.0	29.6 ± 1.4	21.0 ± 2.0	23.5 ± 3.5

Note: Cell lysates were fractionated by sucrose gradient centrifugation and plasmid-encoded 23S rRNA marker mutation A1067U was sequenced. The percentage of plasmid-encoded 23S rRNA in total 23S rRNA was calculated for 50S ribosomal subunits, 70S ribosomes, disomes, and trisomes. Average deviations were calculated in 2–4 independent experiments.

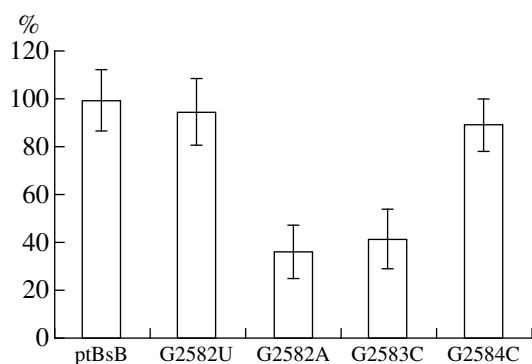


Fig. 2. Protein synthesis in KH-1 cells expressing the mutant 23S rRNA. Incorporation of [14 C]amino acids into peptides during min. was determined and normalized to the [3 H]uridine incorporation. The normalized values of protein synthesis in induced KH-1 cells to uninduced cells are shown as percentages. Average deviations in 2–4 experiments are indicated.

KH-1 but contains the wild-type allele of *pth*, was used as a control.

The expression of the mutant 23S rRNA genes did not affect the colony-forming ability of GM 1674

cells. Thus, in the presence of wild-type Pth, mutant ribosomes do not reduce the number of viable cells. The growth inhibition described above is therefore bacteriostatic. In contrast, inactivation of Pth at 42°C greatly reduced the ability of KH-1 cells expressing G2582A, G2582U, and G2583C to form colonies at 30°C (Figs. 3a–3c). Therefore it was conjectured that the mutations G2582A and G2583C cause an increase in peptidyl-tRNA accumulation rates. KH-1 cells containing ribosomes with the U2584C mutation exhibit the same colony-forming ability as the wild type (Fig. 3d).

For the G2582A and G2583C mutants, a more direct method was used to measure the intracellular concentration of peptidyl-tRNA. Total RNA was prepared from KH-1 cells grown at 42°C and incubated with CuSO_4 to deacylate the aminoacyl-tRNA. The peptidyl-tRNA remains intact in this procedure. A sample of the RNA was treated with purified Pth. Both Pth-treated and untreated RNA samples were aminoacylated with [14 C]leucine. The fraction of peptidyl-tRNA was calculated as the ratio of aminoacylation of the Pth-untreated to the Pth-treated RNA samples.

In KH-1 cells which express plasmid-encoded wild-type 23S rRNA, the fraction of peptidyl-tRNA

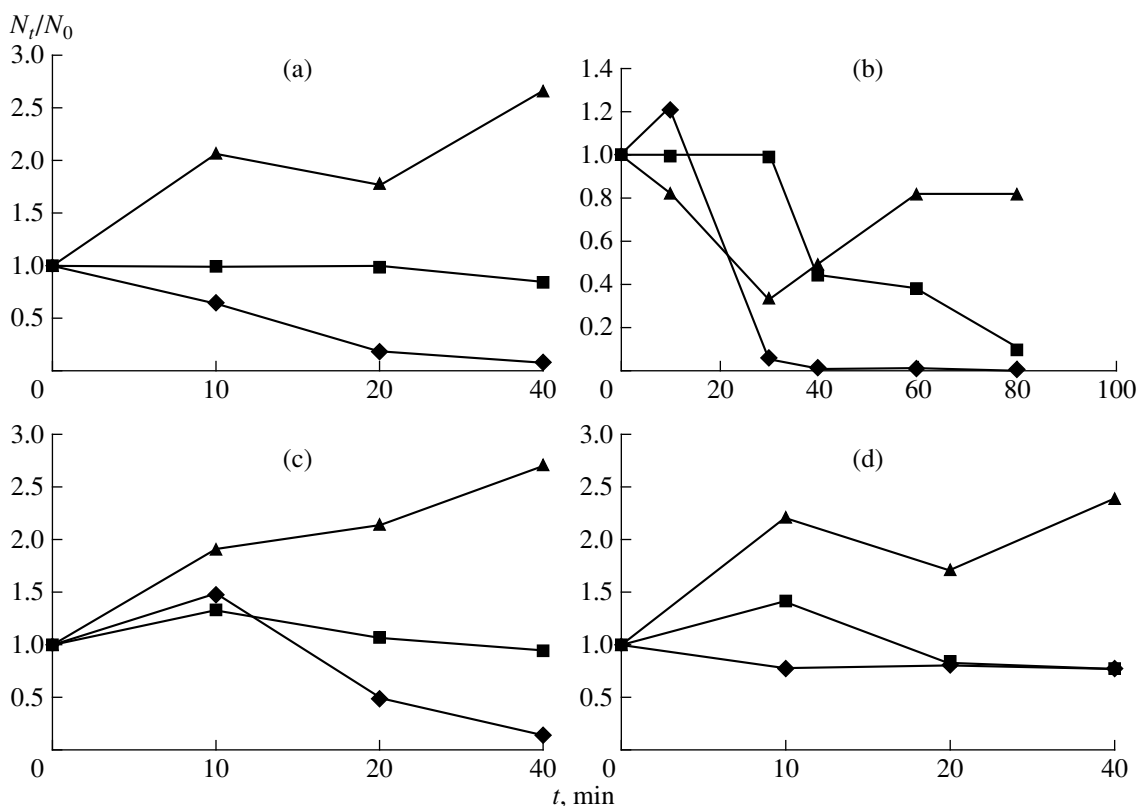


Fig. 3. Effect of mutant 23S rRNA expression on the cell survival at 42°C in KH-1(Pth^{ts}) and GM 1674 (wild-type) strains. Number of viable cells was normalized to time point 0. (◆) Induced KH-1 cells, (■) noninduced KH-1 cells, (▲) induced GM 1674 cells. (a) G2582A, (b) G2583C, (c) G2582U, (d) U2584C.

does not exceed 20% of total tRNA^{Leu} after incubation of cells at 42°C for 80 min. Mutations G2582A and G2583C cause about 50% of total cell tRNA to be masked by peptidyl residues (Fig. 4). These results corroborate those of the colony-forming assay, showing that mutations G2582A and G2583C cause increased peptidyl-tRNA dissociation from the ribosomes.

Nucleotides G2582–U2584 are located at or near the acceptor substrate-binding site in the ribosomal peptidyltransferase center [2]. Thus, the growth phenotype of the mutant 23S rRNA is likely to be caused by a translational defect. Indeed, translational activity of the cells carrying mutant ribosomes with G2582A and G2583C exhibited one-third of translation as compared with the wild-type control. The mutations G2582A (Ü, unpublished results) and G2583C [17] also reduced the ribosomal activity in the poly(U)-dependent *in vitro* translation system. Because the mutant ribosomes constitute only 1/3 of the total ribosome population, the threefold reduction in the translation level *in vivo* is not solely attributable to the reduced activity of the mutant ribosomes. The dominant effect of translation inhibition by the mutant ribosomes must be caused at least in part by inhibition of the wild-type ribosomes in mixed polysomes.

The fact that the expression of the mutant 23S rRNA leads to increased death rates of KH-1 cells at nonpermissive temperature (Fig. 3) and to the increased peptidyl-tRNA level in the cells (Fig. 4) indicates that the mutant ribosomes induce peptidyl-tRNA drop-off *in vivo*. The unequal distribution of the mutant 23S rRNA in sucrose gradient fractions (50S > 70S > disomes > trisomes) implies that the peptidyl-tRNA is released from the mutant ribosomes during early stages of elongation. It has been shown that in cells limited in Pth activity the translational defect can be compensated by overexpression of the tRNA^{Lys} gene, indicating that the limitation for tRNA^{Lys} is responsible for the defective growth [14]. On the other hand, loss of a peptidyl-tRNA from a ribosome could lead to ribosome stalling and thus to retardation of the ribosomes located upstream on the mRNA. The mutant ribosomes can thus inhibit translation also in the presence of functional Pth; therefore, the dominant growth effect and the dominant inhibition of translation are possibly not only caused by the lack of available tRNA.

It is known that the rate of peptidyl-tRNA drop-off is influenced by hyperaccuracy mutations in the ribosomal protein S12, error-prone mutations in ribosomal protein S4, antibiotics streptomycin, neomycin [9, 10], erythromycin, carbomycin, spiramycin [11], and by starvation for amino acids [12]. To our knowledge, we have found the first evidence that mutations in ribosomal RNA can influence peptidyl-tRNA drop-

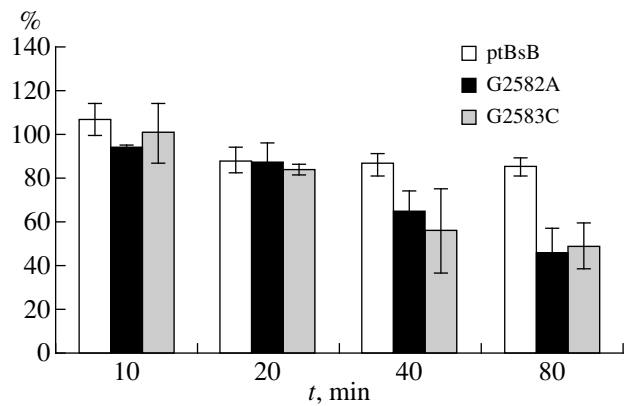


Fig. 4. Time course of accumulation of peptidyl-tRNA at 42°C in Pth temperature-sensitive KH-1 cells. Total cell RNA was extracted at time points indicated and used for aminoacylation assay. The fraction of tRNA that was aminoacylated with radioactive leucine before peptidyl-tRNA was hydrolyzed by Pth treatment is plotted as percentage. Average deviations in 2–4 independent experiments are shown.

off from translating ribosomes, and thus the processivity of translation.

Catalysis, accuracy, and processivity of translation all depend on the correct binding of tRNAs to the ribosome. Mutations in the top region of helix 90 may cause a decrease in tRNA binding stability in the ribosome and thereby influence the catalytic rate, accuracy and processivity of translation. Our results show that tRNA–23S rRNA interaction plays an important role in maintaining the processivity of translation.

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