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Sequestration of specific tRNA species cognate to the last sense codon of an overproduced gratuitous protein

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ABSTRACT

High-level expression of non-functional model proteins, derived from elongation factor EF-Tu by the deletion of an essential domain, greatly inhibits the growth of *Escherichia coli* partly deficient in peptidyl-tRNA hydrolase. High-level expression in wild-type cells has little effect on growth. The inhibitory effect is therefore presumably due to the sequestration of essential tRNA species, partly in the form of free peptidyl-tRNA. The growth inhibitory effect can be modulated by changing the last sense codon in the genes encoding the model proteins. Thus, replacement of Ser by Lys or His at this position increases growth inhibition. The effects of 11 changes studied are related to the rates of accumulation previously observed of the corresponding families of peptidyl-tRNA. Two non-exclusive hypotheses are proposed to account for these observations: first, the last sense codon of mRNA is a prefered site of peptidyl-tRNA drop-off in cells, due to the slow rate of translation termination compared with sense codon translation; secondly, the relatively long pause of the ribosome at the stop codon (of the order of 1 s), results in significant temporary sequestration on the ribosome of the tRNA cognate to the last sense codon.

INTRODUCTION

A significant proportion of ribosomes that initiate translation of an mRNA fail to terminate by the normal mechanism, which involves the recognition of messenger-encoded stop signals, hydrolysis by the ribosome of peptidyl-tRNA and release of the completed polypeptide. Truncated proteins arise at a frequency of $\sim 3 \times 10^{-4}$ per codon, which implies that about one-third of the ribosomes that initiate β-galactosidase synthesis fail to reach the stop signal (1–3). Measurements of the synthesis of β-galactosidase dimers indicate that the frequency of such processivity failures is affected by mutations in genes encoding ribosomal proteins S12, S4 and S5 (3), known to control the accuracy of translation. It is believed that the majority of processivity failures result in the dissociation of peptidyl-tRNA from the ribosome (2), or 'drop-off'. The recycling of these molecules requires peptidyl-tRNA hydrolase (Pth), a ubiquitous enzyme known to be essential for viability in *Escherichia coli* (4,5). In cells deficient in Pth, protein synthesis is arrested and the cells finally die due to starvation for tRNA, which becomes sequestered as peptidyl-tRNA and unavailable for protein synthesis. When deprived of Pth by the transfer to a non-permissive temperature of cells containing a thermosensitive enzyme, $tRNA^{Lys}$ becomes limiting considerably before other tRNA species (6). The reasons for this are not yet fully understood, and may be related to a higher rate of drop-off of peptidyl-tRNA species involving $tRNA^{Lys}$. One possible explanation is that peptidyl-$tRNA^{Lys}$ is a poor substrate for Pth compared with peptidyl-tRNAs that accumulate slowly. Recent measurements, however, show that the differences are not large enough to explain the large variation in accumulation rates (V.Heurgué-Hamard, unpublished results).

Several aspects of peptidyl-tRNA drop-off remain poorly understood. It is unclear what fraction of drop-off events occurs at codons cognate to the tRNA in question, and what fraction occurs at near-cognate codons, as a result of errors of selection of tRNA that escape the proofreading mechanism (7). Studies of the suppression of a thermosensitive Pth mutant showed that drop-off was not merely a passive event, but involved translation factors RRF and RF3 *in vivo* (8,9). However, attempts to demonstrate that mutants affecting RRF and RF3 increased translational processivity using the *lacZ* monomer-dimer approach of Jørgensen and Kurland (2) were unsuccessful (V.Heurgué-Hamard and R.H.Buckingham, unpublished work). One explanation for this unexpected result, apart from technical reasons related to the difficulties of this experimental approach, is that drop-off events involving RRF and RF3 occur predominantly at the beginning or end of mRNAs and would therefore not be detected.

The drop-off of short peptidyl-tRNAs has been the subject of extensive recent study (6,9–14). The expression of very short open reading frames, encoded by 'mini-genes', can lead to the rapid accumulation of peptidyl-tRNA and the arrest of protein synthesis even in cells with normal amounts of Pth. In such cases drop-off does indeed appear to occur at a codon read by the cognate tRNA, namely, the last sense codon encoded by the mini-gene, as the inhibitory effect can be relieved (at least in the limited number of cases that have been studied) by over-production of the tRNA species cognate to the last sense codon. The presence of a stop codon very early in an mRNA favours drop-off and peptidyl-tRNA accumulation for several reasons. These include the catalysis of drop-off by translation factor and the phenomenon of 30S ribosome recycling on short mRNAs (9,14). Another important factor probably arises

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Table 1. Bacterial strains

Strain	Genotype	Reference/construction
FTP4993	*metB*, *glyV*55, *trpA*(AGC233, UGA234)	F.T.Pagel
IB188	*ara*, Δ(*lac–proB*), *nalA*, *metB*	Derivative of XA102 (40)
GY5552	Δ(*lac*, *pro*), *thi*, *strA*, *endA*, *sbcB*15, *hsdR*4 F′*traD*36, *proAB*, *lacI*qZΔM15, *recA*938::Cm	Marsh and Walker (41)
VH7	*supE44*, *thi1*, *thr1*, *leuB6*, *lacY1*, *tonA21*, *pth*(ts), *fadR13*::Tn*10*	Heurgué-Hamard *et al.* (6)
VH287	*ara*, Δ(*lac–proB*), *nalA*, *metB, pth*(ts), *fadR13*::Tn*10*, *recA*938::Cm	This work, IB188 and P1 lysates on VH7 and GY5552
VH733	*metB*, *glyV*55, *pth*(ts)	Heurgué-Hamard *et al.* (8)
VH804	*metB*, *glyV*55, *trpA*(AGC233, UGA234), *recA*::Cm	This work, FTP4993 and P1 lysate on GY5552
VH805	*metB*, *glyV*55, *pth*(ts), *recA*::Cm	This work, VH733 and P1 lysate on GY5552

directly from the slow kinetics of the termination reaction compared with the translation of most sense codons. The limiting factor in the termination reaction is probably the low value of k_{cat}, of the order of 1 s^{-1} (15). The pausing of ribosomes for substantial periods at stop signals has been observed both *in vitro* (16,17) and *in vivo* (18).

Since drop-off is an important phenomenon when the ribosome is paused at the termination signal of very short coding sequences, it is of interest to know whether the drop-off of peptidyl-tRNAs with complete polypeptide chains represents a significant part of total drop-off. The reason for the high rate of peptidyl-$tRNA^{Lys}$ accumulation in cells deprived of Pth (19) is not well understood, but may simply be a reflection of a generally increased frequency of adenosine (and consequently of Lys codons) close to initiation and termination codons (20). Little is known of the distribution of polypeptide chain lengths in peptidyl-tRNA molecules arising by premature peptidyl-tRNA dissociation from the ribosome and we cannot yet say whether the beginnings or ends of coding regions are preferred regions for drop-off to occur.

To search for effects on peptidyl-tRNA accumulation related to the coding sequence just upstream of the stop signal, we have examined the overexpression of a series of model proteins that differ with respect to the last sense codon. The experiments are performed under conditions of limitation for Pth, so that cell growth becomes sensitive to the degree of sequestration of different tRNA species. The results show that the identity of the last sense codon does indeed affect the degree of toxicity due to high-level expression of the model protein and that the effect is related to the rate of accumulation of the corresponding peptidyl-tRNA species described by Menninger (19). Two non-exclusive mechanisms can account for the observations: first, drop-off occurring at a significant level at the last sense codon, and secondly, the relatively slow process of termination and ribosome recycling that sequesters the tRNA corresponding to the last sense codon for a sufficient period that the pool of free, charged tRNA is significantly depleted.

MATERIALS AND METHODS

Bacteria and plasmids

Escherichia coli K12 strains are listed in Table 1. All plasmid constructions expressing ΔEF-Tu and variants of this protein are derivatives of pHD67 (3), which contains the partially deleted *tufB* gene from plasmid pTuB12.1 (21) cloned in the expression vector pTrc99c (22). Plasmid pJMM1 contained a further truncation in the *tufB* gene (encoding ΔΔEF-Tu), and was obtained by digestion of pHD67 with *Eag*I and religation of the plasmid. Variants of both plasmids pHD67 and pJMM1 were constructed by a method employing two PCR steps. To introduce different codons before the stop codon, a first PCR was performed using a mutagenic oligonucleotide covering the stop signal and a second oligonucleotide upstream of the *Eag*I (951) site (see Fig. 1) site in pHD67 or pJMM1. The product of this PCR step was used in a second PCR step in conjunction with a further oligonucleotide downstream of an *Sgf*I site located 181 nucleotides beyond the *tufB* stop codon. The product was restricted with *Sgf*I and *Psh*AI or *Eag*I and used to replace the corresponding fragment in pHD67 or pJMM1 respectively. All variants of these plasmids were verified by sequencing (23). They are listed in Table 3 together with the corresponding final sense codon. Plasmids pVH1 and pJMM19, carrying the *pth* and *lysV* genes, respectively, are derivatives of low-copy-number plasmid pWSK129 (24). Plasmid pJMM19 was made by recloning the *Apa*I–*Bam*HI fragment carrying *lysV* from pVH119 (6) between the same sites in pWSK129; like pVH119 it results in an increase of ~2-fold in the quantity of $tRNA^{Lys}$ in the cell.

Growth conditions

Luria–Bertani (LB) broth was employed as rich medium. Antibiotics were added as necessary at the following final concentrations: kanamycin 50 μg/ml, ampicillin 200 μg/ml. In experiments with thermosensitive Pth strains, precultures were grown at 30°C in LB broth containing appropriate antibiotics. After dilution to an OD_{600} of 0.05, growth was continued at 37°C (or as otherwise indicated in figure legends) to an OD_{600} of 0.5. Synthesis of ΔEF-Tu or ΔΔEF-Tu was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM after a further dilution to an OD_{600} of 0.05. For labelling of proteins with [^{35}S]Cys, cells were grown in M9-glucose medium (25) supplemented with all amino acids except Cys. An overnight culture was diluted to ~2×10^7 cells/ml and labelled for two generations by addition of [^{35}S]Cys (1 Ci/μmol) to a concentration of 15 μCi/ml. IPTG was added to a concentration of 1 mM and labelling continued for 3 h.

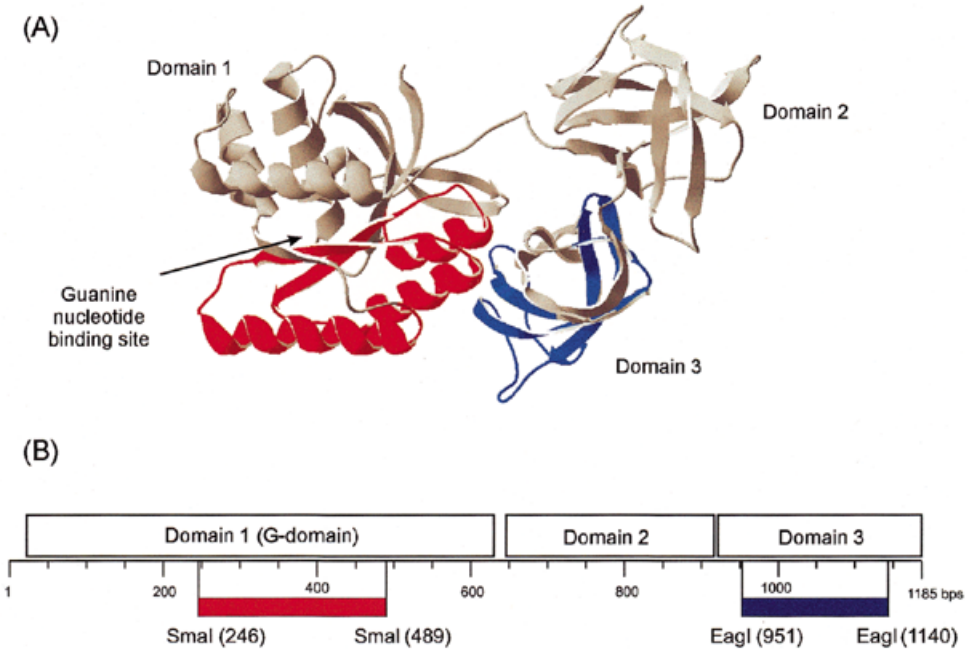

Figure 1. Deleted regions of EF-Tu in model proteins ΔEF-Tu and ΔΔEF-Tu. The deleted regions are shown in relation to the 3D structure of EF-Tu (**A**) and to the *tufB* gene coding sequence (**B**). The region in red is absent from the ΔEF-Tu protein and both this and the region in blue from ΔΔEF-Tu. The model is based on the crystallographic structure of EF-Tu (42) and the sequence is from GenBank, accession no. M30610.

Recombinant DNA techniques and genetic manipulations

General procedures for recombinant DNA techniques, plasmid extraction, agarose gel electrophoresis, etc., were performed as described by Sambrook *et al.* (26). DNA fragments from agarose gels were extracted using Jetsorb gel (Bioprobe) according to the manufacturer's instructions. Phage P1 lysates, transductions and transformations were performed as described by Miller (27).

Quantification of proteins

Induced cultures labelled with [^{35}S]Cys as described above were centrifuged and the cells lysed for 3 min at 100°C in lysis buffer (50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue). The proteins were separated by electrophoresis on 10% polyacrylamide gel as described by Laemmli (28). Gels were dried and the radioactivity in individual bands and complete tracks was determined using a PhosphorImager (Molecular Dynamics).

RESULTS AND DISCUSSION

Overproduction of an inactive protein inhibits growth of Pth-deficient cells

The study of tRNA sequestration and its dependence of mRNA sequence required model proteins that could be expressed at a high level in *E.coli* without deleterious effects on the growth of cells with normal levels of Pth activity. In order to avoid tRNA usage that might affect tRNA pool sizes we chose a gene with codon usage typical of highly expressed genes in *E.coli*, a truncated version of *tufB*, encoding protein synthesis elongation factor EF-Tu (3,29). As shown in Figure 1, the truncated protein, called ΔEF-Tu, lacks part of the GTP-binding motif G3 and motif G4 as a result of the deletion of a *Sma*I–*Sma*I fragment in *tufB* covering nucleotides 248–491 (29). This removes a region of the native sequence corresponding to a well-defined domain in the three-dimensional structure of the protein (30). A further truncation was also made by deleting an *Eag*I–*Eag*I fragment (nucleotides 951–1140), yielding a second protein (ΔΔEF-Tu), lacking about two-thirds of domain 3 (Fig. 1) as well as the GTP-binding motifs.

In plasmid pHD67 encoding ΔEF-Tu, transcription depends on the *trc* promoter and is induced by IPTG (3). Continuous labelling experiments with [^{35}S]Cys or [^{35}S]Met indicate that 3 h after induction of *pth* wild-type cells transformed with pHD67, ~25% of total cell protein is ΔEF-Tu, confirming the observations of Dong *et al.* (3). Under the conditions of our experiments the expression of ΔEF-Tu has little effect on the growth of *pth* wild-type cells. In contrast, in cells partly deficient in Pth, growth is partially or completely inhibited. All experiments in Pth-deficient cells have made use of the thermosensitive Pth mutant isolated by Atherly and Menninger (4), transduced into various genetic backgrounds by selection for a nearby transposon. In liquid culture in rich media, the *pth*(ts) strain VH805 grows at temperatures up to 42°C, and grows at 37°C as fast as the *pth*$^+$ parental strain VH804. When transformed with plasmid pHD67, strain VH805 grows normally at 37°C in the absence of ΔEF-Tu expression, but ceases to grow shortly after induction of the gene carried in pHD67 (triangles, Fig. 2). Cotransformation of cells with a plasmid pVH1 carrying the wild-type *pth* gene suppresses this growth inhibition (not shown). A similar behaviour is seen on plates (Fig. 3A, compare segments pHD67 and pTrc99c). At 30°C, where intracellular Pth activity is less affected by the thermolability

Table 2. Growth inhibition at different temperatures of Pth-deficient cells due to plasmid-directed synthesis of ΔEF-Tu and ΔΔEF-Tu

Strain/plasmid	*pth*(ts)/control		*pth*(ts)/ΔΔEF-Tu		*pth*(ts)/ΔEF-Tu		*pth*$^+$/ΔEF-Tu	
	VH805/pTrc99		VH805/pJMM1		VH805/pHD67		VH804/pHD67	
IPTG:	–	+	–	+	–	+	–	+
30°C	++	++	++	++	++	++	++	++
34°C	++	++	++	++	++	–	++	++
37°C	++	++	++	+	++	–	++	++
39°C	++	++	++	–	++	–	++	++
43°C	–	–	–	–	–	–	++	+

Transformed cells were streaked on to LB-ampicillin plates with or without 1 mM IPTG and incubated at the temperature shown in the first column. The table shows inhibited growth (+) or no growth (–) in comparison with the normal growth (++) of *pth*$^+$ cells transformed with control plasmid pTrc99C.

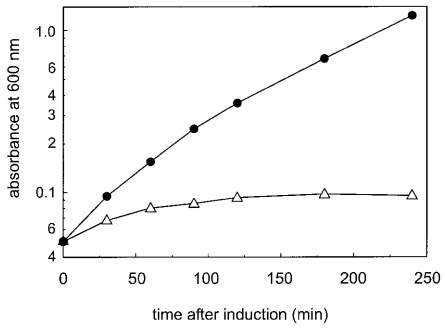

Figure 2. Inhibition of cell growth in partially Pth-deficient strains following induction of ΔEF-Tu synthesis. Tranformants of the Pth thermosensitive strain VH805 carrying plasmid pHD67 encoding ΔEF-Tu were grown at 37°C in LB-ampicillin medium to ~4 × 10^7 cells/ml. Growth was monitored by measurement of OD_{600} as a function of time after induction with 1 mM IPTG; induced culture (open triangles) and non-induced culture (filled circles).

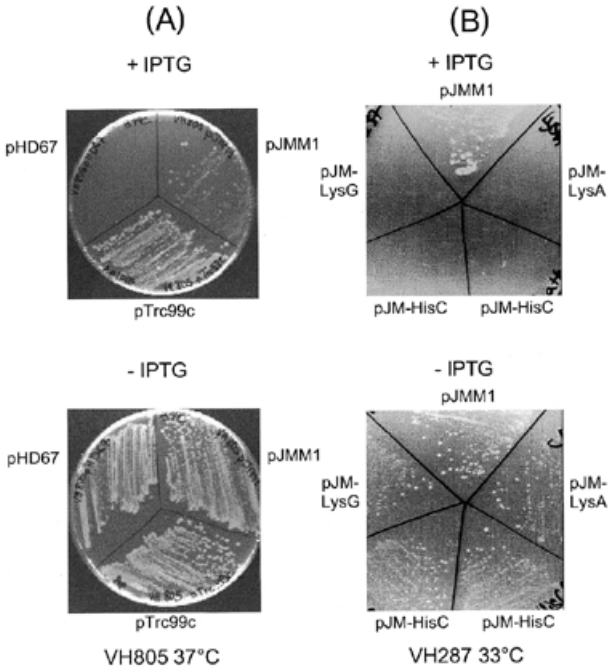

Figure 3. Growth inhibition on solid medium due to ΔEF-Tu or ΔΔEF-Tu synthesis and the effect of mutations in the gene encoding ΔΔEF-Tu. Tranformants of Pth thermosensitive strains with pHD67 or pJMM1 (SerAGC) and variants of the latter plasmid altered in the last sense codon were streaked on LB–agar plates with or without 1 mM IPTG, and incubated at the temperature indicated. Growth on plates is shown for (**A**) transformants of VH805 with plasmids pHD67 and pJMM1, encoding ΔEF-Tu and ΔΔEF-Tu, respectively, and control plasmid pTrc99c; (**B**) transformants of VH287 with plasmid pJMM1 and variants altered in the last sense codon of Δ*tufB* from SerAGC to LysAAA/G or HisCAC/U.

of the enzyme, induction of ΔEF-Tu expression does not affect growth. At temperatures of 34°C and above (in the case of strain VH805), *pth*(ts) cells transformed with pHD67 no longer grow in the presence of IPTG, whereas non-induced cells or cells transformed with the control plasmid pTrc99C grow normally at temperatures up to 39°C (Table 2). The threshold for thermosensitivity is thus lowered by the remarkable magnitude of 9°C when ΔEF-Tu is expressed. In other strain backgrounds the *pth*(ts) mutation leads to more or less thermosensitive growth, and the onset of thermosensitivity of the transformed, induced cells is correspondingly shifted to lower or higher temperatures, respectively.

Two broadly different explanations can account for the observation that overproducing one gratuitous protein can lower the threshold of thermosensitivity in the Pth mutant strain. The overproduction does not lead to a marked increase in the total amount of protein synthesis occurring in the cell. Rather, it diverts part of the cell's capacity for protein synthesis to one protein at the expense of others. Thus, the proportion of any particular protein (including Pth) being synthesised may fall in relation to total protein synthesis as a result of the extra imposed 'protein burden'. In fact, the degree to which the synthesis of different cell proteins is affected by the induction of a plasmid-carried gene is known to be not uniform but to vary according to the mRNA (31). The second class of explanation would depend on particular properties of the mRNA for ΔEF-Tu. Thus, drop-off of peptidyl-tRNA during synthesis of ΔEF-Tu may be higher than the average frequency of drop-off, and increase the total amount of drop-off in the cell. Alternatively, even if the overall amount is not increased, the pattern of tRNAs accumulating as peptidyl-tRNA may change due to particular drop-off points characteristic of the mRNA, and hasten the onset of starvation for an essential tRNA species. Further insight into these and other explanations

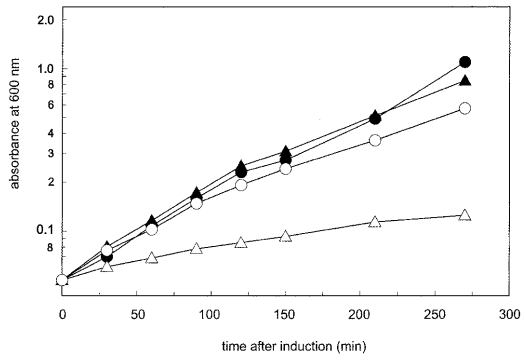

Figure 4. Suppression of growth inhibition due to ΔΔEF-Tu synthesis by overproduction of $tRNA^{Lys}$ from a low-copy-number plasmid. Strain VH805 was cotransformed with plasmid pJMM1 expressing ΔΔEF-Tu and either plasmid pJMM19 carrying *lysV* or the parent plasmid pWSK129 with no insert. Transformants were grown at 38.5°C in LB-ampicillin-kanamycin medium to ~3×10^7 cells/ml, and ΔΔEF-Tu synthesis was then induced with 1 mM IPTG. Growth was monitored by measurement of OD_{600} as a function of time after induction for induced cultures (open circles, pJMM1 and pJMM19; open triangles, pJMM1 and pWSK129) and non-induced cultures (filled circles, pJMM1 and pJMM19; filled triangles, pJMM1 and pWSK129).

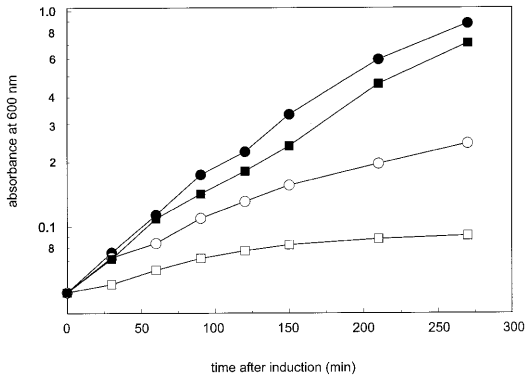

Figure 5. Increased growth inhibition due to ΔΔEF-Tu synthesis on substitution of Lys codons at the last sense position of the ΔΔEF-Tu mRNA. Tranformants of the Pth thermosensitive strain VH805 with plasmid pJMM1 or variants of the plasmid altered in the last sense codon of Δ*tufB* from SerAGC to LysAAA were grown at 38°C in LB-ampicillin medium to ~3×10^7 cells/ml, and ΔΔEF-Tu synthesis was induced with 1 mM IPTG. Growth was monitored by measurement of OD_{600} as a function of time after induction for induced cultures (open circles, pJMM1; open squares, pJM-LysAAA) and non-induced cultures (filled circles, pJMM1; filled squares, pJM-LysAAA).

was obtained by experiments in which specific modifications were made to the ΔEF-Tu mRNA.

Deletion of a C-terminal fragment of ΔEF-Tu reduces the growth inhibition

It has been reported that a region near the C-terminus of EF-Tu, including residue G375, is important for an autoregulatory mechanism of *tufB* expression, and that this regulatory effect is conserved in ΔEF-Tu (32,33). This suggested that the toxicity associated with ΔEF-Tu expression might in part be related to regulatory effects on *tufB* expression. The Δ*tufB* in plasmid pHD67 was therefore modified by deletion of a region of the gene encoding 63 amino acids close to the C-terminus of ΔEF-Tu, including residue G375 of TufB and surrounding residues, resulting in plasmid pJMM1. The deleted fragment corresponds to an *Eag*I–*Eag*I fragment in the Δ*tufB* gene (Fig. 1). The expression of this doubly deleted protein 'ΔΔEF-Tu' remains inhibitory to growth in *pth*(ts) cells, but the threshold temperature for inhibition is shifted upwards by ~5°C. The behaviour of *pth*(ts) cells transformed with plasmid pJMM1 is intermediate between pHD67-transformed cells and cells transformed with the control plasmid pTrc99C (Table 2, columns 6 and 7). Thus, at 34–38°C IPTG induction of the *pth*(ts) strain VH805 transformed with pJMM1 still grows, whereas the same strain transformed with pHD67 ceases growth completely (Table 2; see also Figs 4 and 5). In contrast, at 39°C ΔΔEF-Tu expression completely inhibits cell growth although VH805 transformed with pTrc99C grows as well as the pth^+ parental strain VH804 at this temperature. Other experiments (not shown) show that ΔΔEF-Tu is synthesised with similar translational efficiency to ΔEF-Tu. The difference between the two model proteins could be accounted for by the 'protein burden' type of explanation, as a significant part of the polypeptide chain is removed in this second deletion. It is also possible that the second deletion removes drop-off sites encoded within the *Eag*I–*Eag*I fragment of *tufB*.

Growth inhibition is suppressed by $tRNA^{Lys}$ overproduction

The fact that the growth inhibitory effect of overproducing ΔEF-Tu was seen only in mutant cells partially deficient in Pth strongly suggests that the mechanism of growth inhibition is likely to be the same as in severely Pth-limited cells. Previous work has shown that under semi-permissive conditions for *pth*(ts) strains (at ~42°C in the case of strain VH805), growth is inhibited due to starvation for tRNA and in particular for $tRNA^{Lys}$ (6). If tRNA starvation due to sequestration as peptidyl-tRNA results from the induction of ΔEF-Tu synthesis, then the effects should be at least partly suppressed by the overproduction of $tRNA^{Lys}$ similarly to the original observation (6). In the experiment presented in Figure 4, the *lysV* gene is carried on a low-copy-number plasmid pJMM19 compatible with pJMM1 and results in an ~2-fold increase in the level of $tRNA^{Lys}$ (6). The growth inhibition resulting from the induction of ΔΔEF-Tu synthesis in the strain cotransformed with pJMM1 and the parent plasmid pWSK129 is seen to be almost completely eliminated when the plasmid carries a *lysV* insert.

Changing the last codon of the ΔΔEF-Tu gene modulates the growth inhibition

Recent data suggest that the termination step in protein synthesis catalysed by release factors RF1 and RF2 is slow in comparison with an elongation step (15,18,34). This suggests that the last sense codon might be more prone to peptidyl-tRNA drop-off than the average sense codon of an mRNA. To look for evidence of such an effect, we have introduced mutations that change the last amino acid of ΔΔEF-Tu in pJMM1 (or ΔEF-Tu in pHD67) and looked for modulation of the growth inhibitory effects in Pth-deficient cells. Previous work has identified $tRNA^{Lys}$ as the tRNA isoacceptor most likely to become fully sequestered as peptidyl-tRNA in Pth-limited cells (6). The data of Menninger (19) provide further valuable

information concerning the probable relative rates of accumulation of different peptidyl-tRNA species. Thus, peptidyl-tRNA^{Thr}, peptidyl-tRNA^{His} and peptidyl-tRNA^{Ile} as well as peptidyl-tRNA^{Lys} accumulate rapidly under these conditions, whereas peptidyl-tRNA^{Gly} and peptidyl-tRNA^{Cys} accumulate only very slowly. The rapid accumulation of peptidyl-tRNA^{Lys} does not appear to arise either from an abnormally high rate of peptidyl-tRNA^{Lys} drop-off at Lys codons or from an unusually poor substrate activity of peptidyl-tRNA^{Lys} for Pth (V.Heurgué-Hamard, unpublished results). As the data of Menninger (19) can be difficult to interpret in cases of multiple isoacceptors for an amino acid, we have concentrated our changes on introducing new codons belonging to codon families read by a single tRNA isoacceptor.

Ten new codons have been introduced by site-directed mutagenesis into the site preceding the stop codon in the gene for ΔΔEF-Tu in plasmid pJMM1, in place of the ACG Ser codon found in *tufB*. The presence of a single *Eag*I site upstream of the stop codon (as opposed to two sites in pHD67) facilitated these changes. The relative growth of cells transformed by the variant plasmids was studied both in liquid medium and on plates. In the first method, cells in liquid culture were induced with IPTG at a density of ~4×10^7 cells/ml and growth was monitored until the end of exponential growth or until growth ceased due to the toxic effect of ΔΔEF-Tu induction. Growth experiments showed that four of these modified plasmids led to considerably increased growth inhibition in the *pth*(ts) strain VH805 following induction at 37°C, compared with the parent plasmid pJMM1. Both the Lys codons AAA/G and the His codons CAU/C were associated with increased growth inhibition. Figure 5 shows the growth inhibition following induction of the LysAAA variant of ΔΔEF-Tu under these conditions.

These results were confirmed by comparisons on agar plates. This approach had two experimental advantages. First, it was easy to distinguish faster-growing revertants when they occurred. Secondly, multiple parallel experiments enabled different conditions of growth, notably temperature, to be easily compared. Thus, it was possible to identify temperatures at which cells expressing the normal SerAGC ΔΔEF-Tu grew well on plates whereas neither of the LysAAA/G variants grew visibly at all (Fig. 3B). The Pth mutant strain used in this experiment, VH287, is more thermosensitive than VH805, hence the lower temperature employed. The rare Ile codon AUA was associated with a significant increase in toxicity in ΔEF-Tu but a much smaller effect, if any, in ΔΔEF-Tu. With this exception similar effects were observed with mutant plasmids derived from pJMM1 and from pHD67, encoding ΔΔEF-Tu and ΔEF-Tu, respectively, in the cases where variants were constructed of both parent plasmids, namely, SerAGC, LysAAA, LysAAG and GlyCAU. As expected (see above), the temperature thresholds for thermosensitive growth were always lower in the case of pHD67 and its variants.

Experiments in which tRNA^{Lys} was overproduced in cotransformants of VH805 by pHD67-LysA/G and pJMM19 showed that the suppression of growth inhibition due to the LysV-containing plasmid was much less marked than in the case of the parent plasmid pHD67 (results not shown). Similar experiments with derivatives of pJMM1 where the C-terminal Ser codon was replaced by either the Phe codon UUU or UUC or the Gly codon GGC, showed that none of these changes had any significant effect on the degree of toxicity on induction. The comparison of the inhibitory effects of the entire family of plasmids with altered last sense codons is summarised in Table 3, showing that an increase in inhibition was observed only in the case of ΔEF-Tu variants in which the last amino acid corresponded to one of the three most rapidly accumulating peptidyl-tRNA species identified by Menninger (19).

Table 3. Summary of toxicity of pJMM1-derived plasmids with changed last sense codon

Plasmid	Amino acid	Codon	Growth	Drop-off[a]
pJM/pHD67-LysA	Lys	AAA	–	0
pJM/pHD67-LysC	Lys	AAG	–	0
pJM-HisC	His	CAC	–	2
pJM-HisU	His	CAU	–	2
pHD67-Ile[b]	Ile	AUA	–	2
pJM-PheU	Phe	UUU	0	4
pJM-PheC	Phe	UUC	0	4
pJM-Arg	Arg	CGG	0	6
pJMM1/pHD67	Ser	AGC	0	12
pJM/pHD67-Gly	Gly	GGC	0	18
pJM-CysU	Cys	UGU	0	20
pJM-CysC	Cys	UGC	0	20

Growth under conditions of induction (1 mM IPTG) of strain VH805 transformed with the plasmids shown in first column is shown as similar to (0) or less than (–) relative to transformants with the appropriate control plasmid [line 9: pJMM1 (SerAGC) or pHD67 (SerAGC)] under various experimental conditions (see text).
[a]Drop-off observed by Menninger (19) for amino acid-accepting families of peptidyl-tRNA: time required (min) for the accumulation of 25% of acceptance capacity as peptidyl-tRNA. The lines are ordered with respect to this parameter.
[b]Refers to variant of ΔEF-Tu.

The most striking result of these experiments is that small changes to the ΔΔEF-Tu gene can modulate the growth inhibitory effect to a considerable extent, without changing the level of expression of the gene. This implies that protein burden effects cannot easily provide the full explanation for the effects of overproducing our gratuitous model proteins, but that sequestration of essential tRNA species is an important part of the phenomenon. Secondly, the effect of different codons at the position of the last sense codon is clearly related to the rate at which the corresponding tRNA species was found to accumulate as peptidyl-tRNA by Menninger (19).

The most evident hypothesis to explain these observations is that drop-off of peptidyl-tRNA does indeed occur when the ribosome is paused at the last sense codon. However, another mechanism may also be contributing to the tRNA starvation, due directly to the slow kinetics of termination. In both cases, the particular hierachy observed should be expected, since even before induction of ΔEF-Tu or ΔΔEF-Tu synthesis, the cells are partially starved for Pth and therefore for certain tRNAs (notably, tRNA^{Lys} and tRNA^{His}). The hierachy is hence pre-established by the shortage of Pth. This would explain why only the codons corresponding to tRNAs accumulating rapidly

as peptidyl-tRNA (AAA/G, CAC/U and AUA; see Table 3) result in growth inhibition when introduced at the end of ΔEF-Tu or ΔΔEF-Tu. As might be expected, measurements of the degree to which different tRNA species are sequestered as peptidyl-tRNA under semi-permissive conditions for growth rank in the same way as the rates at which they accumulate under non-permissive conditions (results not shown), confirming the observations of Menninger (19). Four acceptor activities were tested: Lys and His (high accumulation), Gly (low accumulation) and Phe (intermediate accumulation). The second mechanism that may contribute arises directly from the relatively long pause of the ribosome at the stop codon (16,17), of the order of 1 s (15). This could result in temporary sequestration on the ribosome of the tRNA cognate to the last sense codon. A rough calculation shows that ~5% of the ribosomes synthesising the model protein could be paused at the stop codon. This might immobilise ~5% of the total of a tRNA isoacceptor such as $tRNA^{Lys}$ at any moment, a fraction that is small but could be significant in the case of a tRNA already in short supply as a result of sequestration as peptidyl-tRNA due to drop-off.

A variety of attempts has been made to demonstrate contributions to tRNA starvation by sequestration as peptidyl-tRNA on or off the ribosome, using mutations in translation factors that affect the kinetics of termination and drop-off (8,9). In general, the effects are difficult to interpret in an unambiguous manner. Further light has, however, been thrown on the problem by studying the effect of changing codons just upstream of the last sense codon in the mRNAs for ΔEF-Tu and ΔΔEF-Tu.

The last two sense codons modulate tRNA sequestration

Translocation factor EF-G is responsible for moving peptidyl-tRNA from the A-site to the P-site on the ribosome, with the concurrent movement of deacylated tRNA from the P-site to the E-site and translocation of mRNA by one codon (35). Two tRNAs are considered to be bound to the ribosome at any time (36,37), due to anti-cooperativity between the A- and E-sites of the binding of tRNA. Whether binding of RF to the A-site induces tRNA release from the E-site in the way that is thought to occur when tRNA binds in a stable manner to the A-site is unknown. We have introduced Lys and in some cases His codons at positions upstream of the last sense codon to see whether this leads to an effect comparable to their presence as the last sense codon (the *N*th codon). As shown in Table 4, when codon (*N*–1), normally a CTG Leu codon, is replaced by an AAA Lys codon (pJM-KVKS) the inhibitory effect is similar to replacing the *N*th codon by the Lys codon. When both the *N* and (*N*–1) codons are changed to Lys codons (pJM-KVKK), the effect is cumulative. In contrast, replacement of the Lys residue normally present at position *N*–4 by Cys (yielding pJM-CVLS) has no effect. The introduction of Lys at *N*–3 into the latter plasmid (producing pJM-CKLS) is also without effect. Similarly, replacement by Gly of either or both of the two Lys residues in ΔEF-Tu encoded within the *Eag*I–*Eag*I fragment (and therefore absent in ΔΔEF-Tu) has no effect. It should be noted that the presence of Lys residues at both the *N*–3 and *N*–4 positions (pJM-KKLS) does increase growth inhibition. However, this is a known effect due to the presence of two similar adjacent codons read by a tRNA in short supply, which introduces a translational pause (38,39). In conclusion, the introduction of Lys at the two last positions modifies the phenomenon of growth inhibition whereas addition or removal of Lys at various upstream positions does not. While not conclusive, these observations tend to argue in favour of the second explanation for tRNA sequestration, i.e. temporary sequestration on the ribosome. They further suggest that the binding of RF to the A-site in response to a stop signal does not result in the rapid ejection of tRNA from the E-site.

Table 4. Toxicity of pJMM1-derived plasmids with changed last ΔΔEF-Tu tetrapeptide

Plasmid	Terminal tetrapeptide	Relative growth
pJMM1	Lys-Val-Leu-Ser (wild type)	+++
pJM-CVLS	Cys-Val-Leu-Ser	+++
pJM-CKLS	Cys-Lys-Leu-Ser	+++
pJM-KVLK	Lys-Val-Leu-Lys	++
pJM-KKLS	Lys-Lys-Leu-Ser	+
pJM-KVKS	Lys-Val-Lys-Ser	+
pJM-KVKK	Lys-Val-Lys-Lys	0
pJM-KVLH	Lys-Val-Leu-His	++
pJM-KVHH	Lys-Val-His-His	+
pJM-KVHK	Lys-Val-His-Lys	+

Relative rates of growth under conditions of induction (1 mM IPTG) of strain VH805 transformed with the plasmids shown in first column. Temperatures of incubation were chosen between 35.5 and 37°C so as to allow some growth of the slowest-growing strain being plated.

Conclusion

Previous work using Pth-limited cells showed that the translation factors RRF and RF3 were involved in a significant proportion of drop-off events in *E.coli* (6,8,9). However, failure to demonstrate that mutants affecting RRF and RF3 increased translational processivity focused attention on the possibility the the processivity failures involving these factors occurred predominantly at the beginning or the end of polypeptide chain synthesis. The involvement of the two factors in processivity failures early in the translation of an mRNA has been amply borne out by recent *in vitro* experiments (8,9,14). Here, we have attempted to demonstrate drop-off at the last sense codon of an mRNA by looking for effects of changing that codon in a system where altered levels of drop-off are reflected in cellular growth rate. Such effects have been clearly demonstrated. However, the fact that the penultimate sense codon also affects growth under conditions of Pth limitation suggests that drop-off at the last sense codon may not be the only (or indeed the principal) mechanism for the observed phenomenon. We suggest that temporary sequestration of tRNA while the ribosome is paused at a stop signal, either as peptidyl-tRNA at the P-site or deacylated tRNA at the E-site, may contribute to tRNA starvation in a codon-specific manner. This may occur when Pth limitation has already established partial starvation for tRNA species that accumulate preferentially under these conditions, such as $tRNA^{Lys}$ or $tRNA^{His}$.

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