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# Level of rRNA, not tRNA, Synthesis Controls Transcription of rRNA and tRNA Operons in *Escherichia coli*†

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**We have recently proposed a model for the negative feedback control of rRNA and tRNA synthesis in *Escherichia coli* by products of rRNA operons or their derivatives (e.g., nontranslating ribosomes) (S. Jinks-Robertson, R. L. Gourse, and M. Nomura, Cell 33:865–876, 1983). In this paper, we examined the following questions. (i) Are the spacer tRNAs carried within rRNA operons the products responsible for the regulation of rRNA and tRNA transcription? (ii) Are tRNAs capable of regulating their own syntheses? We measured tRNA accumulations in cells containing plasmids with intact or defective rRNA operons or with tRNA operons. From the results obtained, we conclude that neither the tRNAs encoded within rRNA operons nor the tRNAs encoded in non-rRNA operons are capable of controlling rRNA or tRNA transcription. Therefore, the products responsible for the initial step leading to rRNA and tRNA regulation are rRNAs (or their derivatives).**

The control of ribosome synthesis is a longstanding unresolved question in the regulation of gene expression (13). The fraction of ribosomes not actively engaged in protein synthesis is very small. *Escherichia coli* cells attain the number of ribosomes appropriate for the growth rate achieved under a particular set of nutritional conditions, a system termed growth-rate-dependent control. Because the translational feedback control of ribosomal protein synthesis is ultimately dependent on the level of rRNA synthesis (for review, see reference 15), an understanding of the mechanism of rRNA regulation is the key to an understanding of the control of the synthesis of the translational machinery.

Recently, we have proposed a model for the negative feedback regulation of rRNA and tRNA synthesis by nontranslating ribosomes, either directly or through some intermediate (12, 15). The results of rRNA gene dosage experiments were in agreement with the predictions of such a model: (i) the total instantaneous rate of rRNA synthesis was not significantly affected by an increase in gene dosage, and (ii) the accumulations of tRNAs encoded by chromosomal rRNA operons were repressed in the presence of rDNA-containing plasmids, indicating that individual rRNA operon transcription was reduced to keep total rRNA synthesis roughly constant. In addition, the accumulations of other tRNAs (not encoded within rRNA operons) were also repressed by the presence of rRNA plasmids. When large deletions were made in the rRNA coding regions of the plasmids, the regulatory effects were abolished: rRNA synthesis (intact plus defective) increased in a gene dose-dependent manner, and rRNA-encoded and non-rRNA-encoded tRNA accumulations were no longer reduced.

First, although these previous experiments showed that some product of rRNA operons (or some derivative of rRNA, e.g., ribosomes) caused the feedback regulation, the actual repressor was not identified. The experiments ruled out distal rRNA operon-encoded tRNAs as the molecules responsible for the regulation (plasmids deleting a central portion of rrnD, but leaving the $tRNA_1^{Thr}$ gene intact no longer repressed chromosomal rRNA-tRNA synthesis), but the experiments did not exclude the possibility that the spacer tRNAs encoded by rRNA operons ($tRNA_{1b}^{Ala}$, $tRNA_1^{Ile}$, $tRNA_2^{Glu}$) could be the repressors. Second, it was not rigorously proven that the products of tRNA operons do not regulate their own syntheses.

In this report, we demonstrate that rRNAs (or derivatives of the rRNAs, e.g., ribosomes) and not the spacer (or distal) tRNAs are the molecules responsible for the primary event controlling rRNA and tRNA regulation. In addition, we show that although tRNA operons are regulated by rRNA products, tRNAs are not also capable of regulating their own expression.

## MATERIALS AND METHODS

**Strains and plasmids.** The following bacterial strains and plasmids were used. Strain HB101 (=NO2842) was F⁻ *pro leu thi lacY rpsL20 endA recA ara-14 galK2 xyl-5 mtl-1 supE44 hsdR hsdM* (1). Plasmid pNO1301 consisted of a 6,877-base pair (bp) *Nae*I-to-*Bam*HI fragment of pKK3535 (3) ligated into *Nae*I-to-*Bam*HI sites of pBR322 (2). This plasmid was used in our previous work (12). pNO1301ΔStu-Sma (=pNO1314) was a derivative of pNO1301 constructed by *Stu*I-plus-*Sma*I digestion followed by dilution, religation, and transformation. The resulting deletion removes the region coding for bases 45 to 1,383 of 16S rRNA. pMMHA (=pNO2632) was a 5.6-kilobase (kb) *Hin*dIII fragment from pMM1 (17) that was isolated and inserted into pBR322 (T. Shibuya and T. Sekiya, unpublished data). The resulting plasmid contains the gene for $tRNA_{3b}^{Asn}$ together with its promoter and more than 1 kb of DNA upstream of the promoter. The plasmid also contains the gene for an unidentified RNA species we have designated "Z" (see below).

**Media, growth, and labeling conditions.** Strains containing rRNA and tRNA plasmids or derivatives were grown on LB plates (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 ml of 1 N NaOH, and 1.5% agar per liter) containing 50 μg of ampicillin per ml. For labeling experiments, MOS medium (14) supplemented with 0.2% glucose, 0.3 mM $K_2HPO_4$, 1 μg

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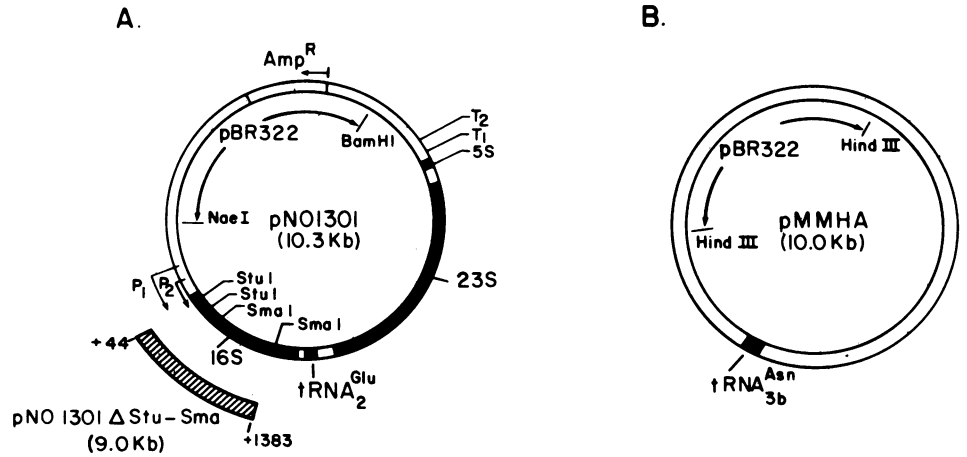


FIG. 1. (A) Structure of pNO1301 and pNO1301ΔStu-Sma. Relevant restriction sites and features are shown. Solid regions represent *rrnB* coding regions. Shaded bar represents a deletion in pNO1301ΔStu-Sma that codes for bases 45 to 1,383 of 16S rRNA. (B) Structure of pMMHA. A 5.6-kb *E. coli* *Hin*dIII fragment contains the gene for $tRNA_{3b}^{Asn}$ and another putative tRNA whose location has not been identified (see text). The orientation of the *Hin*dIII fragment in the plasmid was not determined.

of thiamine per ml, 40 μg of each amino acid per ml, and 100 μg of ampicillin per ml was inoculated with cells taken directly from LB ampicillin plates. Carrier cells for standardizing extraction efficiencies were prepared by uniformly labeling the same strain carrying pBR322 with either $^{33}PO_4$ (50 μCi/ml) or [$^3$H]uridine (100 μCi/ml). Cells carrying pBR322, pNO1301, pNO1301ΔStu-Sma, or pMMHA were grown for several generations and then labeled with $^{32}PO_4$ (25 μCi/ml) from an optical density at 600 nm of 0.10 to 0.50. Therefore, each sample contained the same amount of total cell mass. $^{33}PO_4$, or $^3$H-labeled carrier cells from the control strain were mixed with the $^{32}$P-labeled cells and RNA samples were extracted, electrophoresed in two dimensions, eluted, and counted as described previously (12). After experiments were completed, portions of each strain were plated, rapid plasmid DNA extractions were performed (6), and plasmid DNA was analyzed with restriction enzymes to ensure that the integrity of the plasmid structure was maintained.

**Copy number determinations.** Relative copy numbers of rRNA plasmids in strain HB101 were determined as described previously (12), by filter hybridization of total cellular DNA with saturating amounts of $^{32}$P-labeled 23S rRNA. For pMMHA, ca. 4 pmol of the 5.6-kb tRNA-containing fragment was purified by agarose gel electrophoresis, labeled with [γ-$^{32}$P]ATP with polynucleotide kinase, purified again, and denatured by heating for 5 min at 90°C in 10 mM Tris hydrochloride, pH 8.0. The denatured DNA was rapidly cooled on ice and diluted quickly and at least 20-fold with hybridization solution (100 mM Tris hydrochloride, pH 8.0, 600 mM NaCl, 20 mM disodium EDTA, 0.1% sodium dodecyl sulfate, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 50% deionized formamide) in vials containing filters with DNA from strains harboring either pBR322, pMMHA, or calf thymus DNA. Each vial contained filters from all three strains in triplicate. After hybridization at 42°C for about 16 h, the hybridization mixture was removed, and the filters were batch washed with 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for 2 h with several changes before drying and counting. The copy number obtained in this manner is a relative measure of the 5.6-kb fragment contained in the plasmid per genome; i.e., filters made with DNA from the strain containing pMMHA hybridized 17 times as much radioactive fragment as filters made with equal amounts of DNA from the strain containing only pBR322, irrespective of $^{32}$P-labeled fragment input.

## RESULTS

**Spacer tRNAs do not regulate rRNA and tRNA synthesis.** Since the plasmid deletions in the previous study (12) destroyed both the rRNA and spacer tRNA coding regions, we could not distinguish whether the product involved in the proposed feedback regulation was the rRNA or spacer tRNA. Since the reason that all seven *E. coli* rRNA operons encode spacer tRNAs is not known, a possible explanation for their presence would be that they are in fact responsible for the control of rRNA and tRNA transcription.

To test this hypothesis, we constructed a plasmid (pNO1301ΔStu-Sma; Fig. 1) with a large deletion of all but ca. 200 bp of the 1,542 bp 16S rRNA gene (Fig. 1), which leaves the promoters, spacer $tRNA_2^{Glu}$, the 23S and 5S rRNA genes, and transcription terminators intact. We reasoned that if the spacer tRNA were responsible for the regulation of rRNA and tRNA synthesis, then this plasmid should have an effect similar to that of the intact rRNA plasmid, pNO1301.

Cells containing the intact rRNA plasmid, pNO1301, the defective rRNA plasmid, pNO1301ΔStu-Sma, or a control plasmid, pBR322, were grown for several generations in the presence of $^{32}PO_4$. tRNAs were extracted in the presence of $^3$H- or $^{33}$P-labeled carrier cells and separated on two-dimensional gels, and accumulations were calculated as described in Table 1. The accumulations of tRNAs are a measure of the transcriptional activities of the rRNA and tRNA operons, since mature tRNAs are not degraded at an appreciable rate (18).

In agreement with our previous results, when the number of rRNA operons was increased ca. threefold by introducing multicopy plasmids containing rRNA operons, tRNAs were produced in reduced amounts (Table 1), which were close to the values predicted by the ribosome feedback model (referred to as complete regulation in Table 1). The accumulation of $tRNA_2^{Glu}$ increased, reflecting its presence in a higher percentage of the rRNA operons. On the other hand, in the presence of the extra defective rRNA operons (pNO1301ΔStu-Sma) no such regulatory response was seen; i.e., the accu-

TABLE 1. Accumulations of tRNAs and 5S RNAs from strains containing plasmids carrying intact or defective rRNA operons[a]

| tRNA or 5S RNA | Predicted value | | Observed value | |
|---|---|---|---|---|
| | No regulation | Complete regulation | pNO1301 | pNO1301ΔStu-Sma |
| 5S | 3.00 | 1.00 | 1.00 | 1.28[b] |
| 5SIII | 1.00 | 0.33 | 0.44 | 0.55[b] |
| Trp | 1.00 | 0.33 | 0.45 | 0.84 |
| Glu2 | 4.50 | 1.50 | 1.64 | 3.44 |
| Ala 1B | 1.00 | 0.33 | 0.42 | 0.95 |
| Ile | 1.00 | 0.33 | 0.47 | 0.88 |
| Leu + others | 1.00 | 0.33 | 0.57 | 0.86 |
| Ser 3 | 1.00 | 0.33 | 0.54 | 0.92 |
| Ser 1 | 1.00 | 0.33 | 0.55 | 1.01 |
| Tyr 2 | 1.00 | 0.33 | 0.54 | 1.22 |
| Met/Asn/Thr 4 | 1.00 | 0.33 | 0.50 | 0.99 |
| Val 1 | 1.00 | 0.33 | 0.49 | 0.87 |
| Phe | 1.00 | 0.33 | 0.55 | 1.17 |
| Y | 1.00 | 0.33 | 0.61 | 1.02 |
| Plasmid copy number[c] | 14 | 14 | 14 | N.D. |
| Growth rate[d] | | | 0.88 | 0.84 |

[a] Exponentially growing cells were labeled for two or three generations with $^{32}PO_4$ (25 μCi/ml), and equal amounts of each $^{32}P$-labeled sample were mixed with the same amount of carrier cells that had been uniformly labeled with $^{33}PO_4$ or [$^3$H]uridine. RNA was extracted from the mixed cells and analyzed by two-dimensional electrophoresis. After autoradiography, spots corresponding to individual tRNAs or 5S RNAs were cut from the gels and the $^{32}P/^{33}P$ or $^{32}P/^3H$ ratios were determined. The ratios given are relative values obtained with HB101 (pBR322) as the control and were calculated as [$^{32}P/^{33}P$ (or $^3H$) in $tRNA_x$ (experimental)]/[$^{32}P/^{33}P$ (or $^3H$) in $tRNA_x$ (control)]. The relative tRNA and 5S RNA values predicted by two different regulatory models (no regulation and complete regulation of rRNA synthesis) are given. For both models, the theoretical values were calculated by using a relative plasmid copy number of 14 (21 operons total, including the 7 chromosomal operons) a value obtained by hybridization of $^{32}P$ rRNA to total DNA from strains with and without pNO1301 as described previously (12).

[b] 5S RNA and its sequence variant 5SIII (10) are stable only when incorporated into ribosomes (18). Therefore, the apparent overproduction of 5S RNA in the uncontrolled case (pNO1301ΔStu-Sma) is not so high as would be predicted if the 5S RNA were stable. Likewise, the apparent underproduction of 5SIII in this case results from the fact that ribosomes do not distinguish between the electrophoretic variants and utilize some of the excess 5S RNA at the expense of the 5SIII species.

[c] Although plasmid copy number was not measured for the strain containing pNO1301ΔStu-Sma, copy numbers of similar deletion plasmids were measured and found to be the same as for pNO1301 (data not shown).

[d] Growth rates are relative to HB101 containing pBR322, which in this medium grew at a rate of 1.15 generations per h at 37°C.

mulations of the rRNA operon-encoded and non-rRNA operon-encoded tRNAs were the same as in the control. As predicted, if the product responsible for the regulation was no longer encoded by the plasmid, the spacer $tRNA_2^{Glu}$ was significantly overproduced. Therefore, the spacer tRNA cannot be sufficient to control rRNA and tRNA synthesis.

**$tRNA_{3b}^{Asn}$ does not regulate its own synthesis.** Since we unexpectedly found that products of rRNA genes control expression not only of rRNA but also of tRNA operons, we decided to test whether an additional feedback system exists for control of tRNA operons. That is, although rRNA products are sufficient to control tRNA operon expression, it is conceivable that products of tRNA operons could also feed back to control themselves. Therefore, we decided to test whether tRNA synthesis is gene dose dependent or independent.

For this purpose, we utilized a plasmid (pMMHA) containing the gene for $tRNA_{3b}^{Asn}$, obtained from T. Sekiya (Fig. 1b). A parent of this plasmid was originally identified by hybridization as carrying the *E. coli* gene for $tRNA_{3b}^{Asn}$ (17). pMMHA contains a 5.6-kb *Hin*dIII fragment subcloned into the *Hin*dIII site of pBR322. A section of ca. 900 bp that includes and surrounds the region of the hybridizing tRNA has been analyzed by DNA sequencing and confirms the identity of the gene as that for $tRNA_{3b}^{Asn}$ (T. Shibuya and T. Sekiya, unpublished).

When a strain containing pMMHA was analyzed for tRNA accumulations in the same way as described for the strains containing extra rRNA operons, it was found that the strain grossly overproduced a tRNA that migrated to the position of $tRNA_{3b}^{Asn}$. There was no significant effect on other tRNAs. Since $tRNA_{3b}^{Asn}$ comigrates with $tRNA^{Met}$ and $tRNA_4^{Thr}$ in this gel system, the apparent overproduction was only 8- to 10-fold rather than the 17-fold expected for complete gene dosage dependence. However, correction for the relative abundances of the three tRNAs in this spot (see legend to Table 2) (9, 20) makes it clear that $tRNA_{3b}^{Asn}$ transcription is gene dose dependent, i.e., there is no feedback regulation of $tRNA_{3b}^{Asn}$ synthesis.

In addition, a second spot that migrated in the general region of several other tRNAs was found to be strongly overproduced in strains containing pMMHA (Fig. 2, Table 2). This spot (Z), presumably an unidentified tRNA encoded somewhere within the 5.6-kb DNA fragment on pMMHA, was oversynthesized by about 16-fold, very close to the value expected for gene dose dependence. Therefore, extrapolating from these results and from other data in the literature (4, 5, 7, 11, 16), we conclude that tRNA synthesis is gene dosage dependent in *E. coli*; products of tRNA genes do not regulate their own syntheses or the syntheses of other tRNAs or rRNAs. Rather, tRNA synthesis is regulated by products of rRNA genes, possibly assembled but nontranslating ribosomes (see below).

## DISCUSSION

The data presented here complement studies presented previously (12) and provide further evidence for the ribosome feedback regulation model. The proposed mechanism differs in significant respects from earlier models, as discussed previously (12, 15). By this model, products of rRNA operons, most likely free nontranslating ribosomes, feed back either directly or indirectly, e.g., by activating some hypothetical factor, to negatively control rRNA and tRNA transcription.

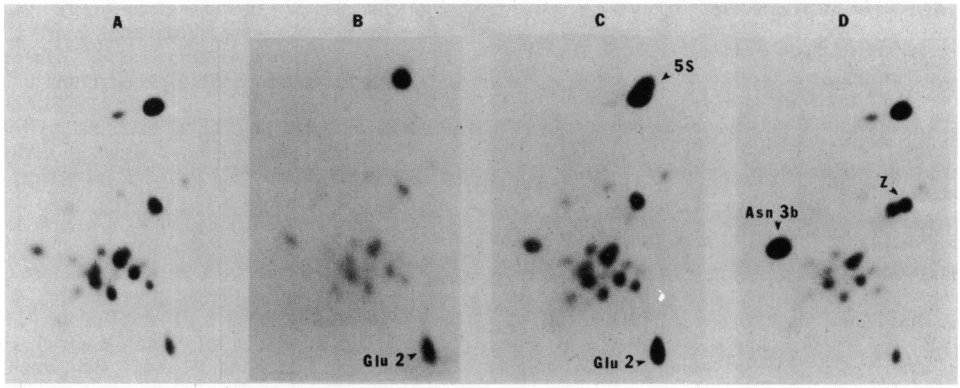


FIG. 2. Two-dimensional gels of small RNAs isolated from strains containing (A) pBR322, (B) pNO1301, (C) pNO1301ΔStu-Sma, and (D) pMMHA. Spots have been identified previously 12. Spots relevant to the present work are indicated. "Z" is an unidentified RNA species, presumably a tRNA, encoded by pMMHA (see text).

We showed that the spacer $tRNA_2^{Glu}$, and by inference other spacer tRNAs ($tRNA_1^{Ile}$ and $tRNA_{1B}^{Ala}$) encoded by rRNA operons, are not the factors responsible for this regulation. Previously, we have shown that defective rRNA operons containing distal tRNA genes but lacking 16S and 23S rRNA genes were not sufficient to regulate rRNA or tRNA synthesis (12). The present experiments with the defective 16S rRNA plasmid also show that 23S rRNA (or 5S RNA) is not sufficient to supply the feedback signal. Furthermore, since regulation is destroyed by deleting the 16S rRNA gene, 16S rRNA must be a part of the regulatory mechanism. Experiments in which only the 23S rRNA gene was deleted showed that 16S rRNA itself is not sufficient (data not shown). For the reasons cited below, however, we cannot be certain whether both subunits (as well as both rRNAs) are necessary for the postulated feedback mechanism.

TABLE 2. Accumulations of tRNAs and 5S RNAs from strains containing plasmids carrying tRNA operons[a]

| tRNA or 5S RNA | Predicted value (no regulation) | Observed pMMHA/pBR322 ratio[b] |
|---|---|---|
| 5S | 1.00 | 0.95 |
| 5SIII | 1.00 | 0.97 |
| Trp | 1.00 | 1.07 |
| Glu2 | 1.00 | 1.04 |
| Ala 1B | 1.00 | 0.99 |
| Ile | 1.00 | 0.94 |
| Leu + others | 1.00 | 0.97 |
| Ser 3 | 1.00 | 0.91 |
| Ser 1 | 1.00 | 0.87 |
| Tyr 1,2 | 1.00 | 1.10 |
| Met/Asn3b/Thr4 | 10.1[c] | 8.2 |
| Val | 1.00 | 0.87 |
| Phe | 1.00 | 0.92 |
| Y | 1.00 | 0.94 |
| Z | 17.00 | 15.7 |
| Plasmid copy number[d] | 17 | 17 |
| Growth[e] | | 0.83 |

[a] Described in the text and in Table 1, footnote *a*.

[b] Ratios are the means of two experiments.

[c] Predicted value for the $tRNA_{3b}^{Asn}$, $tRNA_m^{Met}$, $tRNA_4^{Thr}$ spot was determined based on the data of Ikemura (9). The abundances of $tRNA_{3b}^{Asn}$, $tRNA_m^{Met}$, and $tRNA_2^{Gly}$ relative to $tRNA_1^{Leu}$ were assigned values by Ikemura of 0.6, 0.3, and 0.15, respectively. Since $tRNA_4^{Thr}$ is cotranscribed with $tRNA_2^{Gly}$ (8), $tRNA_4^{Thr}$ was assumed to have an abundance of roughly the same value for purposes of this calculation. Therefore, for a copy number of 17, gene dosage dependent synthesis would predict $[(0.6 \times 17) + 0.3 + 0.15]/(0.6 + 0.3 + 0.15) = 10.1$.

[d] Copy number was determined as described in the text.

[e] Growth rate of HB101 containing pMMHA is relative to the same strain containing pBR322.

Ribosomal protein synthesis is controlled primarily through a feedback system that acts at the level of translation (for a review, see reference 15). Because of this system and the interspersion of genes for small and large subunit protein genes in the same operons, the syntheses of some ribosomal proteins found in 30S subunits are dependent on the concentration of 23S rRNA, and the syntheses of some ribosomal proteins from the 50S subunit are dependent on the concentration of 16S rRNA. In strains carrying plasmids deleting either 16S or 23S rRNA, assembly of both subunits is most likely somewhat affected. Because deletion of either subunit rRNA would most likely influence the assembly of both subunits, we cannot be certain from the present experiments whether both subunits are necessary for the postulated feedback regulation.

Several other investigators have noted that certain tRNAs are overproduced in *E. coli* when their gene copy number is increased (4, 5, 7, 11, 16). These overproductions were all relative to other cellular tRNAs, however, and it is formally possible that the tRNAs were not overproduced but that the others were underproduced. The experiments reported in this paper, however, are normalized only to total cell mass and therefore exclude arguments that could be leveled against relative measurements. The results shown in Table 2 and similar results using λ lysogens containing the genes for $tRNA_3^{Thr}$, $tRNA_4^{Thr}$, $tRNA_2^{Tyr}$, and $tRNA_2^{Gly}$ (our unpublished experiments) confirm the general conclusion of the previous investigators and show in a quantitative manner that tRNA synthesis is gene dose dependent.

Although these data do not rigorously prove that all tRNAs are synthesized in a gene dose-dependent manner, we note that probably all are repressed by the presence of extra rRNA operons. In various experiments, we examined accumulations of over 25 different tRNA spots with similar results (Table 1 and unpublished data). Therefore, as concluded previously (12), it appears that the regulation of tRNA synthesis is controlled by the regulatory system that has evolved to regulate the synthesis of ribosomes. This then would explain why both rRNA and tRNA synthesis rates display similar responses to changes in nutritional conditions (growth-rate-dependent control) (19).

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