The EMBO Journal vol.9 no.13 pp.4359-4366, 1990

Growth rate dependence of transfer RNA abundance in *Escherichia coli*

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Communicated by C.G.Kurland

We have tested the predictions of a model that accounts for the codon preferences of bacteria in terms of a growth maximization strategy. According to this model the tRNA species cognate to minor and major codons should be regulated differently under different growth conditions: the isoacceptors cognate to major codons should increase at fast growth rates while those cognate to minor codons should decrease at fast growth rates. We have used a quantitative Northern blotting technique to measure the abundance of the methionine and the leucine isoacceptor families over growth rates ranging from 0.5 to 2.1 doublings per hour. Five tRNA species that are cognate to major codons ($tRNA_e^{Met}$, $tRNA_1^{fMet}$, $tRNA_2^{fMet}$, $tRNA_1^{Leu}$ and $tRNA_3^{Leu}$) increase both as a relative fraction of total tRNA and in absolute concentration with increasing growth rates. Three tRNA species that are cognate to minor codons ($tRNA_2^{Leu}$, $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$) decrease as a relative fraction of total RNA and in absolute concentration with increasing growth rates. These data suggest that the abundances of groups of tRNA species are regulated in different ways, and that they are not regulated simply according to isoacceptor specificity. In particular, the data support the growth optimization model for codon bias.

Key words: codon preferences/growth dependence/major codons/minor codons/tRNA abundance

Introduction

There are 79 tRNA genes distributed throughout the *Escherichia coli* genome in 40 different operons (Komine *et al.*, 1990). These genes specify the structures of 46 different amino acid acceptor species. Detailed studies of the relative concentrations of the different tRNA species have suggested that they are regulated in a manner that is characteristic of the organism rather than its physiological state (Ikemura, 1981a,b; Skjold *et al.*, 1973). In particular, a striking correlation has been observed between on the one hand a subset of tRNA isoacceptors that are present in relatively high concentrations, and on the other hand, their cognate codons, which are used preferentially to code for the amino acids of the highly expressed proteins of *E.coli* (Grantham *et al.*, 1980a,b, 1981; Chavancy and Garel, 1981; Ikemura, 1981a,b, 1985; Gouy and Gautier, 1982; Grosjean and Fiers, 1982; Ikemura and Ozeki, 1983).

This correlation has led to the identification of the so called major codon preference strategy in which an optimal subset of codons and their cognate tRNA species are thought to be used to support the high expression levels of the major proteins (Chavancy and Garel, 1981; Ikemura, 1981; Grosjean and Fiers, 1982). Likewise, it has been suggested that rarely used codons together with their low concentration cognate isoacceptor species are used preferentially to limit the expression levels of the proteins at the opposite end of the expression scale (Robinson *et al.*, 1984; Varenne *et al.*, 1984; Bonekamp *et al.*, 1985; Varenne and Lazdunski, 1986).

There is, indeed, evidence that the major proteins are translated faster than other proteins and that the elongation rate at major codons is faster than that at other codons (Pedersen, 1984; Sørensen *et al.*, 1989). Nevertheless, there is also mounting evidence that the expression levels of individual genes are not regulated by codon usage (reviewed in Andersson and Kurland, 1990).

In general, the average rate at which a particular mRNA is translated will not influence the number of copies of the corresponding protein made unless that particular mRNA represents a major fraction of the total mRNA pool of the cell. Thus, the number of proteins copied from a particular mRNA species will depend on the number of ribosomes that translated each mRNA copy and the number of copies of the mRNA in question. For a minor mRNA species the average rate at which it is translated has no significant effect *per se* on either the number of ribosomes that translate it or on the number of times it is transcribed. In contrast, variations in the average translation rate for a mRNA species that is a major fraction of the total mRNA pool will directly influence the number of ribosomes available to translate that mRNA, and accordingly, under this condition the average translation rate can modulate protein expression levels. Since all of the mRNA species of a cell normally are minor species, the regulation of the expression levels for individual genes by modulating the average rates of translation is normally not feasible (Andersson and Kurland, 1990).

Nevertheless, there is a way around this constraint even for minor mRNA species and this depends not on the average translation rate but on the translation rates at the beginning of the mRNA. Thus, it has been suggested that codon usage modulates gene expression levels by influencing the queueing of ribosomes onto individual mRNA species (Liljenström and von Heijne, 1987). In such a model the number of ribosomes that can initiate translation of a mRNA is limited by ribosomes that block the initiation sequences because they pause at the beginning of the mRNA. Clearly, it is in principle possible to have pause sites for ribosomes anywhere in the mRNA sequence. However, the further they are from the initiation sequences, the greater is the number of ribosomes that can begin to translate the mRNA before the initiation sites are blocked. Therefore, the further the pause site is from the initiation sequences, the smaller is the queueing effect on the expression level for that protein species. In the extreme, there is a distance downstream from the initiation sequence beyond which the presence or absence

of a pause site will no longer limit the number of ribosomes that can initiate translation. Therefore, an analysis of the frequencies of codon usage in the initial sequences of different genes seems most relevant to queueing models. Indeed, the striking observation is that there is a non-random codon preference in the initial sequences of all of the bacterial genes analysed (Bulmer, 1988). However, both strongly expressed and weakly expressed genes are characterized by similar non-random initial codon strings. Therefore, there is no support for the idea that ribosome queueing regulates gene expression in *E.coli* (Bulmer, 1988).

Finally, it has been shown that the rare codons in the mRNA species that code low expression level proteins are not under strong selective pressure (Sharp and Li, 1986). In addition, short consensus sequences in the beginning segments of bacterial coding sequences have been identified, and these are complementary to sequences in the 5′ and the 3′ termini of 16S rRNA (Petersen *et al.*, 1988; Sprengart *et al.*, 1990). These sequences are likely to be involved in the mRNA interactions with ribosomes during initiation. More to the point, such consensus sequences would be vulnerable to disruption by the insertion of minor codons in the sorts of experiments done previously to demonstrate the infleunce of 'slow' codons on gene expression levels (Robinson *et al.*, 1984; Bonekamp *et al.*, 1985; Chen and Inouye, 1990). Thus, there seems little support for the suggestion that rare, slow codons, are used to limit the expression level of the corresponding genes. Instead, it seems very likely that minor codons are simply a reflection of the mutation pressures on coding sequences (Sharp and Li, 1986).

In contrast, there is evidently strong selective pressure for retaining major codons in the genes for highly expressed proteins (Sharp and Li, 1987). This is consistent with the interpretation of the major codon preference strategy as a global strategy to maximize the growth rates of bacteria at high growth rates rather than to modulate the expression levels of individual genes (Kurland, 1987; Andersson and Kurland, 1990). Such a global strategy is based on a theory of growth rate optimization in which the components of the cell are arranged so that the rates of translation normalized to the mass investment of the translation apparatus are maximized (Ehrenberg and Kurland, 1984).

Such an optimization at the highest growth rates can be obtained if the genes that encode those proteins that dominate the composition of the rapidly growing cells are made up preferentially of a subset of codons. According to this strategy, these preferred codons can be translated at high rates by a smaller total concentration of tRNA molecules than that required to translate at the same rates a random assortment of codons. At the lowest growth rates the protein population of the cell is heterogeneous, and the major proteins are far less dominant. This means that a more even distribution of codons is used in the mRNA population expressed at the lower growth rates. An optimal arrangement of the tRNA concentrations at the lower growth rates would require a larger amount of all tRNAs relative to ribosomes than at the highest growth rates as well as an adjustment of the individual isoacceptor concentrations to a more even distribution in order to match a less specialized distribution of codons in the mRNA population (Ehrenberg and Kurland, 1984; Kurland, 1987; Andersson and Kurland, 1990).

In effect, this growth optimization strategy requires a systematic variation of the relative concentrations of different isoacceptor species as a function of growth rate. Here, the tRNA species that are cognate to major codons should increase and the tRNA species cognate to minor codons should decrease as the growth rate increases. The present study was initiated to test this prediction. At the outset we knew that there were variations in the relative concentrations of different unidentified tRNA species (Fournier, personal communication). Our problem was to identify the different isoacceptor species and to quantitate their variations in different growth media. The present data show that tRNA species such as $tRNA_1^{Leu}$, $tRNA_3^{Leu}$, $tRNA_e^{Met}$ and $tRNA_{1,2}^{Metf}$ increase both in relative and absolute concentration when the growth rate increases. In contrast, tRNA species such as $tRNA_2^{Leu}$, $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ decrease drastically in the same growth rate range. The details of these systematic variations support the growth-optimization model.

Results

We have relied on Northern blotting to obtain quantitative estimates of the amounts of individual tRNA species present in unfractionated bacterial RNA prepared from bacteria cultured in various growth media. The particular blotting methods we used are described in detail by Smith and Yarus (1989) and the small variations that we introduced are presented in Materials and methods. The oligomeric deoxynucleotide probes we used had chain lengths of 18–22; each was selected for complementarity to non-conserved regions in a particular tRNA (see Materials and methods). The complementarity of the selected oligonucleotide probes was tested by computer with all known sequences for tRNA and rRNA species in *E.coli*. In general, there were at least eight non-complementary positions between the sequence of a DNA probe and any alignment with a non-cognate tRNA.

The specificity of the DNA probes was tested experimentally by hybridization to tRNAs that had been fractionated with a high resolution two-dimensional gel system (see Figure 1). All of the DNA probes, except the one cognate to the two *N*-formylmethionyl $tRNA^{fMet}$ species, hybridized with only a single electrophoretic component. The probe specific for the *N*-formylmethionyl $tRNA^{fMet}$ hybridized with two spots, which correspond to the two forms of this species that have either an A or a G at position 47 (Komine *et al.*, 1990).

Each of the radioactive DNA probes was individually calibrated in blotting titrations with purified cognate tRNA as described in Materials and methods. The probes could then be used to estimate the amounts of the cognate tRNA species by titration of samples of bacterial RNA in the same sort of blotting experiments. We consistently observed that all of the probes, with the exception of that for initiator tRNA, hybridize with only a single component in either one-dimensional or high resolution two-dimensional electrophoretic fractionations of total bacterial RNA (see Figure 1). Thus, we were unable to detect any sign of unprocessed precursors. This is consistent with the results of labelling experiments suggesting that the steady state quantities of the tRNA precursors are vanishingly small (Ikemura *et al.*, 1975). Likewise, we were unable to detect signs that the tRNA species studied here are heterogeneous with respect to degree of secondary modification.

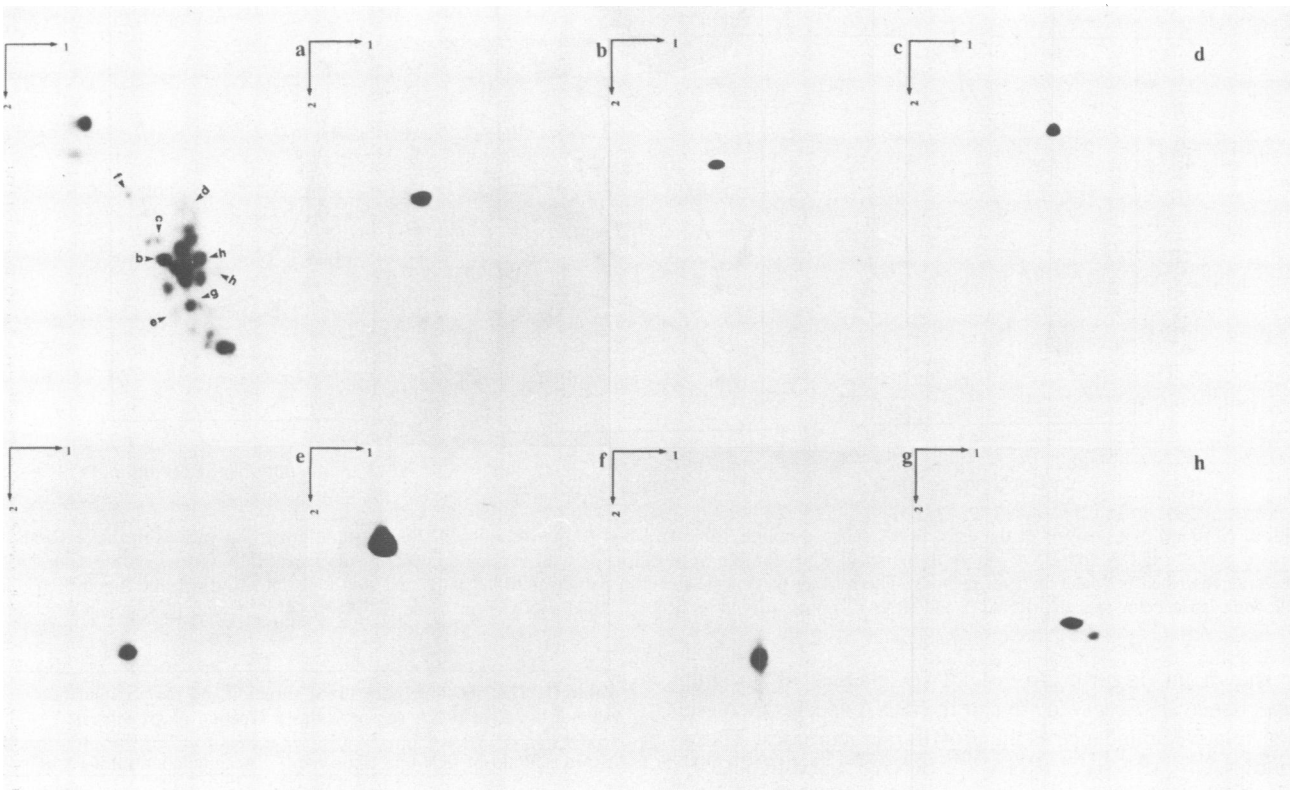

Fig. 1. Autoradiographs of two-dimensional gel pattern of small RNA and the Northern blot hybridization with seven specific ^{32}P-end-labelled DNA probes. The arrows indicate the gel dimensions and the origin of electrophoresis. Panel **a**: small RNA labelled *in vivo* as described by Ikemura and Ozeki (1977), was fractionated by a two-dimensional gel system (see Materials and methods). Arrowheads indicate the tRNAs that were hybridized to the probes (panels **b**–**h**), as described in Materials and methods. The probes were specific for $tRNA_1^{Leu}$ (panel **b**), $tRNA_2^{Leu}$ (panel **c**), $tRNA_3^{Leu}$ (panel **d**), $tRNA_4^{Leu}$ (panel **e**), $tRNA_5^{Leu}$ (panel **f**), $tRNA_e^{Met}$ (panel **g**), $tRNA_{1,2}^{fMet}$ (panel **h**).

Finally, we note that there are two conventions in the literature concerning the nomenclature of the five leucine tRNA isoacceptors, which depends on whether the tRNA species are biochemically or genetically defined (see Thorbjarnardottir *et al.*, 1985). Here, we follow the genetic convention as described in Table I.

Relative tRNA distributions

The tRNA levels were determined in two different ways. The first of these involved measurement of the relative amounts of particular isoacceptor species in a sample of so called small RNA, which is a chromatographic subfraction of total RNA that contains 5S rRNA, 4.5S RNA and tRNA (see Materials and methods). The tRNA and 5S rRNA are the predominant RNAs in the small RNA fraction. The 4.5 S RNA makes up ~1–2% of this fraction (Hsu *et al.*, 1984), and it is accordingly negligible. The 5S rRNA makes up a variable part of the small RNA fraction under different growth conditions. This variation was measured with the aid of a complementary DNA probe that had been calibrated in titrations with a pure 5S rRNA standard. We find that bacteria growing at the lowest growth rate we studied (0.5 doublings/h) yield a small RNA fraction containing 8% 5S rRNA, and that this increases to 13% at the highest growth rate we studied (2.1 doublings/h). We used the 5S rRNA data to calculate the amount of total tRNA present so that the results of the individual tRNA titrations can be expressed as a percentage of total tRNA.

Table I. Identity of the eight tRNA subspecies

tRNA[a,b]	Anticodon (5′–3′)	Predicted codon recognition (5′–3′)	RSCU[c]	
			low	high
Leu1 (4)	CAG	CUG	2.20	5.50
Leu2 (1)	GAG	CUU, CUC	0.70, 0.70	0.10, 0.20
Leu3 (1)	UAG	CUA, CUG	0.30, 2.20	0.04, 5.50
Leu4 (1)	CAA	UUG	0.90	0.10
Leu5 (1)	UAA	UUA, UUG	1.20, 0.90	0.10, 0.10
fMet1 (2)	CAU	AUG	1.00	1.00
fMet2 (1)	CAU	AUG	1.00	1.00
Mete (2)	CAU	AUG	1.00	1.00

[a,b]The tRNA and/or tDNA sequences are derived from Sprinzl *et al.* (1989) and Komine *et al.* (1990). The number of corresponding tRNA genes is shown in parenthesis.
[c]RSCU (Sharp and Li, 1986) represents the relative synonymous codon usage: the observed frequency of usage of a particular codon divided by the frequency expected if all synonymous codons are used with equal frequency. The RSCU values are shown for genes with low expression levels (low) and for genes with high expression levels (high).

The data describing the fraction of total tRNA invested in individual isoacceptor species as a function of growth rate

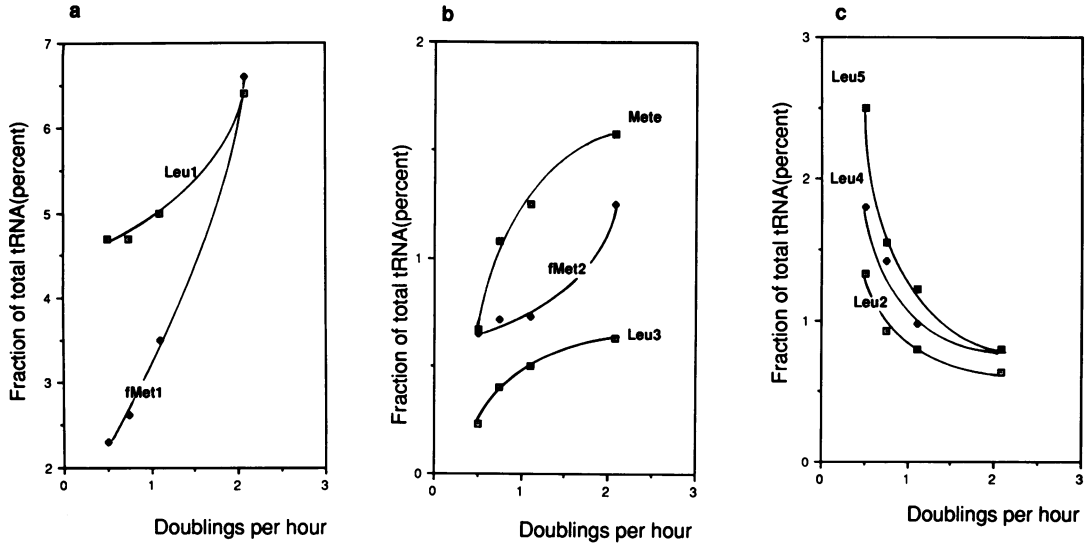

Fig. 2. Northern blot analysis of the eight tRNA subspecies (see Table I). Total RNA was isolated from bacteria grown on various media. Hybrids formed between tRNA and DNA probes in the small RNA sample were calibrated with varying amounts of the pure tRNA species on the same filters as described in Materials and methods. The relative percentage for each particular tRNA is plotted as a function of growth rate for the following isoacceptor species; $tRNA_1^{Leu}$ and $tRNA_1^{fMet}$ in **a**, $tRNA_3^{Leu}$, $tRNA_2^{fMet}$ and $tRNA_e^{Met}$ in **b** and $tRNA_2^{Leu}$, $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ in **c**.

is shown in Figure 2(a–c). Two classes of growth dependence are observed: five of the tRNA species increase their relative abundance as the growth rate increases. These are $tRNA_1^{Leu}$, $tRNA_3^{Leu}$, $tRNA_1^{fMet}$, $tRNA_2^{fMet}$ and $tRNA_e^{Met}$, for which the relative increases are 1.4- to 2.9-fold. In contrast, three tRNAs decrease their abundance as the growth rate increases. These are $tRNA_2^{Leu}$, $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$, for which the decreases are 2- to 3.1-fold. These data show that groups of individual tRNA species are regulated in different ways that are dependent on the growth state of the bacteria.

Ribosome-normalized variations

The second sort of measurement we carried out involved one dimensional electrophoresis on a horizontal agarose gel of unfractionated bacterial RNA followed by simultaneous titrations with the aid of DNA probes for both 16S rRNA and individual tRNA species Here, the probes were calibrated with 16S rRNA and tRNA standards which were run in the same gels as the unfractionated test samples. In this way we take advantage of the fact that 16S rRNA is present in one copy per ribosome (Kurland, 1960) and that the amounts of free 16S rRNA in the steady state are negligible (Lindahl, 1975). This suggests that all free 16S rRNA measured from the cells, is ribosome associated *in vivo*. Furthermore, the fraction of total ribosomes actively involved in peptide chain elongation *in vivo* is 80% (Forchhammer and Lindahl, 1971). Accordingly, the normalization of the amounts of individual tRNAs to the 16S rRNA provides both an internal standard as well as a measure of physiological interest (see below).

The data obtained in this way for the leucine and methionine isoacceptors are summarized in Table II. Comparison of the estimates for the individual isoacceptors relative to each other at each particular growth rate reveals excellent agreement between the data obtained from the total RNA and those from the small RNA fraction. Since these are independent estimates they support the interpretation that the variations we observe for the different tRNA species are not due to selective loss of one or the other component during the preparation of the RNA fractions.

Table II. The ribosome normalized variation and the accumulation rates of the eight tRNA subspecies as a function of growth rate

tRNA	Ratio of tRNA to ribosome				Accumulation rates ($\times 10^9$)[a]			
	0.5/h	0.8/h	1.1/h	2.1/h	0.5/h	0.8/h	1.1/h	2.1/h
Leu1	0.40	0.39	0.33	0.33	16.4	26.7	37.8	96.4
Leu2	0.15	0.10	0.07	0.03	6.0	6.8	8.0	9.0
Leu3	0.02	0.03	0.03	0.03	0.8	2.1	3.4	9.0
Leu4	0.20	0.12	0.07	0.03	8.2	8.2	9.0	9.0
Leu5	0.25	0.13	0.08	0.03	10.2	9.0	9.0	9.0
fMet1	0.23	0.24	0.25	0.25	9.4	16.0	29.0	75.0
fMet2	0.07	0.06	0.05	0.05	2.9	4.1	5.7	15.4
Mete	0.10	0.10	0.09	0.07	4.1	6.8	10.3	21.1

[a]Amount of tRNA accumulated per doubling time and cell mass.

The normalization to rRNA provides an additional perspective on the growth rate dependent variations of the tRNA because the ratio of total tRNA to rRNA is also a growth rate dependent parameter (Kjeldgaard and Kurland, 1963; Bremer and Dennis, 1987). This normalization accentuates the decrease in the abundance of the minor leucine isoacceptors ($tRNA_2^{Leu}$, $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$) that attends the increase in the growth rate. In particular, their ratios to ribosomes decrease between 5-fold and 9-fold, and at the highest rate converge to a common value of 0.03. In contrast, the ribosome-normalized abundances for the other five tRNAs ($tRNA_1^{Leu}$, $tRNA_3^{Leu}$, $tRNA_1^{fMet}$, $tRNA_2^{fMet}$ and $tRNA_e^{Met}$) are constant or very nearly constant at all growth rates.

Discussion

We have studied the abundance of eight tRNA species as a function of bacterial growth rates. These eight species represent two contrasting isoacceptor families. The methionine family was chosen because in both the initiation

Table III. The intracellular concentration of the eight tRNA subspecies as a function of growth rate

Growth rate doubling/h	Cell volume nl/10^6 cells[a]	$[tRNA]_i$; M ($\times 10^{-6}$)[b]							
		Leu1	Leu2	Leu3	Leu4	Leu5	fMet1	fMet2	Mete
0.5	0.22	15.0	5.7	0.8	8.0	9.4	8.1	2.6	3.8
0.8	0.36	15.1	4.0	1.2	4.8	5.2	9.6	2.4	4.0
1.1	0.57	14.6	3.0	1.3	3.1	3.5	10.9	2.2	4.0
2.1	1.31	26.0	2.4	2.4	2.4	2.4	20.3	4.1	5.7

[a]The cellular volume was estimated from the density values described by Churchward *et al.* (1981) and from the cell mass variation with growth rate as described by Bremer and Dennis (1987).
[b]The number of tRNAs per cell was calculated from our measurements (see Table II) and from the data of Bremer and Dennis (1987). The concentration values were then calculated from the moles and the cell volumes.

and the elongation modes these three tRNA species are cognate to only one codon. The antithesis is provided by the leucine family which contains five tRNA species that translate six different codons (see Table I). The data show that all three of the methionine acceptors and two of the leucine acceptors ($tRNA_1^{Leu}$ and $tRNA_3^{Leu}$) make up an increasing fraction of the total tRNA as the growth rate increases, while the three remaining leucine acceptors ($tRNA_2^{Leu}$, $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$) represent a decreasing fraction of the total in the same range of growth rates. These data are in agreement with our expectations.

The prediction is that the relative abundance of tRNA species that translate major codons should increase while those that translate minor codons should decrease as the growth rate increases. Since there is only one codon for methionine, AUG is by definition a major codon and the behaviour of all the members of this family is straightforward, and consistent with the expectation. The leucine family is far more interesting.

The calculations of Sharp and Li (1986) clearly identify CUG as the only major leucine codon used by *E.coli* (see Table I). The three isoacceptors ($tRNA_2^{Leu}$, $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$) that translate minor leucine codons decrease in relative abundance as the growth rate increases. Not surprisingly, the unique major leucine acceptor ($tRNA_1^{Leu}$) that translates CUG increases in abundance as the growth rate increases. The fascinating isoacceptor species is $tRNA_3^{Leu}$, which can translate the minor codon CUA as well as the major codon CUG. This minor tRNA species increases in relative abundance as the growth rate increases. Such behaviour suggests that it is the cognate codon and not the characteristic abundance of an isoacceptor species that determines its response to changes in the growth state of the bacteria.

The measurements of the ribosome-normalized tRNA abundances reveals another distinctive aspect of the growth rate dependence of the different isoacceptors. Those species that decrease in relative amounts at the faster growth rates are obviously present in sharply lower amounts per 16S rRNA as the ribosome abundance increases with the growth rate. However, the more striking correlation is that those isoacceptors that increase in relative amount maintain at all growth rates a relatively constant relationship to the ribosome abundance. Such behaviour suggests that there may be at least two distinguishable modes for the expression of different tRNA genes: one that tightly couples the expression of some tRNA species to that of rRNA and the other that decouples the expression level of the other tRNA species from that of rRNA.

Another way of viewing this phenomenon emerges from calculations of the net accumulation rate for each tRNA as a function of growth rate. This parameter is calculated as follows: the mass ratio of the individual tRNA species to 16S rRNA was measured (see Table II) and this converted to the molar ratio of each tRNA to total ribosomes. The concentrations of ribosomes per cell mass have been estimated by Bremer and Dennis (1987); from their figures and ours the individual tRNA concentrations per cell mass can be calculated. Finally, the growth rate is used to calculate the rate of tRNA accumulation per mass of bacteria per unit time for each of the eight isoacceptors.

The calculations summarized in Table II show that the net accumulation rates per mass of bacteria for $tRNA_2^{Leu}$, $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ are nearly constant in the growth range we studied. In contrast, $tRNA_1^{Leu}$ and $tRNA_3^{Leu}$ increase over the whole growth range as do the three methionine isoacceptors. In other words, the two classes of tRNA are distinguishable by the fact that the one is produced at a virtually constant rate per cell mass, while the production of the other accelerates together with that of the rRNA species.

As we note in Table I, the genes for the major tRNA species that we have studied are found in multiple copies on the bacterial genome, while the minor tRNA species are represented by single genes. This is a general pattern (Komine *et al.*, 1990) and it is tempting to believe that it has something to do with the ways that the different tRNA species are accumulated under different growth conditions. Likewise, the same data suggest that the only tRNA genes to be found within the rRNA operons of *E.coli* are those corresponding to the major tRNA species. Here too we might guess that this arrangement would facilitate a coupling between the abundance of some major tRNA species and ribosomes at different growth rates. Nevertheless, the present data, while useful in defining the patterns of RNA accumulation at different growth rates, do not permit us to draw direct conclusions about the mechanisms that regulate the accumulation of the different RNA species.

We note in passing that as for other components of the translation system (Bremer and Dennis, 1987; van der Meide *et al.*, 1983), the initiation factor 2 (IF2) and the ribosome levels have been found to increase coordinately with cell growth rate so that their molar ratio is maintained close to 0.3 IF2 molecules per ribosome (Howe and Hershey, 1983). Likewise, we have observed here that the sum of the initiator tRNA species is held at a molar ratio close to 0.3 per ribosome at all growth rates (see Table II). Such tight coordinate regulation that leads to equal initiator tRNA and IF2 concentrations under all growth conditions illustrates the exquisite precision with which the expression levels of the

components of the translation system are maintained.

Our expectations for how the abundance of individual tRNA species would respond to changes in the growth rates is based ultimately on a kinetic analysis of the translation system in which a key role is played by the concentrations of ternary complexes (Ehrenberg and Kurland, 1984). According to this analysis an optimal strategy to maximize the efficiency of translation at the highest growth rates would be to use a subset of codons to code the proteins that are produced in large amounts and to adjust the concentrations of individual tRNA isoacceptors to this codon preference (Kurland, 1987; Andersson and Kurland, 1989). Therefore, we require a calculation of the concentrations of the isoacceptors at the different growth rates to test our predictions.

As mentioned above we could calculate the individual tRNA to bacterial mass ratio with the aid of the data of Bremer and Dennis (1987). These figures could be converted to tRNA concentration with the aid of the cellular volume measurements of Churchward *et al.* (1981). As can be seen in Table III, there is a 6-fold increase of cell volume over the 4-fold range of growth rates that we have studied. The corresponding concentrations have been calculated from these figures for the eight tRNA species. The isoacceptor concentrations range from 0.8 to 26 $\times$ 10^{-6} M. The average concentration is 6.8 $\times$ 10^{-6} M at the lowest growth rate and 8.2 $\times$ 10^{-6} M at the highest growth rates. The important points in the present context are that the five isoacceptors that translate the preferred codons increase monotonically in concentration with the growth rate while the three that translate minor codons decrease over the same range (Table III). Although it is too soon to be certain that our codon preference strategy has been definitively verified, the data obtained so far do seem encouraging.

Finally, these variations in the concentrations of isoacceptors may be relevant to the growth rate dependent variations of the translation rates for different mRNA species observed by Pedersen (1984), who noted a marked increase of the elongation rate for major proteins and a more modest one for a few minor proteins when the media was enriched. A more definitive analysis of the consequences of tRNA isoacceptor variation on translation rates will require data about the concentrations *in vivo* of the remaining tRNA species as well as information about the kinetics of their interactions with the ribosomes. At the present time we have good kinetic characterizations in translation *in vitro* for one tRNA species, $tRNA^{Phe}$ (e.g. Diaz *et al.*, 1986). In the absence of more relevant data, we have used the rate constants describing the interaction of Phe$-tRNA^{Phe}$ ternary complex with poly(U) programmed ribosomes *in vitro* to calculate a very tentative kinetic for the interaction *in vivo* between ribosomes and ternary complexes containing the Leu and Met isoacceptors. This calculation together with the data in Table III suggests that at the highest growth rates $tRNA_1^{Leu}$ comes within 80–90% saturation of its cognate codons and $tRNA_e^{Met}$ comes close to 50% saturation at the highest growth rates. At the lowest growth rates the abundance of the major $tRNA^{Leu}$ and $tRNA^{Met}$ isoacceptor species together have been reduced, but these levels still exceed the concentrations of the individual minor species (see Table III). Accordingly, these data suggest that at the lowest growth rates, all codons are translated at rates far from the maximum.

Materials and methods

Bacterial strains

The bacterial strain used for RNA determinations was *E.coli* W1485, which was derived from W3100 (Guyer *et al.*, 1981). Cells were grown in M9 supplemented with vitamin B1 (0.01 mM), $FeCl_3$ (0.03 mM), $CaCl_2$ (0.1 mM) and carbon sources as indicated: acetate, 0.4%; succinate, 0.4%; or glucose 0.4%. In addition, rich medium contained 20 amino acids, purines and pyrimidines as described by Neidhardt *et al.* (1974). Cells were grown aerobically at 37°C under vigorous shaking in a water bath. To maintain cells in a steady state of balanced growth for at least 10 generations, log phase cultures were serially diluted into fresh, prewarmed medium. Cultures were harvested on ice at an A_{450} close to 0.8, centrifuged and washed with buffer (25 mM Tris–HCl pH 7.4, 60 mM KCl, 10 mM $MgCl_2$ and 20% sucrose).

Preparation of RNA

Circa 6 mg (dry weight) cells were resuspended in 200 μl of the above buffer without sucrose but containing instead 4 mg/ml lysozyme. This suspension was incubated for 10 min at room temperature, and the cells were lysed by freezing in a dry ice–ethanol mixture and then thawing them at 45°C; this was done three times. To the lysed cells was added 340 μl detergent solution (25 mM Tris–HCl pH 7.4, 60 mM KCl, 10 mM $MgCl_2$, 0.6% Brij58, 0.2% Na-deoxycholate, 0.02% SDS) and 60 μl phenol. After mixing with a vortex, the suspension was incubated on ice for 15 min. Then 600 μl phenol was added, and the nucleic acids were extracted from the protein mass. The water layer was then re-extracted with phenol. The interphase and the phenol layers were re-extracted again to ensure good yield. Finally the water layers were combined, and used as a sample to determine the levels of 16S rRNA and tRNA species (see section on Northern hybridization).

The recovery of total RNA for the crude extracts was >85% at all growth states. The estimates of recovery were done according to the method of Ryals *et al.* (1982). Small RNA (tRNA, 5S rRNA, 4.5S RNA) was recovered from the crude extracts as follows: the total RNA in the combined water layers was precipitated in 2.5 volumes of ethanol containing 50 mM NaAc. The RNA pellet was dried, and dissolved in 2 ml of buffer containing 10 mM Tris, pH 7.5, 10 mM $MgCl_2$ and 0.1 M NaCl, and then it was applied to a DEAE-cellulose column (bed vol. 200 μl), that had previously been equilibrated with 15 ml of the same buffer. The column was washed with 20 ml of buffer. Small RNA was then eluted with 2 ml buffer that had been adjusted to 1 M NaCl. The RNA was ethanol precipitated as described above. This protocol yields the so called small RNA which is predominantly tRNA, but it also contains 5S rRNA and 4.5S RNA.

The leucine and the methionine tRNAs, used as standards in the Northern hybridization assays (see section of Northern hybridization) were purified partially on benzoylated DEAE-cellulose columns (Gillam *et al.*, 1967) and subsequently chromatographed on Sepharose 4B with reverse $(NH_4)_2SO_4$ gradients (Holms *et al.*, 1975). The leucine and the methionine accepting activities were identified by standard amino acylation tests and collected. The purity of the isoacceptor tRNAs was tested on gels, and if needed the tRNAs were further purified to homogeneity on high concentration polyacrylamide–bis gels (see section on Electrophoretic fractionation). The identity of the tRNA subspecies was determined by Northern hybridization with specific DNA probes (see section of Northern hybridization). The $tRNA_3^{Leu}$ was first identified on transfer blots from 2D gels before it was purified from the gels.

Electrophoretic fractionation

The RNA species in the crude cell extracts, and those in the small RNA samples, were fractionated on horizontal 2.5% agarose gels with recycling TAE pH 7.5 buffer system (Tris–acetate 40 mM, EDTA 1 mM). Various amounts of total RNA in the crude extracts, or 2–3 μg small RNA were diluted into 10 μl formamide plus 2 μl loading buffer type III (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol) before being applied to the gel. Electrophoresis was carried out at 150 V for 1–2 h.

Both the identification and the purification of the tRNA standards as well as the quantitative estimations of the two initiator $tRNA^{fMet}$ species could be done by electrophoresis in polyacrylamide gels of different compositions. The 2D gel system was particularly useful to provide high resolution of RNA components for the detection of slowly processed tRNA precursors as well as for testing the specificity of probes. In certain cases 2D gels were used to purify tRNAs to homogeneity.

The two initiator tRNAs could be separated on one-dimensional 12% polyacrylamide 7 M urea gels. In general the 2D gel system employed in the first dimension a 10% polyacrylamide gel containing 7 M urea (15 $\times$ 18 $\times$ 0.10 cm), and in the second dimension a 20% polyacrylamide

gel containing 4 M urea (15 × 18 × 0.10 cm). The ratio of acrylamide to bisacrylamide was 19:1. The first dimensional gels were polymerized at 4°C overnight and then prerun at 80 V for 4 h at 4°C in TBE (100 mM Tris pH 8.3, 100 mM boric acid, 2 mM EDTA). Just prior to electrophoresis the samples were diluted in formamide-dye solution to a final concentration less than 1 μg/1 μl. The electrophoresis was carried out at 150 V for 20 h at 4°C. The second dimensional gel was cast with the first dimensional gel slice, which had been rotated 90°, positioned on the top of the gel plate. The 20% gel was poured to a level ~1 mm below the gel slice. Finally the 10% gel was sealed with 10% polyacrylamide gel solution lacking urea. After 4 h polymerization at room temperature electrophoresis was first carried out at 180 V for 4 h and then at 240 V in the same buffer for 32 h at room temperature.

Northern hybridization

The amounts of the tRNAs, 5S rRNA and the 16S rRNA were determined by Northern hybridization. The method we used differs slightly from the method of Smith and Yarus (1989).

The RNA was fractionated on gels, as described above, and transferred to an Amersham Hybond-N hybridization filter in a Trans-blot cell. The gels were incubated for 15 min in the transfer buffer, 25 mM sodium phosphate pH 6.5, before the electrotransfer. For quick tests we used a Bio-Dot slot format blotting apparatus. 1–3 μg of RNA sample was diluted in 400 μl of 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) and then spotted on Hybond-N filter, which had been prewetted first in water and then in 20 × SSC. RNA was then fixed to the membrane by UV crosslinking on a transilluminator for 9 min. The filters were prehybridized for 4 h at 60°C in 10 ml of 900 mM NaCl, 90 mM Tris pH 7.5, 9 mM EDTA, 1% SDS, 1 mg/ml polyvinylpyrrolidine (mol. wt 40 000), 1 mg/ml ficol (mol. wt 400 000) and denatured salmon sperm DNA (>200 μg/ml). The hybridization was carried out for 24 h, in 5–10 ml of the same solution plus 20–30 pmol 5′ ^{32}P-labelled DNA probe. The hybridization was initiated at 60°C and the temperature was gradually lowered to room temperature. The filters were then rinsed first twice in 6 × SCC and then once in 3 × SCC plus 0.5% SDS, at room temperature. The filter was finally exposed to Kodak XAR film. The labelled bands were excised from the filter and the radioactivity was measured in a liquid scintillation counter by standard techniques. In some cases we used a scanning counter to analyse the autoradiograms.

The tRNA specific probes used were synthetic DNA oligonucleotides with chain lengths of 18–22 that were complementary to the TF stem, the variable loop, anticodon stem and anticodon loop sequences of the tRNAs studied (Leu1: 5′-CCCCCACGTCCGTAAGGACA-3′, Leu2: 5′-CCCGTAAGCCCTATTGGGCA-3′, Leu3: 5′-CACCTTGCGGCGCCAGAA-3′, Leu4: 5′-CCGGCACGTATTTCTACGG-3′, Leu5: 5′-CCCGCACAGCGCGAACGCCG-3′, fMet1,2: 5′-CGGGTTATGAGCCCGACGA-3′, Mete: 5′-CCTGTGACCCCATCATTATGAG-3′). The 16S rRNA specific DNA probe was an 18mer complementary to a conserved region from positions 562–579 (Noller and Woese, 1981), 5′-TGCGCTTTACGCCCAGTA-3′. The 5S rRNA probe was a 25mer which matches all 5S rRNA species of *E.coli*, 5′-TACGGCGTTTCACTTCTGAGTTCGG-3′.

The amounts of the individual tRNA species were normalized to that of 16S rRNA in the crude cell extracts by quantitative estimates of the corresponding hybridized DNA probes obtained in a single blotting experiment. In this way, the 16S rRNA could function as an internal standard for our different measurements; this obviated the need to have 100% recovery of our samples. In order to calibrate the radioactive DNA probes a titration was performed with a series of predetermined quantities of purified RNA species that were hybridized in the same blotting sheet. These quantitative titrations provided us with a characteristic specificity activity from which the amounts of each RNA species could be estimated on the basis of the amounts of the corresponding radioactive probe that were recovered in the blotting sheets.

The detection limit of the probes was ~1 ng of pure tRNA. We found that our titration curves yielded linear correlations between radioactivity in probes and pmol of RNA over the range of 10–90 ng of pure RNA. We observed that the reproducibility of the absolute tRNA determinations in the small RNA samples was within a 10% standard deviation for all the species that were studied. On the other hand, when the tRNA levels are normalized to 16S rRNA, the standard errors of the mean tended to be <6%, which further testifies to the usefulness of the 16S rRNA internal standard.

Chemicals and enzymes

Samples of 16S rRNA and 5S rRNA were purchased from Boehringer. [^{32}P]ATP used for labelling the DNA probes and the Hybond-N membranes were purchased from Amersham. The polynucleotide kinase that was used to label the probes was purchased from Pharmacia. Formamide, phenol and the components used for the electrophoresis were of ultrapure grade, purchased from IBI (International Biotechnologies, Inc.).

Acknowlegements

We are grateful to Skip Fournier for sharing unpublished data and protocols with us. We are indebted to Mike Yarus for his generous hospitality and to him as well as D.Smith for their time, their interest as well as the knowledge that helped to set us on the right road. This work was supported by The Swedish Cancer Society and The Natural Sciences Research Council.

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Received on June 27, 1990; revised on September 17, 1990