

Origins of minigene-dependent growth inhibition in bacterial cells

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The expression of very short open reading frames in *Escherichia coli* can lead to the inhibition of translation and an arrest in cell growth. Inhibition occurs because peptidyl-tRNA hydrolase fails to recycle sufficiently rapidly peptidyl-tRNA released from ribosomes at the stop signal in competition with normal termination, causing starvation for essential species of tRNA. Previous studies have shown that the last sense codon, the strength of the Shine–Dalgarno sequence and the nature and context of the stop codon affect the toxicity associated with mini-gene expression. Here, several important parameters are studied as a function of the length of the mini-gene coding sequence. The rate of peptidyl-tRNA drop-off catalysed by translation factors decreases dramatically for peptides longer than a hexamer. The probability that ribosomes recycle without dissociation of the mini-gene mRNA varies strongly with the length of the coding sequence. The peptidyl-tRNA hydrolase *rap* mutant, unlike the wild-type enzyme, is highly sensitive to the length and sequence of the peptide. Together, these parameters explain the length dependence of mini-gene toxicity.

Keywords: mini-gene/peptidyl-tRNA/release factor/
ribosome recycling/translation termination

Introduction

During normal protein synthesis in bacterial cells, ribosomes attach to mRNA at the ribosome binding site, translate the open reading frame (ORF) until a termination codon is reached, and with the aid of codon-specific termination (or release) factors (RF), release the completed polypeptide chain into the cytoplasm by hydrolysis on the ribosome of the ester linkage between the polypeptide and tRNA (Hershey, 1987; Buckingham *et al.*, 1997). Another reaction can, however, compete with normal termination, whereby peptidyl-tRNA dissociates from the ribosome before hydrolysis of the ester bond (Menninger, 1976; Hernández *et al.*, 1997; Heurgué-Hamard *et al.*, 1998). The peptidyl-tRNA released is then hydrolysed by the enzyme peptidyl-tRNA hydrolase (Pth), allowing the tRNA to be charged by the cognate

aminoacyl-tRNA synthetase and reutilized in protein synthesis. Premature dissociation of peptidyl-tRNA from the ribosome ('drop-off') is a normal accompaniment of protein synthesis, and Pth is essential to cell growth (Atherly and Menninger, 1972; Schmitt *et al.*, 1997).

Recent work has shown that two sets of proteins catalyse drop-off. One set is composed of three factors involved in the process of ribosome recycling (Karimi *et al.*, 1999): termination factor RF3, ribosome recycling factor RRF and elongation factor EF-G, the last of which primarily catalyses the translocation of peptidyl-tRNA from A- to P-site (Haenni and Lucas-Lenard, 1968). The initiation factors IF1 and IF2 comprise a second set of proteins stimulating drop-off (Karimi *et al.*, 1998). Mutations reducing the level of expression of RRF, and others inactivating RF3, reduce drop-off *in vivo* (Heurgué-Hamard *et al.*, 1998). *In vitro*, the three proteins RF3, RRF and EF-G can stimulate the drop-off of dipeptidyl-tRNA at least 30-fold (Dinçbas *et al.*, 1999). Although the experiments *in vivo* offer convincing evidence for the physiological significance of RRF and RF3 in the drop-off reaction, it remains unknown, however, whether their role in stimulating drop-off is restricted to short peptides or whether a wide range of chain lengths is concerned.

When the frequency of drop-off exceeds the capacity of Pth to recycle the tRNAs sequestered as peptidyl-tRNA, starvation for essential tRNA isoacceptors occurs, leading to an inhibition of protein synthesis and eventually to cell death. This is believed to account for the shutdown in protein synthesis that takes place when a temperature-sensitive *pth* mutant is shifted to 43°C (Atherly and Menninger, 1972). A related phenomenon appears when certain mutants of *Escherichia coli*, *rap*, are infected with bacteriophage λ . The *rap* locus is identical to *pth*, and *rap* mutants exhibit reduced Pth activity for model substrates (Garcia Villegas *et al.*, 1991; Schmitt *et al.*, 1997). These mutants are unable to maintain normal bacteriophage vegetative growth, due to the expression of very short, phage-encoded ORFs, 'mini-genes', encoded by the *bar* loci of phage λ . When the *bar* regions are carried on plasmids, the induction of their expression can kill *rap* mutant cells. The transcription of the *barI* and *barII* regions yields RNA molecules containing Shine–Dalgarno (SD)-type sequences, followed by the translation initiation codon AUG, a single additional sense codon and a translational stop signal. Further experiments indicate that the transcripts are translated, producing fMet-Ile-tRNA^{Ile}, but that dipeptide release brought about by termination factors RF1 and RF2 is partially defective, leading to dipeptidyl-tRNA^{Ile} drop-off and starvation for tRNA^{Ile} (Hernández-Sánchez *et al.*, 1998).

Recent studies have shown that multiple parameters related to the sequence of the mini-gene transcript contribute to the growth inhibitory effect (Dinçbas *et al.*,

Table 1. Parameters related to peptidyl-tRNA accumulation and toxicity index I_{tox} for different peptidyl-tRNAs

Plasmid	Peptidyl-tRNA	Pth activity ($\times 10^{-5}$) k_{cat}/K_m ($M^{-1}s^{-1}$)		Dissociation rate ($\times 10^2$) s^{-1}		Termination by RF1 k_{cat}/K_m ($\mu M^{-1}s^{-1}$)		Termination by RF2 k_{cat}/K_m ($\mu M^{-1}s^{-1}$)		Recycle number ^a < n > _{rec}	I_{tox}^b Pth wild type	
		Pth (<i>rap</i>)	Pth wild type	No factors	RRF, EF-G, RF3			Pth (<i>rap</i>)				
SD7	fMK-tRNA ^{Lys}	0.195 (± 0.012)	14.5 (± 0.5)	0.13 (± 0.01)	1.71 (± 0.17)	70 (± 2)	2.00 (± 0.42)	>250	>3.5			
SD8	fMFK-tRNA ^{Lys}	0.590 (± 0.031)	16.1 (± 1.1)	0.11 (± 0.01)	1.17 (± 0.05)	70 (± 10)	2.18 (± 0.47)	27 (± 9)	1.0			
SD9	fMFIK-tRNA ^{Lys}	0.489 (± 0.037)	15.8 (± 1.9)	0.23 (± 0.02)	2.07 (± 0.11)	97 (± 5)	4.65 (± 1.0)	39 (± 16)	1.21 (± 0.56)			
SD10	fMITK-tRNA ^{Lys}	0.594 (± 0.027)	21.4 (± 2)	0.23 (± 0.01)	1.90 (± 0.01)	89 (± 6)	6.8 (± 1.6)	21 (± 10)	0.58 (± 0.32)			
SD11	fMITQK-tRNA ^{Lys}	0.100 (± 0.011)	10.2 (± 1.6)	0.13 (± 0.01)	1.46 (± 0.19)	71 (± 10)	7.2 (± 1.5)	47 (± 15)	0.45 (± 0.18)			
SD12	fMITQLK-tRNA ^{Lys}	0.755 (± 0.025)	20.0 (± 2.0)	0.08 (± 0.02)	0.27 (± 0.03)	77 (± 8)	10.4 (± 2.3)	0.74 (± 0.24)	0.028 (± 0.011)			
SD13	fMITQLTK-tRNA ^{Lys}	1.525 (± 0.06)	23.6 (± 1.5)	0.16 (± 0.02)	0.33 (± 0.06)	86 (± 12)	8.9 (± 1.9)	0.26 (± 0.09)	0.017 (± 0.007)			

Values are given with the standard deviation from the mean, calculated from at least three determinations.

^aRecycle number is the average number of times a ribosome translates a mRNA without the 30S subunit leaving the mRNA.

^bThe definition of relative toxicity index is given in the text; it is calculated using an average of the k_{cat}/K_m values for RF1 and RF2, weighted according to their relative concentrations (1:5) in the cell (Adamski *et al.*, 1994). The dissociation rate used is that in the presence of factors RRF, RF3 and EF-G.

Values are normalized to that for SD8 with wild-type Pth.

1999). Thus, when the relatively weak ribosome binding sites of the natural *bar* SD regions are replaced by an SD sequence that interacts strongly with 16S rRNA, mini-gene expression can be lethal even in cells with wild-type Pth, confirming observations by Tenson *et al.* (1999). In part, this is due to the increased probability that ribosomes retranslate the same mini-mRNA without dissociating from it after peptide release. Three further parameters expected to affect the rate of peptidyl-tRNA accumulation, namely, termination efficiency, rate of drop-off and rate of hydrolysis of the resulting peptidyl-tRNA by Pth, were shown to vary according to the last sense codon in the mini-mRNA, the stop codon present and the nucleotide following the stop codon (Dinçbas *et al.*, 1999).

Here, we study the effect of the length of mini-genes on the parameters affecting peptidyl-tRNA accumulation. The rate of peptidyl-tRNA drop-off increases dramatically for peptidyl-tRNA species <7 amino acids long, due to a greater stimulation of drop-off by RF3, RRF and EF-G. In addition, the rate of translation termination catalysed by RF2 increases when the number of mini-gene codons varies from 2 to 8. The variation in activity of wild-type Pth according to the length of the peptide is weak, but this becomes an important factor in the case of the Pth *rap* mutant. Finally, the probability that ribosomes recycle without leaving the mini-mRNA varies strongly with the length of the coding sequence. Taken together, these parameters provide an explanation for the fact that toxic mini-genes preferentially encode very short peptides.

Results

The development of an *in vitro* translation with purified components that is able to translate short synthetic mRNAs has made it possible to isolate ribosomal complexes paused with a stop codon in the A-site and containing a peptidyl-tRNA of known composition in the P-site (Freistroffer *et al.*, 1997). Such termination complexes are obtained by initiating translation on mini-gene transcripts using initiation factors and fMet-tRNA^{Met}, and translating the short ORF in the presence of all other necessary components but in the absence of termination factors. The paused complexes may then be purified from other components by gel filtration and used to study the kinetics of termination or drop-off of peptidyl-tRNA (Heurgué-Hamard *et al.*, 1998; Dinçbas *et al.*, 1999).

Pth activity for different substrates

Previous work (Dinçbas *et al.*, 1999), in which the activity of Pth (*rap*) was measured on peptidyl-tRNA substrates differing by the nature of the tRNA species and the last amino acid, showed that fMet-Lys-tRNA^{Lys} was the least and fMet-Phe-tRNA^{Phe} the most efficiently cleaved among the substrates studied. The rates of hydrolysis differed by a factor close to four. Two constructs showed furthermore that the cleavage efficiency could be greatly improved merely by increasing the length of the peptidyl-tRNA by two amino acids. We have now studied more systematically the activity of both the *rap* mutant of Pth and the wild-type enzyme on substrates varying in peptide length from 2 to 8 amino acids with Lys as the last amino acid, the smallest being fMet-Lys-tRNA^{Lys}. This peptidyl-tRNA arises from the translation of a mini-gene shown to be the

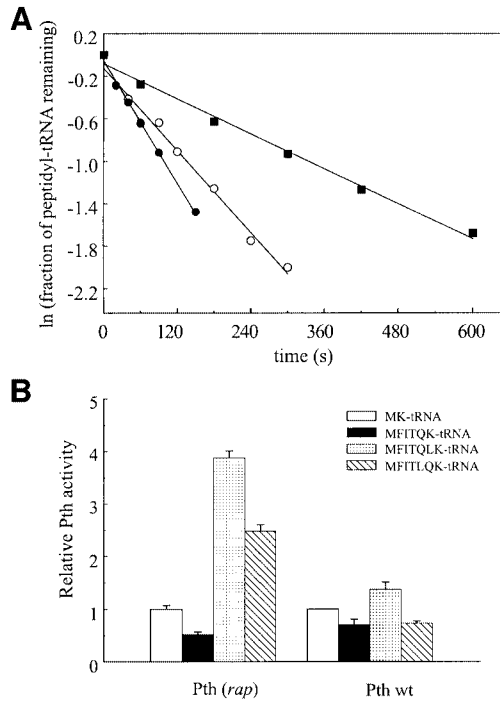


Fig. 1. Pth (*rap*) activity on different peptidyl-tRNAs. (A) Hydrolysis of fMet-Lys-tRNA^{Lys} (filled squares), fMet-Phe-Ile-Lys-tRNA^{Lys} (open circles), fMet-Phe-Ile-Thr-Gln-Leu-Lys-tRNA^{Lys} (filled circles) in the presence of 0.13 μM Pth (*rap*). (B) Hydrolysis of different peptidyl-tRNA^{Lys} in the presence of 0.13 μM Pth (*rap*) or 4 nM Pth wild type (in competition experiments). Relative activities are given using an activity of 1 for fMet-Lys-tRNA^{Lys} hydrolysis.

most toxic among a substantial set when expressed in the *pth* (*rap*) mutant. The Pth substrates were obtained from release complexes consisting of 70S ribosomes bound to mRNA, with the stop codon UAA located at the A-site and peptidyl-tRNA at the P-site. The complexes were incubated with RRF, RF3 and EF-G, known to promote efficient dissociation of peptidyl-tRNA (Heurgué-Hamard *et al.*, 1998; Dinçbas *et al.*, 1999). The k_{cat}/K_m values for the Pth (*rap*) mutant and wild-type Pth are shown in Table I (columns 3 and 4, respectively) and a typical experiment in Figure 1A. The k_{cat}/K_m values for wild-type Pth and Pth (*rap*) cleavage of fMK-tRNA^{Lys}, to which other values are normalized, were estimated at $1.45 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $1.95 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, respectively.

Pth (*rap*) cleaved the longest peptidyl-tRNA fMFITQLTK-tRNA^{Lys} with an efficiency almost eight times higher than the dipeptidyl-tRNA fMK-tRNA^{Lys}. Within this set the cleavage efficiency increased by a factor of three with the transition from two to three amino acids, remained almost constant until the length reached five amino acids, and then increased again. However, anomalous behaviour was observed for fMFITQK-tRNA^{Lys} (Table I, line 5), which is cleaved even more poorly than fMK-tRNA^{Lys}. An attempt was made to see whether the C-terminal dipeptide sequence might be responsible by synthesizing a new mRNA (02.MFITLQK.Oa; see Materials and methods for mRNA nomenclature) differing from 02.MFITQLK.Oa by inversion of the two codons next to the last. A significantly reduced Pth (*rap*) activity was indeed observed towards the new substrate (relative activity of

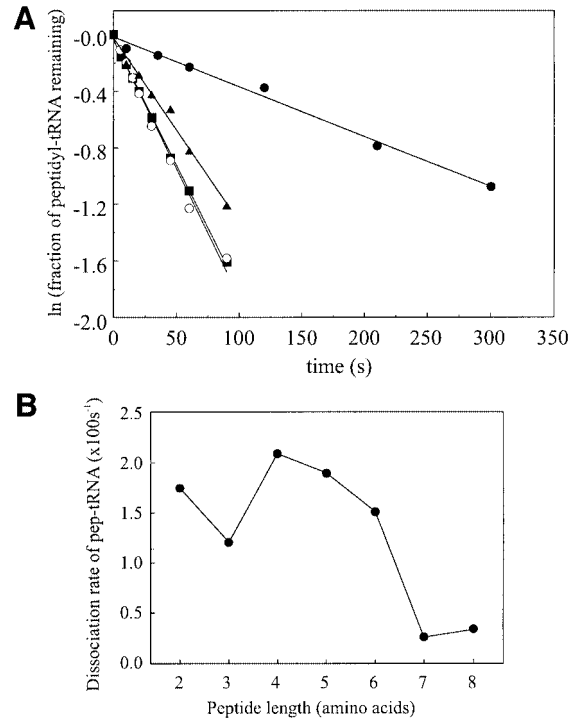


Fig. 2. (A) Dissociation of peptidyl-tRNA from ribosomal complexes in the presence of 1 μM RRF, RF3 and EF-G: fMet-Phe-Ile-Thr-Gln-Leu-Lys-tRNA^{Lys} (filled circles), fMet-Phe-Ile-Thr-Gln-Lys-tRNA^{Lys} (filled triangles), fMet-Phe-Ile-Lys-tRNA^{Lys} (open circles), fMet-Lys-tRNA^{Lys} (filled squares). (B) Dissociation of peptidyl-tRNA stimulated by RRF, RF3 and EF-G at 1 μM concentration as a function of peptide length of peptidyl-tRNA^{Lys}.

2.5 versus 3.9, Figure 1B), but not to the extent of fully explaining the anomalous behaviour of fMFITQK-tRNA^{Lys}. The very inefficient cleavage of this substrate may be due to a combination of peptide sequence (Gln-Lys as the last amino acids) and peptide length.

The activity of wild-type Pth is much less affected by peptide length, but is qualitatively similar (Table I, column 4). The large differences reported previously by Shiloach *et al.* (1975) in the case of wild-type Pth activity towards (Gly)_n-Val-tRNA of different length was not apparent in our experiments. As in the case of the *rap* mutant of Pth, we observed that fMFITQK-tRNA^{Lys} was inefficiently cleaved. In this case inverting the two amino acids next to the last of fMFITQLK-tRNA^{Lys} also decreased the relative activity, by ~2-fold (relative activity of 0.73 versus 1.37, Figure 1B).

Drop-off of peptidyl-tRNA with varying peptide length

Drop-off rates of peptidyl-tRNA were measured *in vitro* from a series of release complexes with peptidyl-tRNA in P-site and a UAA stop codon in A-site (see Materials and methods) as previously described (Heurgué-Hamard *et al.*, 1998), in the absence of normal termination. The essential difference between the peptidyl-tRNA species studied was the length of the peptide. The same last sense codon, stop codon and downstream sequence were used throughout the series to avoid effects when a sense codon replaced the stop codon, a different stop codon was used, or the A-site codon was changed (Heurgué-Hamard *et al.*, 1998;

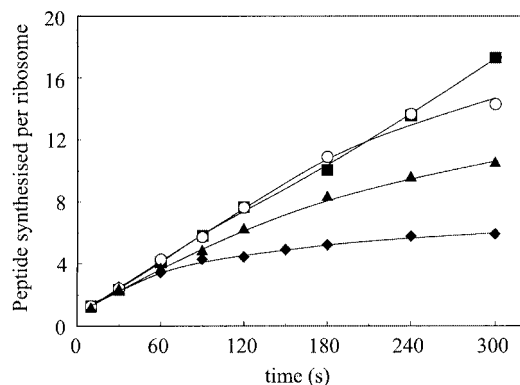


Fig. 3. Recycling of ribosomes on mRNA 02.MK.Oa (filled squares), 02.MFIK.Oa (open circles), 02.MFITQK.Oa (filled triangles) and 02.MFITQLTK.Oa (filled diamonds) in the presence of chase mRNA. The curves were analysed (see Materials and methods) to determine the average number of times $\langle n \rangle_{rec}$ that an mRNA is translated before it leaves the ribosome and is replaced with another mRNA.

Dinçbas *et al.*, 1999). Drop-off for each peptidyl-tRNA was measured in either the presence or the absence of factors RRF, RF3 and EF-G known to stimulate drop-off (Heurgué-Hamard *et al.*, 1998). The results are shown in Figure 2A and Table I (columns 5 and 6). In the absence of translation factors, the drop-off rate does not depend regularly on peptide length. Thus, the dissociation rate of fMFITQLTK-tRNA^{Lys} is almost the same as that for fMK-tRNA^{Lys}. In contrast, the dissociation rate of peptidyl-tRNA in the presence of RRF, RF3 and EF-G depends strongly on peptide length, decreasing dramatically when it reaches 7 amino acids (Figure 2B). The low value for tripeptidyl-tRNA in relation to dipeptidyl- and tetrapeptidyl-tRNA indicates a dependence on sequence of both factor-catalysed and non-catalysed dissociation (Table I, lines 1–3).

Termination of translation of mini-genes

Termination rates catalysed by RF1 and RF2 were measured from the same release complexes used to measure drop-off rates of peptidyl-tRNA. When two release complexes compete for limited amounts of RF, it is possible to estimate relative k_{cat}/K_m values for the termination reaction (Pavlov *et al.*, 1998). Absolute values for the dipeptidyl-tRNA substrate were determined as described previously (Dinçbas *et al.*, 1999), and used to calculate k_{cat}/K_m values for the remaining substrates (Table I, columns 7 and 8). Significant variations were observed for RF2, but not for RF1, termination becoming more efficient as peptide length increases. As in the case of the previous parameters, the peptide sequence may play an active role, in line with reports that the amino acids present at the C-termini of proteins affect peptide release (Björnsson *et al.*, 1996).

Recycling of ribosomes on mini-mRNAs

Previous work showed that minigene toxicity could not be fully understood by consideration of only the parameters described above: Pth activity, drop-off rate and termination efficiency (Dinçbas *et al.*, 1999). Efficiency of translation was clearly a further critical point, especially when the strength of the SD sequence was different. Although minigene toxicity was first described in the case

Table II. Effect of mini-gene expression on cell growth: growth rates (generations/h) of Xac *rap* and Xac strains at 37°C following IPTG induction

Plasmid	Mini-gene overexpressed	Xac <i>rap</i> 0.1 mM IPTG	Xac 1 mM IPTG
SD7	02.MK.Oa	no transformant	1.24
SD8	02.MFK.Oa	0.50	1.91
SD9	02.MFIK.Oa	0.63	1.92
SD10	02.MFITK.Oa	1.49	2.06
SD11	02.MFITQK.Oa	1.02	2.05
SD12	02.MFITQLK.Oa	2.29	2.12
SD13	02.MFITQLTK.Oa	2.32	2.11
pTrc99c	none	2.33	2.12

pTrc99c is the control vector without insert.

of *bar* mRNA expression in the *pth* (*rap*) mutant, it is not restricted to Pth-deficient strains (Dinçbas *et al.*, 1999; Tenson *et al.*, 1999). Toxicity depends to a considerable extent on the nature of the SD sequence, which in the λ *bar* sequences is poorly complementary to 16S RNA. Replacement of the *bar* SD sequence by a more efficient one, like SD 002 (Calogero *et al.*, 1988) makes the *bar1* minigene toxic in wild-type Pth cells, pointing to the efficiency of translation as an important parameter.

In the *in vitro* system it is possible to measure the ribosomal recycling time (Pavlov *et al.*, 1997a,b), which represents the average time for a ribosome between two initiations on mRNA, and includes the time required for initiation, elongation, termination and recycling. Initiation may take place either on the same mRNA molecule, or on another molecule following dissociation of the 30S subunit from the mRNA. However, if a second messenger with a different sequence is added in excess after initiation on the first mRNA, the probabilities of these alternatives may be measured. Previously, we showed that the probability of reinitiating translation on the same mRNA molecule was almost 100% for a messenger with SD 002, and 40% for a weaker SD from the λ *bar1* gene, a large difference that could explain the longer recycling time for messengers with weak SD sequence. We have now measured this probability for the series of different mRNAs, all possessing the same strong SD 002 but containing from two to eight sense codons. These results are shown in Table I, column 8 and in Figure 3. They show that the mRNA encoding fMK will on average be translated >100 times before leaving the ribosome, and reveal a strong inverse relationship between this recycling number and length of the mini-gene coding sequence.

Growth inhibition due to expression of mini-genes of varying length: relation with a toxicity index (I_{tox}) defined from biochemical parameters

Mini-genes were cloned in the pTrc99c vector (Amann *et al.*, 1988) downstream of the *trc* promoter, which allows expression to be induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG). The vector carries the *lacI^q* gene to maintain repression in the absence of IPTG. The effect of inducing mini-gene expression was studied both in the *pth* (*rap*) mutant and in wild-type bacteria by measurement of growth rates (generations by hour) in rich medium (Table II). As expected, mini-gene expression is more inhibitory to growth in the *pth* (*rap*) strain than the

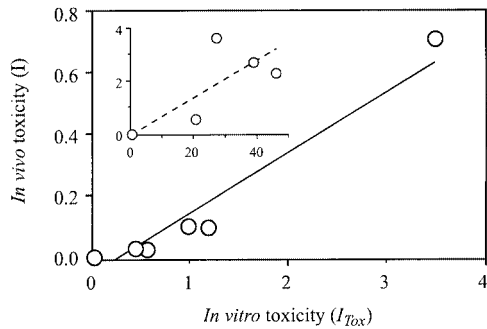


Fig. 4. Comparison of *in vivo* toxicity with I_{Tox} determined by the different biochemical parameters in strain Xac and Xac(*rap*); for the latter, see insert to figure. The index $I_{\text{Tox}} = \text{const} \langle n \rangle_{\text{rec}} \frac{k_{\text{drop}}}{k_{\text{c}} k_{\text{Pth}}}$ and the *in vivo* toxicity index I are described further in the text.

wild-type strain, and a lower level of expression, obtained at a sub-saturating level of IPTG (0.1 mM) was required in this case to allow a more progressive change in growth rate over the series of mini-genes.

Among the mini-genes studied, that encoding fMet-Lys is the most toxic. It was impossible to transform the Xac *rap* strain with plasmid SD7, as previously reported in the case of the minigene with the same SD sequence but encoding fMet-Ile (Dinçbas *et al.*, 1999). In both wild-type and *rap* strains, the toxicity decreases (growth rate increases) with mini-gene length. In the *rap* strain, plasmid SD11, encoding fMFITQK, is again an exception, and appears more toxic than SD10 encoding fMFITK.

In the *pth* wild-type strain the inverse relationship between peptide length and growth inhibition is still observed, but is less striking: a higher toxicity of the gene encoding the dipeptide fMK (plasmid SD7) is evident, but it is barely possible to distinguish any of the remaining plasmids by their effect on growth in liquid medium. Strains transformed with plasmid SD7 cease growing after 2 h (2.5 generations), whereas the remaining transformed strains continue growing for ~5 h (10 generations). On solid medium (LB agar), the relationship between peptide length and growth is also very clear in both Xac and Xac *rap* strains (results not shown). For example, in the presence of 1 mM IPTG no overnight growth at 37°C is observed in the case of transformants of strain Xac by plasmids SD7, SD8 or SD9. The detailed biochemical and growth rate analysis we report here has been obtained with a set of peptidyl-tRNAs with varying length but fixed sequences. Some of these *in vivo* experiments have, however, been repeated with peptidyl-tRNAs of a different sequence, a subset of fMIEKLTk-tRNA. The dependence of toxicity on length was very similar to the series based on fMFITQLK (L.Mora, unpublished results).

It was shown previously (Dinçbas *et al.*, 1999) that *in vivo* toxicity can be predicted from *in vitro* results with the aid of a toxicity index that estimates the total rate of cellular drop-off normalized to Pth activity in terms of biochemically measurable parameters. An extended version of the index (Materials and methods) contains the rate of initiation of transcription of mini-genes (k_{T}), the average number $\langle m \rangle_{\text{TR}}$ of times that a particular mRNA is bound to ribosomes and translated rather than degraded, the average (or 'recycle') number $\langle n \rangle_{\text{rec}}$ of times that a transcript is recycled without leaving its 30S subunit, the

rate of drop-off of peptidyl-tRNA (k_{drop}), $k_{\text{cat}}/K_{\text{M}}$ for the interaction between release factors RF1 or RF2 and the ribosome (k_{S}), $k_{\text{cat}}/K_{\text{M}}$ for the interaction between peptidyl-tRNA and Pth (k_{Pth}) according to

$$I_{\text{Tox}} = k_{\text{T}} \langle m \rangle_{\text{TR}} \langle n \rangle_{\text{rec}} (k_{\text{drop}}/k_{\text{S}} \cdot 1/k_{\text{Pth}})$$

Defined this way, I_{Tox} becomes approximately proportional to the fraction of tRNA sequestered in the peptidyl-tRNA form.

The *in vitro* experiments described above yield data for $\langle n \rangle_{\text{rec}}$, k_{S} , k_{drop} and k_{Pth} for the different mRNA and peptidyl-tRNA, and for the comparisons of different *in vivo* situations it is assumed that the other parameters in I_{Tox} do not vary from case to case. Relative I_{Tox} values (with fMK-tRNA^{Lys} and wild-type Pth as a reference) are indicated in Table I and may be compared with the growth rate data presented in Table II. In the Xac *rap* strain, the smallest mini-gene is highly toxic, whereas mini-genes encoding hepta- and octa-peptides exhibit no toxicity. The higher toxicity of plasmid SD11 compared with SD10 in Xac *rap* is clearly reflected in the toxicity index, and is due to low Pth cleavage efficiency. To compare growth inhibition with I_{Tox} , we have defined an index of *in vivo* toxicity as:

$$I = (\mu_0/\mu) - 1$$

where μ_0 is the growth rate in the presence of the parent plasmid pTrc99c, and μ the growth rate with the mini-gene under study. When growth is not impaired, μ will tend to μ_0 and I to 0. Figure 4 shows that there is an approximately linear relation between *in vivo* and *in vitro* toxicity in the case of the *pth* wild-type strain. This is much less true for the *pth* (*rap*) strain, in which the levels of inhibition are much higher, even at 0.1 mM IPTG. It was clear from experiments at lower IPTG concentrations (results not shown) that the order of inhibition by different plasmids varied according to the level of mini-gene expression. Thus, SD9 was less inhibitory than SD8 at 0.1 mM IPTG (Table II) but more inhibitory than SD8 at 10 μM IPTG, whereas the parameters measured *in vitro* predict that they should be similar (Table I).

Discussion

The drop-off of short peptidyl-tRNA molecules from the ribosome is of interest for at least two reasons. The first, of more general importance, concerns peptidyl-tRNA drop-off near the beginning of normal genes in the absence of in-frame termination codons. It should be recalled that the strong stimulation of short peptidyl-tRNA drop-off by RRF, EF-G and RF3 occurs irrespective of whether the codon in the ribosomal A-site is a sense or a stop codon (Heurgué-Hamard *et al.*, 1998). The possible physiological interest of this phenomenon will be discussed below. The second reason is to understand the mechanism of mini-gene toxicity.

The clearest example of mini-gene expression known to impair bacterial physiology concerns the λ mini-genes *barI* and *barII* (Ontiveros *et al.*, 1997; Hernández-Sánchez *et al.*, 1998), both of which encode the dipeptide fMet-Ile. Recent studies by Tenson *et al.* (1999) showed that mini-gene toxicity was preferentially associated with very short ORFs. These authors selected *in vivo* toxic mini-genes from a library encoding peptides of up to 5 amino acids in

length and obtained preferentially those with in-frame stop codons (i.e. shorter than pentapeptide).

The accepted explanation for mini-gene toxicity depends on an accumulation of peptidyl-tRNA corresponding to the last codon of the mini-gene, causing subsequent starvation for this tRNA. Specific accumulation of this particular peptidyl-tRNA and suppression of toxicity by overexpression of the corresponding tRNA isoacceptor has been shown experimentally for the natural or synthetic mini-genes encoding fMet-Ile, fMet-Lys and fMet-Arg (Ontiveros *et al.*, 1997; Tenson *et al.*, 1999).

Our primary purpose in the experiments described here was to understand why toxic mini-genes are small. To do so, we measured on a series of constructs varying in length the biochemical parameters likely to modify the pool of peptidyl-tRNA in the cell. Four important quantities that determine the cellular pool of peptidyl-tRNA that were directly measured *in vitro* as a function of peptide length are: (i) the average number of times an mRNA is translated before it is degraded, here scored as the average number of times, $\langle n \rangle_{\text{rec}}$, an mRNA is translated without leaving the 30S subunit; (ii) the rate of peptidyl-tRNA dissociation from the ribosome at each translation round; (iii) normal termination catalysed by release factors, the principal reaction competing with drop-off; and (iv) Pth activity, which allows recycling of peptidyl-tRNA thereby avoiding tRNA sequestration. The results we report here confirm that mini-gene toxicity is inversely correlated with the length of the coding sequence, and identify four reasons for this. For the series of mini-genes studied, those exceeding four sense codons in length did not affect the growth of *pth* wild-type cells, and those exceeding six sense codons (with one exception) did not affect the growth of *pth* (*rap*) cells. The use of a toxicity index, scoring the total flux towards the free peptidyl-tRNA pool, normalized to peptidyl-tRNA hydrolase activity, helped to identify the particular parameters associated with the growth inhibitory behaviour of individual mini-genes. Thus, the high toxicity due to fMet-Lys synthesis is caused essentially by a combination of three parameters: an inefficient Pth activity towards fMK-tRNA^{Lys}; a high rate of dissociation catalysed by RRF, EF-G and RF3 of this dipeptidyl-tRNA from the ribosome; and a high efficiency of mini-gene translation (large $\langle n \rangle_{\text{rec}}$). As the peptide encoded increases in length beyond five amino acids, the rate of factor-catalysed drop-off decreases dramatically, to <20% of the value for short peptidyl-tRNAs. The phenomenon may be amplified by the fact that RF2-dependent peptide release shows the opposite dependence on length, increasing in efficiency as the peptide chain lengthens. This variation is not, however, evident for RF1, which is much more active than RF2 though present at a 5-fold lower concentration in *E.coli* K12 strains (Adamski *et al.*, 1994). In contrast, over the range of peptide lengths studied, uncatalysed peptidyl-tRNA drop-off shows no general dependence on length.

Like factor-catalysed drop-off, $\langle n \rangle_{\text{rec}}$ decreases strikingly with peptide length. Previous experiments with dipeptide synthesis had shown that the degree of complementarity of the SD sequence to the 16S rRNA was of primary importance to the magnitude of $\langle n \rangle_{\text{rec}}$ (Dinçbas *et al.*, 1999). Here we show how $\langle n \rangle_{\text{rec}}$ varies as a function of the number of amino acids encoded by a

mini-gene, from >100 for dipeptide down to ~6 for octapeptide synthesis. This parameter is clearly of great importance, and the results are relevant not only to mini-gene translation, but also to the more general problem of translational coupling between genes cotranscribed from multicistronic operons, where short distances separate the termination codon of one gene and the ribosome binding site of another (Adhin and van Duin, 1990; Draper, 1996). In our calculation of the toxicity index we assumed that mRNAs are protected from degradation when they are on the 30S subunit, in accordance with previous results that frequently translated mRNAs are protected from degradation (Iost and Dreyfus, 1995). Our data suggest an inverse relationship between the length of the coding sequence and mRNA lifetime, as indeed found experimentally for a set of mini-genes with varying lengths and with a strong SD sequence (L.Mora, unpublished results).

In the case of *pth* (*rap*) cells, a third factor becomes crucial, as the hydrolase activity of this mutant, unlike that of the wild-type enzyme, shows a strong positive correlation with peptide length. An exception was nevertheless observed in the case of the hexapeptidyl-tRNA studied, which was even less rapidly hydrolysed than fMet-Lys-tRNA^{Lys} by both wild type and the *rap* mutant of Pth. Accordingly, peptide sequence can modulate the overall dependence on peptide length, a finding that is worthy of further study. A recent report suggests that residue Arg133 in Pth, which is mutated to His in Pth (*rap*), is one of two positively charged residues that interact with the 5'-phosphate of elongator peptidyl-tRNA (Schmitt *et al.*, 1997; Fromant *et al.*, 1999). We suggest therefore that a weakened interaction with this 5'-phosphate in tRNA may require compensation by a longer peptide chain. If all Pth molecules in our preparations are active, wild-type Pth is ~75-fold more efficient than the *rap* mutant in hydrolysing fMet-Lys-tRNA^{Lys}, and remains 10-fold more efficient in the case of octapeptidyl-tRNA.

Previous work from Shiloach *et al.* (1975) showed a higher dependence on length of the activity of wild-type Pth on other peptidyl-tRNAs. Thus, the k_{cat}/K_m value for Gly-Val-tRNA was 20 times lower than for (Gly)₂-Val-tRNA and 55 times lower than for (Gly)_n-Val-tRNA where *n* is three or larger. These differences between their results and ours could be due to the nature of the peptide chain, less hydrophobic in case of the peptidyl-tRNAs we have studied. However, additional experiments with substrates such as fMet-Gly-tRNA yielded values of k_{cat}/K_m close to those we report here for other peptidyl-tRNAs (V.Heurgué-Hamard, unpublished results).

The importance of the length of the coding sequence in mini-genes highlights the role of the termination signal. Compared with the translation of sense codons by ternary complex (aminoacyl-tRNA·EF-Tu·GTP), RF-dependent release of the polypeptide chain is quite slow (Freistroffer *et al.*, 1997, 2000), which can explain why the tRNA translating the last sense codon is the species that accumulates preferentially as peptidyl-tRNA. In support of this, changes in the choice of stop codon and its downstream context (factors affecting the kinetics of termination) have significant effects on mini-gene toxicity (Dinçbas *et al.*, 1999).

Two further effects of a stop codon are specific to mini-genes. When present early in the coding sequence (before

Protein purifications

Bacterial elongation factors, initiation factors, and other components needed for the preparation of ribosomal complexes paused at the stop codon of mini-gene mRNA were prepared as described previously (Ehrenberg *et al.*, 1990; Freistroffer *et al.*, 1997; Dinçbas *et al.*, 1999; Karimi *et al.*, 1999). RF2 was prepared from W3110 cells according to Tate and Caskey (1990), RF1 was purified from MRE600 cells as described by Dinçbas *et al.* (1999), and wild-type and *rap* mutant Pth was as described by Dinçbas *et al.* (1999).

In vitro assays

Determination of k_{cat}/K_m values for RF1 and RF2. Relative k_{cat}/K_m values for hydrolysis of the different peptidyl-tRNAs in the ribosomal P-site by RF2 were measured in competition experiments (Pavlov *et al.*, 1998). Ribosomal complexes paused at a stop codon were prepared as described previously (Freistroffer *et al.*, 1997; Pavlov *et al.*, 1998). Two differently labelled ribosomal release complexes in standard factor mix (typically 1 pmol of each), were set to compete for a limiting amount of release factor (typically 0.2 pmol). The extents of hydrolysis of the peptidyl-tRNAs were analysed as described previously (Dinçbas *et al.*, 1999) and data were evaluated according to Pavlov *et al.* (1998). Absolute values of k_{cat}/K_m for RF1 and RF2 were determined as described by Dinçbas *et al.* (1999), using ribosome-release complexes programmed with the messenger encoding the dipeptide MK. k_{cat}/K_m values for RF1 and RF2-dependent termination for the other constructs were calculated using relative values obtained in competition experiments.

Determination of absolute values of k_{cat}/K_m for wild-type Pth and Pth (*rap*). Ribosome release complexes were prepared as above with ribosomes programmed with 02.MK.Oa mRNA. Ribosomal release complexes were incubated for 30 min at 37°C with RRF, RF3 and EF-G; 75 pmol of each; in 70 µl of standard factor mix for dissociation of peptidyl-tRNAs from the ribosomal P-site. Following incubation, 5 µl of Pth (*rap*) (10 pmol) or Pth (wild type) (0.3 pmol) were added to 70 µl of the release complex mix. Samples were withdrawn at different times and the reaction was stopped by cold TCA precipitation. The k_{cat}/K_m was determined from the linear relationship with time of the remaining fraction $r(t)$ of peptidyl-tRNA:

$$\ln r(t) = - (k_{cat}/K_m) \cdot [Pth] \cdot t$$

The concentrations of active Pth were approximated to the total Pth concentrations measured as described by Bradford (1976).

Determination of Pth (*rap*) activity. Reactions were done as indicated above, but stopped by adding HCOOH at a final concentration of 10%. Samples were spun in an Eppendorf centrifuge at 14 000 r.p.m. for 15 min. The pellet was dissolved in 200 µl of 0.5 M KOH and incubated for 15 min at room temperature for hydrolysis of the oligopeptide from peptidyl-tRNA, whether on or off the ribosome. A volume of 10 µl of concentrated HCOOH (98–100%) was added to the dissolved sample for precipitation of large RNA and large proteins. Samples were centrifuged at 14 000 r.p.m. for 15 min and the soluble peptides were separated on an RP18 (Waters) column equilibrated with various concentrations of methanol (MK: 18%, MFK: 34%; MFIK and MFITK: 42%; MFITQK: 36%; MFITQLK, MFITLQK and MFITQLTK: 50%) and 0.1% trifluoroacetic acid. Radioactivity was monitored by on-line radiometry (Ramona). Due to high K_m values for Pth (*rap*) interaction with peptidyl-tRNAs, k_{cat}/K_m for the hydrolysis of free peptidyl-tRNAs could be measured under these conditions.

Determination of relative k_{cat}/K_m values of Pth wild type. Relative k_{cat}/K_m values for hydrolysis of the different free peptidyl-tRNAs by Pth were measured in competition experiments. Two differently labelled ribosomal release complexes (typically 1 pmol of each) (Freistroffer *et al.*, 1997; Pavlov *et al.*, 1998) in standard factor mix were incubated for 30 min at 37°C with RRF, RF3 and EF-G at a final concentration of 1 µM each to promote peptidyl-tRNA dissociation. Peptidyl-tRNAs were then set to compete for a limiting amount of Pth (typically 0.6 pmol). The extents of hydrolysis of the peptidyl-tRNAs were analysed by withdrawing aliquots at different time points for quenching in equal volumes of 20% ice-cold formic acid. After centrifugation at 14 000 r.p.m. in an Eppendorf centrifuge for 12 min at 4°C, the radioactivity in the supernatant was counted in Aquasafe 300 plus scintillation fluid (Zinsser). Data were evaluated by plotting the natural logarithm of the peptidyl-tRNA fraction remaining in function of time.

Drop-off rate constants. Peptidyl-tRNA drop-off experiments were as described (Heurgué-Hamard *et al.*, 1998). Dissociation rate constants

were measured in the presence of EF-G (1 µM), RRF (1 µM), RF3 (1 µM) or in the absence of translation factors. Translation factors were pre-warmed together with Pth (wild type) (120 pmol) for 2 min at 37°C in standard factor mix buffer. An equal volume of ribosomal release complex, pre-warmed for 1 min at 37°C, was added to the factor mix. The reaction was stopped at specified times by addition of HCOOH to a final concentration of 10% and the peptides released by KOH treatment were analysed by HPLC as described above [see determination of Pth (*rap*) activity].

Probabilities of dissociation of mRNA from ribosomes after termination. Recycling experiments on seven different mRNAs coding for fMK, fMFK, fMFIK, fMFITK, fMFITQK, fMFITQLK and fMFITQLTK were done as described previously (Pavlov *et al.*, 1997a,b; Dinçbas *et al.*, 1999) with the following modifications: factor mix 1 contained per 60 µl modified quantities of these components: 0.3 mg of tRNA^{bulk} (prepared as described by Ehrenberg *et al.*, 1990), 2 nmol of EF-Tu, 200 pmol of EF-Ts, 750 pmol of chase mRNA, which contained SD 002 and encoded fMFITR but contained no stop codon. Data were analysed as described by Dinçbas *et al.* (1999).

Bacterial strains, plasmids and bacteriophage

The *E.coli* K12 strains (Xac, Xac *rap*) and B strain (VH998) used for overexpression of Pth (*rap*) are described in Dinçbas *et al.* (1999). Minigenes were cloned from the constructions used in the *in vitro* experiments (see Table II for plasmid names). PCRs were cut by *KpnI* and *PstI* and cloned into pTrc99c digested by the same enzymes. All plasmid constructions were sequenced by the method of Sanger *et al.* (1977).

Growth conditions

Luria Broth (LB) medium was supplemented according to the requirements. Antibiotics were added at the following final concentration: 50 µg/ml kanamycin; 200 µg/ml ampicillin. When induction was necessary to express mini-genes, 1 M IPTG was added to LB plates or liquid medium to the indicated final concentration. Growth was monitored either by streaking transformed strains on plates or in most cases in liquid medium. An overnight culture was diluted to an OD₆₀₀ of 0.05 and grown until 0.5 in LB–ampicillin medium without IPTG. This culture was again diluted to 0.05 grown in LB–ampicillin medium containing the IPTG concentration indicated. Absorbance at 600 nm was monitored as a function of time for at least 6 h. All growth measurements were done at 37°C.

Recombinant DNA manipulations and genetic manipulations

General procedures for DNA recombinant techniques, plasmid extraction, etc. were performed as described by Sambrook *et al.* (1989). Purification of fragments on agarose gel was by Jetsorb gel extraction (Bioprobe). Phage P1 lysates, transductions and transformations were performed as described by Miller (1992).

Modification of toxicity index to include recycling of ribosomes

In the following treatment, k_T is the rate of initiation of transcription of a mini-gene, k_{TR} is the rate of initiation of translation of an mRNA, k_d is the rate constant of degradation of a free mRNA (it will be assumed that ribosome-bound mRNA is not degraded) and it follows immediately that $\langle m \rangle_{TR} = k_{TR}/k_d$ is the average number of times that a chosen mRNA binds from its free state to any ribosome and becomes translated one or several times. P is the probability that an mRNA leaves the 30S subunit after termination with release factor, $[RF]k_S$ is the total rate of termination by release factor and k_{drop} is the rate of drop-off of peptidyl-tRNA. The probability of drop-off per ribosome cycle is $P_{drop} = k_{drop} / (k_{drop} + [RF]k_S)$.

When $k_{drop} \ll k_S[RF]$, P_{drop} can be replaced by $k_{drop}/[RF]k_S$ and for relative I_{tox} values, the release factor concentration can often be neglected. Defining the toxicity index as the total flux to the peptidyl-tRNA pool of a certain tRNA isoacceptor normalized to the peptidyl-tRNA hydrolase activity and assuming that P is the same when peptide synthesis is terminated by release factors and drop-off, gives

$$I_{tox} = k_T \langle m \rangle_{TR} \langle n \rangle_{rec} (k_{drop}/k_S \cdot 1/k_{Pth}) \quad (\text{Eqn 1})$$

since then $1/P = \langle n \rangle_{rec}$, the average number of times that an mRNA is translated before it leaves the ribosome and is replaced with another molecule.

With the same general definition of I_{tox} but assuming instead that an mRNA leaves the 30S subunit with certainty after a drop-off event then the toxicity index would instead be

$$I_{\text{Tox}} = k_T \langle m \rangle_{\text{TR}} \cdot 1/[1 + (1/P_{\text{drop}} - 1) \cdot P] \cdot 1/k_{\text{Pth}} \quad (\text{Eqn } 2)$$

When $P_{\text{drop}} \ll P$ equations (1) and (2) converge except that the latter also contains the concentration of release factor 1 or 2 in the denominator. Preliminary experiments indicate that P is the same after termination with release factor and by drop-off and we have therefore chosen Equation (1) for main text and data evaluation.

The global *in vivo* life time ($1/k_D$) of an mRNA can be expressed in biochemically measurable parameters as

$$1/k_D = \langle m \rangle_{\text{TR}} \cdot \langle n \rangle_{\text{rec}}/k + 1/k_d \quad (\text{Eqn } 3)$$

where k is a rate constant for ribosome recycling, i.e. the inverse of the time per ribosome cycle when the 30S and mRNA remain associated. The first term in Equation 3 describes the average time that an mRNA is protected by ribosomes assuming that the protection time in the first ribosome cycle is the same as in subsequent ones. The second term is the average time that an mRNA spends in free state.

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