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The growth defect in *Escherichia coli* deficient in peptidyl-tRNA hydrolase is due to starvation for Lys-tRNA^{Lys}

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The existence of a conditional lethal temperature-sensitive mutant affecting peptidyl-tRNA hydrolase in *Escherichia coli* suggests that this enzyme is essential to cell survival. We report here the isolation of both chromosomal and multicopy suppressors of this mutant in *pth*, the gene encoding the hydrolase. In one case, the cloned gene responsible for suppression is shown to be *lysV*, one of three genes encoding the unique lysine acceptor tRNA; 10 other cloned tRNA genes are without effect. Overexpression of *lysV* leading to a 2- to 3-fold increase in tRNA^{Lys} concentration overcomes the shortage of peptidyl-tRNA hydrolase activity in the cell at non-permissive temperature. Conversely, in *pth, supN* double mutants, where the tRNA^{Lys} concentration is reduced due to the conversion of *lysV* to an ochre suppressor (*supN*), the thermosensitivity of the initial *pth* mutant becomes accentuated. Thus, cells carrying both mutations show practically no growth at 39°C, a temperature at which the *pth* mutant grows almost normally. Growth of the double mutant is restored by the expression of *lysV* from a plasmid. These results indicate that the limitation of growth in mutants of *E.coli* deficient in Pth is due to the sequestration of tRNA^{Lys} as peptidyl-tRNA. This is consistent with previous observations that this tRNA is particularly prone to premature dissociation from the ribosome.

Keywords: GroESL/peptidyl-tRNA hydrolase/processivity/ribosome/tRNA^{Lys}

Introduction

The probability of abortive polypeptide chain termination is amongst the most important factors that have shaped the evolution of the machinery for protein synthesis, and determined the optimum level for the precision of translation (Kurland and Ehrenberg, 1985). The process occurs at a significant frequency, appears to be unavoidable but is energetically extremely expensive for the cell. Abortive termination may arise in different ways: one of these, dissociation of peptidyl-tRNA from the ribosome, leads to an intermediate that must be recycled to allow reutilization both of amino acids and tRNA.

A crucial enzyme in the recycling process, peptidyl-tRNA hydrolase (Pth; EC 3.1.1.29), is a ubiquitous and essential protein whose role is believed to be that of a scavenger of peptidyl-tRNA molecules that arise normally during the course of protein biosynthesis. Two main hypotheses have been advanced to explain why this function should be essential to the cell (Menninger, 1976; Atherly, 1978). It has been suggested that peptidyl-tRNA may poison protein biosynthesis, perhaps by the formation of defective initiation complexes or by interfering with ribosome recycling. Alternatively, protein biosynthesis may be inhibited by starvation for a special subpopulation of tRNAs that is used often and whose cellular concentration is rate limiting for normal protein biosynthesis (Chapeville *et al.*, 1969).

A physiological role has been proposed for peptidyl-tRNA dissociation from the ribosome, the principal reaction giving rise to the substrate for Pth: that of reducing the synthesis of polypeptide chains that are normal in length but contain errors (Menninger, 1977). This hypothesis has been called the 'ribosomal editor' and supposes that peptidyl-tRNA dissociation from the ribosome, or 'drop-off', occurs frequently as a result of erroneous incorporation of an amino acid into the growing polypeptide chain, following a failure of proofreading in the ribosomal A-site. The rationale underlying the hypothesis is that the weak interaction between a codon and the anticodon of a non-cognate tRNA fails to be maintained at some point between the peptidyl transfer to the non-cognate aminoacyl-tRNA and the subsequent peptidyl transfer, thereby leading to drop-off. The value of such a process as a cellular strategy for error correction has been questioned by Kurland and Ehrenberg (1985) on the grounds that the energetic cost to the cell of correcting errors this way would be far greater than the cost of living with the errors.

Several measurements have been made of processivity failures during translation. Thus, Manley observed an average premature termination rate of 3×10^{-4} per codon during *lacZ* translation, a possible underestimate since short truncated chains would have escaped detection (Manley, 1978). Slightly lower but comparable rates of 2 and 2.5×10^{-4} were found by Jørgensen and Kurland (1990) and Dong and Kurland (1995), using a quite different method. Both the contribution of termination errors at sense codons (Jørgensen *et al.*, 1993) and that of terminations resulting from frameshift errors (Atkins *et al.*, 1972; Ryden-Aulin and Hughes, 1990) appear to be very small. Thus, peptidyl-tRNA drop-off and the translation of truncated mRNA (also yielding peptidyl-tRNA) must contribute the major part of premature termination events. Jørgensen and Kurland (1990) conclude that, in the particular case of β-galactosidase, about two-thirds of these incomplete chains result from drop-off during elongation rather than from the translation of

Table I. Chromosomal and plasmid-borne suppressors of thermosensitive *pth* mutant strain

Strain/plasmid	Position (min)	Characterization
VH27	27.1	secondary L107F mutation in Pth
VH41	43.2	mutation in *glyW*: C35U change in $tRNA^{Gly}_3$ anticodon
VH22	94	suppressor mutation between *lysU* and *groESL*
VH23	27.5	suppressor mutation between *hemA* and *hns*
pVH39	94.2	4 kb fragment carrying the *groESL* operon
pVH80/81	54.3	*Pst*I fragments carrying the *valU–lysV* operon

VH27 is a temperature-resistant pseudo-revertant derived from VH7; VH41, VH22 and VH23 are similar revertants derived from VH21 (see Table III). The chromosomal positions shown are linear with respect to the physical map (Rudd, 1992).

truncated mRNA molecules. Direct measurements of peptidyl-tRNA drop-off (Menninger, 1976) suggest an average value of 4×10^{-4} per codon, consistent with the overall level of processivity errors and the notion that drop-off is the major contributor to these events.

The study of peptidyl-tRNA drop-off and the role of Pth have been facilitated greatly by the isolation of mutants affecting *pth*, the gene encoding the enzyme. One mutant, temperature sensitive in growth and Pth activity (Atherly and Menninger, 1972), showed an arrest in protein biosynthesis and a rapid accumulation of peptidyl-tRNA following a shift to non-permissive temperatures (Menninger, 1976). An *Escherichia coli* mutant (*rap*) that fails to allow normal growth of wild-type bacteriophage λ (Henderson and Weil, 1976) has also been shown to affect *pth* (Garcia Villegas *et al.*, 1991). The transcription of certain inessential regions of λ, called *bar*, causes inhibition of protein synthesis and cell death in *rap* mutant strains, for reasons still poorly understood.

Following preliminary studies by Gingrich and Menninger (1978) and Anderson and Menninger (1987), we have sought phenotypic suppressors of the temperature-sensitive *pth* mutation, *pth*(ts), in order to gain insight into the phenomenon of peptidyl-tRNA drop-off and the cellular role of Pth. We show here that the temperature-sensitive phenotype may be suppressed by the expression from a low copy number plasmid of a gene for $tRNA^{Lys}$. We propose that starvation for this species of tRNA is the principal reason for the inhibition of protein synthesis in cells limited in Pth activity.

Results

Several classes of suppressor of thermosensitive mutants affecting pth

Pseudo-revertants of the temperature-sensitive allele of *pth* (Atherly and Menninger, 1972) are obtained readily by selection at non-permissive temperature (Gingrich and Menninger, 1978; Anderson and Menninger, 1987). By this approach, we have obtained several distinct classes of suppressor. Co-transduction experiments with P1 phage using transposons of known position in the neighbourhood of the *pth* gene at 27.1 min on the *E.coli* chromosome allowed suppressors to be classified as: (i) non-transducible with *pth* and hence distant from the gene; (ii) very highly linked to *pth* and probably resulting from mutations within the gene; and (iii) showing intermediate levels of co-transduction with *pth* and hence nearby but extragenic. The nature of one probable intragenic suppressor, in strain VH27 (see Table I), was confirmed by cloning the *pth* gene and performing complementation analysis of a *pth*(ts) mutant, and finally by determination of the gene sequence. A nucleotide transversion G→T in the gene results in a L107F change in the protein, six residues away from the G101D mutation in Pth that confers thermosensitive growth (Garcia Villegas *et al.*, 1991). Assays of Pth activity in cell extracts from this intragenic pseudo-revertant showed the restoration of ~50% of wild-type activity.

The chromosomal positions of two extragenic suppressors distant from *pth* were mapped by obtaining nearby mini-Tn*10* transposon insertions and determining their positions with the aid of probes prepared from the sequence adjacent to the transposon (Heurgué-Hamard *et al.*, 1995). One such insertion, located at 43.2 min on the physical map (Rudd, 1992), was 64% transducible with a *uvrC*::Tn*10* mutation (Singer *et al.*, 1989), itself found to be 99.5% co-transducible with the *pth* suppressor in pseudo-revertant VH41 (see Table I). This suggested that the suppressor might be a tRNA missense suppressor derived from *glyW*, a hypothesis that was confirmed by sequencing the gene. The C→T transversion found in the nucleotide corresponding to the central position of the anticodon should lead to a tRNA able to insert Gly at GAC Asp codons, thereby restoring the wild-type sequence of Pth.

Using a similar approach in pseudo-revertant VH22, a second extragenic suppressor was shown to be located close to 94 min. The position of this mutation was defined more closely by co-transduction experiments and was shown to lie between *lysU* and *groESL* (Table I). The episome F112, known to cover the region between *uxuAB* and *metB* on the *E.coli* chromosome, was introduced into the pseudo-revertant VH22 but did not prevent growth at 43°C, suggesting that the suppressor mutation is dominant to the wild-type allele. Attempts were therefore made to clone the suppressor near 94 min, selecting for plasmids that restored growth at 43°C. As described below, these experiments did not allow the cloning of the suppressor allele, but led us to obtain two multicopy suppressors of the *pth* thermosensitive strain, one of which has provided considerable insight into the molecular events that lead to the inhibition of protein synthesis and finally cell death in the *pth* mutant strain.

Isolation of a multicopy suppressor of thermosensitive Pth strain VH20

During attempts to clone the extragenic suppressor in pseudo-revertant VH22, it became apparent that multicopy suppressors of the thermosensitive mutation could also be obtained. In one such attempt, a *Pst*I digest of whole

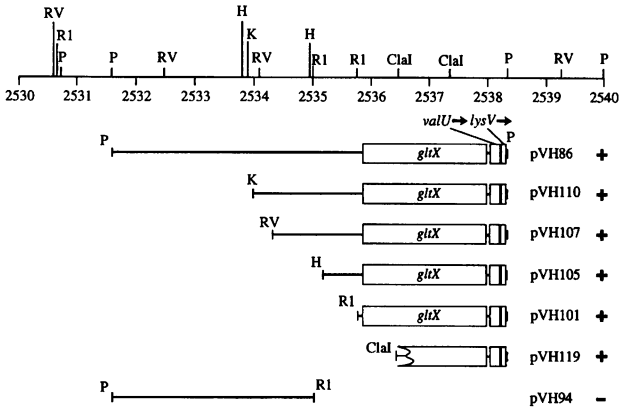

Fig. 1. Chromosomal localization and subcloning of sequences conferring *pth*(ts) suppressor activity. The insert in plasmid pVH86 possessing *pth*(ts) suppressor activity (see text) was subjected to restriction enzyme digestion and subcloned in plasmid vector pWSK29. The resulting plasmids shown here were tested for suppression activity, indicated as + or – in the column to the right. H = *Hin*dIII, K = *Kpn*I, P = *Pst*I, R1 = *Eco*RI, RV = *Eco*RV. The numbers correspond to the physical map positions in version 6 of EcoMap (Rudd, 1992).

chromosomal DNA from a pseudo-revertant strain VH271 (derivative of VH22; see Table III), known to harbour a suppressor mutation close to 94 min on the chromosome, was cloned into pWSK29, a plasmid of copy number 6–8. Transformants of the thermosensitive *pth* strain VH20 were plated at 42°C to select for plasmids able to suppress thermosensitivity. Two colonies were picked and used individually for plasmid preparations and further rounds of transformation of VH20. These transformations yielded exclusively thermoresistant transformants, indicating that the plasmids, named pVH80 and pVH81, carried a suppressor of the thermosensitive *pth* mutation.

Identification of the cloned suppressor

Restriction enzyme analysis of plasmids pVH80 and pVH81 revealed that each contained two *Pst*I inserted fragments, of which one, ~6 kb in length, was common to the two plasmids. This fragment was subcloned into the same vector to yield pHV86, which retained the suppressor activity. The origin of this fragment was determined by Southern hybridization to chromosomal restriction fragments produced by the eight enzymes of the *E.coli* physical map (Kohara *et al.*, 1987; Heurgué-Hamard *et al.*, 1995), and by partial sequence analysis, and shown to be derived from the 54 min region of the chromosome, using the coordinates of Rudd (1992), far from the locus of the 94 min suppressor and thus not related directly to the latter (see Figure 1). Several genes of relevance to protein synthesis are present on the 6 kb fragment, notably *gltX*, encoding glutamyl-tRNA synthetase and the *valU–lysV* operon, which contains three genes for $tRNA^{Val}$ and one gene for $tRNA^{Lys}$ (Brun *et al.*, 1990a,b). Progressive subcloning of the sequences present in this insert indicated that neither the sequences downstream of *gltX* (to the left of the gene in Figure 1), nor the integrity of the *gltX* gene itself, was necessary for the suppressor activity. Thus, each of the plasmids pVH101, 105, 107, 110 and 119 maintained suppressor activity, whereas plasmid pVH94 did not. These observations suggested that expression of all or part of the plasmid-

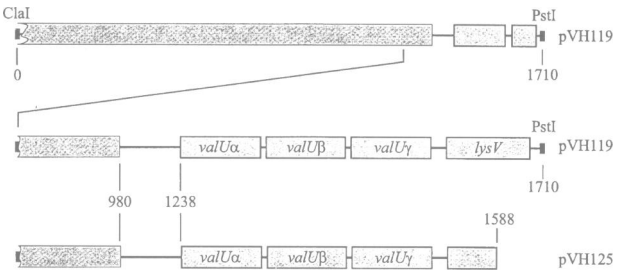

Fig. 2. Deletion analysis of plasmid carrying *pth*(ts) suppressor activity. Plasmid pVH119 (see text and Figure 1) was subjected to progressive deletions from the *Pst*I end with exonuclease III, yielding plasmid pVH125, no longer able to suppress *pth*(ts). Numbering is in base pairs from the *Cla*I end of the insert. A larger deletion removed *valU*β, *valU*γ and part of *valU*α (plasmid pVH124; not shown).

carried *valU* operon was responsible for suppression. To determine the required sequence more exactly, the *Cla*I–*Pst*I insert in pVH119 was subjected to progressive deletion with exonuclease III from the *Pst*I end. Partial removal of the *lysV* gene, the last-transcribed gene of the *valU* operon, as in plasmid pVH125 (see Figure 2), leaving the three genes for $tRNA^{Val}$ intact, was sufficient to destroy the suppressor activity.

Overexpression of valU–lysV(SuUAA/G) does not suppress pth(ts)

The necessity for an intact *lysV* gene in the plasmid insert to suppress the thermosensitivity of *pth*(ts) strains suggested that an increase in the cellular level of $tRNA^{Lys}$ was the significant factor in suppression. If this need for additional $tRNA^{Lys}$ were to overcome the sequestration of the tRNA in the form of peptidyl- $tRNA^{Lys}$, as seemed likely, then the requirement should only be satisfied by $tRNA^{Lys}$ able to read normal Lys codons and not by an altered $tRNA^{Lys}$ that had lost this ability. In order to test this prediction, we made use of an ochre suppressor (*supN*) strain shown by Uemura *et al.* (1985) to carry a mutation in *lysV*, leading to an altered anticodon sequence. The 1 kb *Cla*I–*Pst*I fragment carrying the *valU* operon (see Figure 1) was therefore cloned from a PCR-amplified fragment into pWSK29 from either a wild-type strain or the *lysV*(SuUAA/G) strain GE875, yielding plasmids pVH153 and pVH154 respectively. Transformants of VH20 by plasmid pVH154 grew at 30°C, though more slowly than the control strains transformed by plasmid with no insert or by pVH153, probably due to the effect of overproducing the ochre suppressor tRNA. As expected, however, suppression of thermosensitivity was observed only with plasmid pVH153.

Expression of lysV from pVH153 increases $tRNA^{Lys}$ levels 2- to 3-fold

Since we propose that an increase in the level of $tRNA^{Lys}$ is responsible for restoring the growth of *pth*(ts) strains at 43°C, it was of interest to quantitate the increase due to the presence in the cell of plasmids carrying the *valU* operon. Methods have been developed for measuring tRNAs (Smith and Yarus, 1989; Emilsson and Kurland, 1990; Emilsson *et al.*, 1992) that depend on hybridizing labelled DNA probes to Northern blots of tRNA species after separation by polyacrylamide gel electrophoresis. The quantity of $tRNA^{Lys}$ in cell extracts was determined

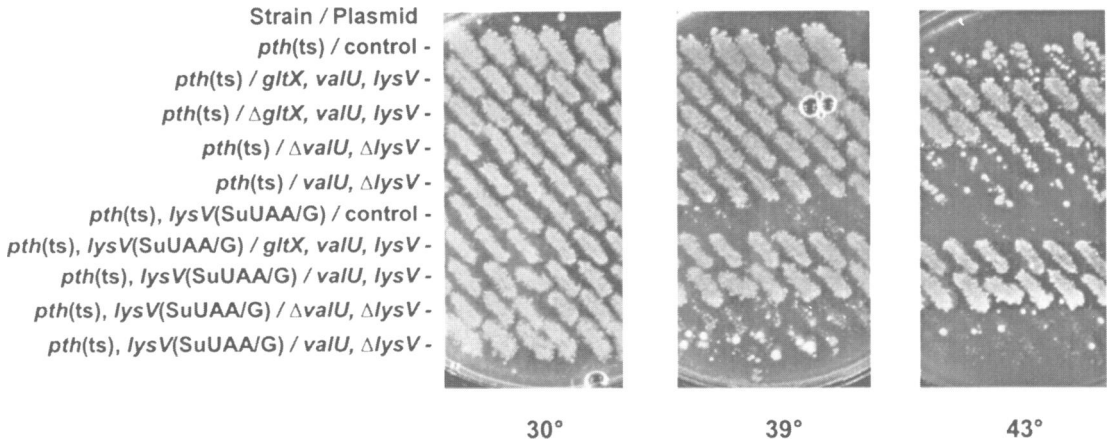

Fig. 3. Growth at different temperatures of *pth*(ts) and *pth*(ts), *lysV*(SuUAA/G) strains transformed with plasmids carrying the *valU* operon, partially deleted derivatives or control plasmid; the relevant genotype is shown in the column: strain/plasmid. The *pth*(ts) and *pth*(ts), *lysV*(SuUAA/G) strains C600pth(ts) and VH591 respectively were transformed by the parent plasmid pWSK29 without insert; plasmid pVH86, carrrying *gltX* and the *valU* operon; VH119, carrying the *valU* operon; or plasmids pVH124 or 125, deleted for the *lysV* part of the *valU* operon. Each row contains several patches representing separate colonies from the same transformation, which were patched on to an LB ampicillin plate and replicated to further plates for incubation at 30, 39 or 43°C.

relative to $tRNA^{Leu}_1$, the major isoacceptor of Leu in the cell. The ratio of these two tRNAs was found to increase 2- to 3-fold in extracts from strain C600 transformed by plasmid pVH153 compared with control strains.

Thermosensitivity due to pth(ts) is increased in lysV(SuUAA/G) strains

The observation that thermosensitive growth of *pth*(ts) strains may be suppressed by overproducing $tRNA^{Lys}$ suggested that the reverse might also be true, namely, that decreasing the level of $tRNA^{Lys}$ in the cell should increase the degree of thermosensitivity. The nonsense suppressor mutation *lysV*(SuUAA/G) was therefore introduced into a *pth*(ts) strain, and growth on plates was studied at various temperatures. The double mutant grew as well at 30°C as the control strain carrying the *pth*(ts) mutation alone, but failed to grow at 39°C, a temperature at which the control strain grew normally (see Figure 3). The *lysV*(SuUAA/G) mutation could be complemented by plasmids carrying wild-type *lysV*, thus the double mutant strain VH591 transformed by plasmids pVH86 or 119 grew normally at 39°C (Figure 3). This figure also shows the suppression of the thermosensitivity of strain C600pth(ts) at 43°C by plasmids pVH86 and 119. The growth of isolated or semi-isolated colonies as opposed to patch growth on regions of the plates at 39 or 43°C (see Figure 3) is indicative of host reversion to temperature resistance.

Levels of $tRNA^{Lys}$ in pseudo-revertants VH22 and VH23

The locations of chromosomal suppressors of *pth*(ts) thermosensitivity in strains VH22 and VH23 have been determined approximately, although the genes involved remain unidentified (see above). It was of interest nevertheless to measure the levels of $tRNA^{Lys}$ in these pseudo-revertants in view of the possibility that suppression might be due to an indirect effect of the mutations on the amount of this tRNA. Measurements were performed in two ways: by probing Northern blots of tRNA preparations with specific probes as described above or by determining the aminoacylation capacity *in vitro* for different amino acids of tRNA purified from the pseudo-revertants and appropriate control strains. Both methods indicated that the ratio of $tRNA^{Lys}$ to $tRNA^{Leu}_1$ increased in strains VH22 and VH23, by average factors of 1.6 compared with the parent strain VH21. While smaller than the increase observed in strains transformed by plasmid pVH153 carrying the *valU* operon, such an increase might be sufficient to relieve inhibition of protein synthesis caused by the sequestration of $tRNA^{Lys}$ as peptidyl-$tRNA^{Lys}$.

Lack of suppression of pth(ts) by control plasmids carrying other tRNA genes

The suppression of *pth*(ts) by overproduction of $tRNA^{Lys}$ suggested that growth inhibition under non-permissive conditions was due to a specific shortage of this tRNA. To test this conclusion further, several plasmids carrying other tRNA genes were introduced into the *pth*(ts) strain (see Table II). Only plasmid pHV86, carrying the *lysV* gene, suppressed thermosensitive growth. Thus, of the 11 tRNA species examined, only the overproduction of $tRNA^{Lys}$ appears to be associated with suppression.

Suppression of pth(ts) by overexpression of groESL

As well as yielding the *valU* operon, attempts to clone the chromosomal suppressor in revertant VH22 resulted in the cloning of a 13 kb fragment of the chromosome shown by Southern mapping to originate from the 94 min region. Subcloning of this insert by *Eco*RI digestion and finally by partial *Sau*3AI digestion showed that suppression was retained by a 4 kb fragment cloned in plasmid pWSK129. This insert contained the entire *groESL* operon. By cloning a similar region from the non-suppressor strain Y1, suppression was shown not to depend on any mutation within the cloned region, a conclusion confirmed by using plasmid pOF12 (Fayet *et al.*, 1986), carrying a wild-type *groESL* region on an 8 kb *Eco*RI fragment. Futhermore, co-transduction experiments using a kanamycin-resistant cassette inserted immediately downstream of the *groEL* gene (Zeilstra-Ryalls *et al.*, 1993) as selected marker indicated that the 94 min chromosomal suppressor muta-

Table II. Plasmids employed for overexpression of tRNA species

tRNA	Gene	Plasmid	Reference
$tRNA^{Arg}_3$	*argX*	$ptRNA^{His}CCA$	O'Connor *et al.* (1993)
$tRNA^{His}$	*hisR*	$ptRNA^{His}CCA$	O'Connor *et al.* (1993)
$tRNA^{Leu}_1$	*leuT*	$ptRNA^{His}CCA$	O'Connor *et al.* (1993)
$tRNA^{Lys}$	*lysV*	pVH86	this work
$tRNA^{Phe}$	*pheV*	pPP15	Delamarche *et al.* (1987) and Caillet *et al.* (1985)
$tRNA^{Pro}_3$	*proM*	$ptRNA^{His}CCA$	O'Connor *et al.* (1993)
$tRNA^{Ser}_5$	*serW*	pTH211	Cummings *et al.* (1994)
$tRNA^{Thr}_2$	*thrW*	pTH2	Komine and Inokuchi (1990)
$tRNA^{Thr}_3$	*thrT*	pBSBA2	Springer *et al.* (1989)
$tRNA^{Thr}_4$	*thrU*	pBSAR1	Springer *et al.* (1989)
$tRNA^{Val}_1$	*valU*	pVH94	this work

The tRNA genes except for those carried by $ptRNA^{His}CCA$ were derived from *E.coli*, and the overproduction of the corresponding tRNAs has been described previously. The four tRNAs expressed from plasmid $ptRNA^{His}CCA$, carrying the *hisR* tRNA cluster from *Salmonella typhimurium*, are identical in sequence to their counterparts in *E.coli*. Expression of the cluster in *E.coli* was measured by hybridization using a probe specific to $tRNA^{His}$, which was found to be overexpressed 7-fold. Parent plasmids were $ptRNA^{His}CCA$, pACYC184; pVH plasmids, pWSK29; pPP15 and pTH211, pBR322; pTH2, pUC118; pBS plasmids, Bluescript (Stratagene).

tion in revertant VH22 was clearly distinct from the *groESL* operon. We conclude, therefore, that overexpression of the chaperonins GroES and GroEL is able to suppress *pth*(ts) thermosensitivity.

Discussion

The isolation of a temperature-sensitive mutant of *pth* by Atherly and Menninger (1972) opened the way to genetic studies of premature dissociation of peptidyl-tRNA from the ribosome, a possibility that has, however, remained largely unexplored. Isolation of suppressors of the thermosensitive phenotype may give rise to altered levels of components of the protein synthetic machinery or to mutant components. Characterization of one such mutant indicated that the rate of peptidyl-tRNA drop-off was reduced ~5-fold, but the gene involved has not been identified (Anderson and Menninger, 1987). One cold-sensitive pseudo-revertant was found to contain a large deletion in the *argA* region, but the gene of relevance to Pth function is unknown (Atherly, 1979).

In the experiments described here, we have identified several types of suppressor of Pth thermosensitivity. The missense suppressor derived from *glyW* is a classical missense suppressor that restores the amino acid sequence of the wild-type protein by inserting Gly at position 101. The suppression resulting from the overproduction of the chaperonins GroES and GroEL is consistent with their role in stabilizing thermolabile folding intermediates of proteins (Van Dik *et al.*, 1989; Gordon *et al.*, 1994). The type of second site mutation exemplified by the L107F change in Pth will be of increasing interest when crystallographic structural information about the protein becomes available. However, the observation of most relevance to the phenomenon of premature termination is the identification of a tRNA species which, when overproduced in the cell, restores growth at 43°C to cells limited in Pth activity. Our measurements show that an overproduction of ~2-fold is sufficient to obtain suppression of thermosensitivity. This level of overproduction, measured relative to the concentration of $tRNA^{Leu}_1$ by tRNA-specific probes, is consistent with the presence of three genes coding for $tRNA^{Lys}$ in the cell, and the use of a plasmid estimated to be present in 6–8 copies. The fact that a reduction in the quantity of $tRNA^{Lys}$ in the cell, obtained by converting one of the genes encoding the tRNA to a nonsense suppressor, leads to an increased thermosensitivity of cell growth is consistent with this interpretation. This observation, and the fact that overproduction of the nonsense suppressor species rather than the wild-type tRNA does not suppress thermosensitivity, suggests that the overproduced $tRNA^{Lys}$ exerts its effect by functioning normally as a reader of Lys codons. It is extremely unlikely that overproduced $tRNA^{Lys}$ is acting as a missense suppressor of the mutation in *pth*(ts). An alternative hypothesis would be that overproduction of $tRNA^{Lys}$ somehow increases expression of the *pth* gene. The activity of Pth in cell extracts from the suppressed *pth*(ts) mutants we describe here (with the exception of the intragenic revertant) is too low for reliable measurement by the enzymatic assay. However, no effect of overproducing $tRNA^{Lys}$ was observed on β-galactosidase production from a *pth–lacZ* translational fusion (our unpublished results), which argues against this possibility. We interpret the experiments described here to mean that the inhibition of growth at 43°C of the *pth*(ts) mutant arises primarily as a result of starvation for $tRNA^{Lys}$ in the cell.

The wide variability in the rates of accumulation of different tRNAs as peptidyl-tRNA following transfer of a *pth*(ts) strain to non-permissive temperature was described by Menninger (1978). Amongst amino acid-accepting families, Lys was shown to be the fastest accumulating in the cell, and Gly the slowest, differing by a factor of ~30. Since the accumulation of individual isoaccepting species of tRNA was not studied, with the exception of Leu and Ile isoacceptors, it was not possible to conclude that $tRNA^{Lys}$ was the tRNA accumulated fastest as peptidyl-tRNA, and hence the species for which starvation was likely to occur first. Our observations strongly suggest that this is actually the case. Other amino acid-accepting families shown by Menninger to accumulate rapidly after a shift to non-permissive temperatures include His and Thr. However, we do not observe suppression as a result of overproducing $tRNA^{His}$, any of three Thr isoacceptors or several other tRNAs, consistent with our conclusion that the effect is specific to $tRNA^{Lys}$.

The reason for the disparity between the rates of accumulation of different peptidyl-tRNAs in the cell when

Table III. *Escherichia coli* bacterial strains

Strain	Genotype	References
C600pth(ts)	*supE44, thi1, thr1, leuB6, lacY1, tonA21, pth*(ts) *zch::*Tn*10*	Garcia-Villegas *et al.* (1991)
CAG18565	MGG1655 *nupC3146*::Tn*10kan*	Singer *et al.* (1989)
GE875	*argG292*(UAG), *relA1, melA7, leu290*(UAA), *his292, supN23 (HfrPO2A)*	Uemura *et al.* (1985)
OFB819	*galE, zje::kan*	Zeilstra-Ryalls *et al.* (1993)
VH7	*supE44, thi1, thr1, leuB6, lacY1, tonA21, pth*(ts), *fadR13::*Tn*10*	this work
VH20	*supE44, thi1, thr1, leuB6, lacY1, tonA21, pth*(ts), *recA56, srl-1300::*Tn*10*	this work
VH21	*trpR, lacZU118, pth*(ts), *zcg::Tn10, pth*(ts)	this work
VH22	temperature-resistant pseudo-revertant of VH21	this work
VH271	VH22 *zje::kan*	this work
VH4	*supE44, thi1, thr1, leuB6, lacY1, tonA21, pth*(ts)	this work
VH558	GE 875 *nupC3146*::Tn*10kan*	this work
VH591	C600 *pth*(ts) *supN23, nupC3146*::Tn*10kan*	this work
Y1	*trpR, lac*ZU118	Yanofsky and Soll (1977)

Pth is limiting is not clear. Different peptidyl-tRNAs may be better or worse substrates for the enzyme. Although diacetyl-lysyl-tRNA^{Lys} was the best substrate amongst those tested by Menninger *et al.* (1973), oligolysyl-tRNA^{Lys} was a very poor substrate. Thus, it is possible that some naturally arising peptidyl-tRNA^{Lys} substrates may be cleaved slowly by Pth. The rates of dissociation from the ribosome of different species may also vary widely. Central to this problem is to understand the balance between drop-off events involving cognate peptidyl-tRNA–codon interactions and those involving non-cognate interactions. This question can be considered both at the level of overall ribosome function and at the level of individual tRNAs. The likelihood of drop-off is probably vital in determining the level of selectivity of the ribosome. A high selectivity will entail a relatively loose tRNA–mRNA–ribosome interaction that will be accompanied by a significant level of drop-off involving cognate interactions, as well as a high level of drop-off involving non-cognate interactions. A lower selectivity will decrease the probability of drop-off, but will lead to more non-cognate tRNAs binding to the ribosome and escaping the proofreading step at the A-site, and hence more drop-off events involving non-cognate interactions.

The evolutionary pressures on ribosome structure that influence the tightness of the tRNA–mRNA–ribosome interaction presumably operate on tRNA genes and metabolic processes such as the post-transcriptional modification of tRNAs, as well as on ribosomal components. Both the propensity for misreading and the consequences of misreading events vary according to the species of tRNA: whether, for example, a species reads codons of a single codon family or one of two, three, four or six codons. It is possible only to speculate about the features of tRNA^{Lys} that make it particularly prone to drop-off in *E.coli*. The tRNA is thought to make a particularly unstable interaction with AAG codons, a property that is necessary for efficient translational frameshifting in the *dnaX* gene in *E.coli* (Tsuchihashi and Brown, 1992). The reading properties of tRNA^{Lys} may be influenced by a unique conformation of the anticodon loop, due to the presence of two post-transcriptional modifications, 5-methylaminomethyl-2-thiouridine at position 34 and N^6-threonylcarbamoyladenosine at position 37. The conformation of the anticodon loop when the tRNA is involved in a codon–anticodon interaction, or indeed in the form of a ternary complex with EF-Tu–GTP, is unknown but, when free in solution, model studies show that an interaction between the two modified bases at positions 34 and 37 disrupts the normal stacked helical conformation of the three anticodon bases and leads to an unusual structure in which only U35 and U36 are present in the normal configuration for interaction with mRNA (P.F.Agris, personal communication). It may be of interest to study the rates of drop-off of tRNA^{Lys} in modification-deficient mutants of *E.coli*. It is expected that further insight will come from the identification of chromosomal mutants that act as suppressors of the thermosensitive *pth* mutant. Thus, the results presented here suggest that the chromosomal suppressors located near 27.5 and 94 min may act in an essentially similar way to the *lysV* multicopy suppressor by increasing the concentration of tRNA^{Lys} in the cell; they thereby underline the significance of this suppressor.

Materials and methods

Bacteria and plasmids

Escherichia coli K12 strains are listed in Table III. VH4 was obtained from C600pth(ts) by selection of fusaric acid-resistant colonies on CTF plates containing chlortetracycline and fusaric acid (Maloy and Nunn, 1981). The Tn*10kan* transposon from CAG18565 was transduced by phage P1 to strain GE875, producing VH558, and the continued presence of *lysV*(SuUAA/G) (*supN23*) was tested by growth on minimal medium supplemented with histidine. VH591 was obtained from C600pth(ts) by co-transduction (frequency 83%) of Tn*10kan* and *lysV*(SuUAA/G) with a P1 lysate on VH558. Strain OFB819 was the source of the Kan^R cassette at *zje* in VH271. All plasmid constructions were derivatives of pWSK29 or pWSK129 (Wang and Kushner, 1991). Plasmids pVH153 and 154 were constructed with the aid of PCR-amplified fragments covering the *valU* operon, obtained using two oligonucleotides, 5′-GGATGACTCGCTTCGCTC-3′ upstream of the *valU* operon terminator, and 5′-CGGACGCAGATGTTTTTT-3′ in the *gltX* gene. The 975 bp *ClaI–PstI* fragment was cloned in each case between the same sites of pWSK29. Plasmid pOF12 (Fayet *et al.*, 1986), contained the *groESL* operon on an 8.1 kb *Eco*RI fragment. The *pth* gene was cloned from potential intragenic revertants after amplification by PCR using oligonucleotides 5′-GAATTCAATGGCACCGACGAAAATAC-3′ and 5′-AAGCTTCAAACCGACGATACCGCA-3′. Fragments were cloned into the *Eco*RV site of pWSK129 after treatment with terminal transferase and dTTP (Marchuk *et al.*, 1991).

Growth conditions and mutant selection

Luria broth (LB) medium (Sambrook *et al.*, 1989) or minimal Vogel–Bonner medium (Vogel and Bonner, 1955) was supplemented according to requirements. Antibiotics were added at the following final concentrations: tetracycline 15 μg/ml; kanamycin 50 μg/ml; ampicillin 100 μg/ml. Pseudo-revertants of *pth*(ts) were selected by plating dilutions of strains VH21 and VH7, grown in LB medium at 30°C, on LB agar at 43°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 DNA fragments on agarose gel was by Jetsorb gel extraction (Bioprobe). Phage P1 lysates, transductions and transformations were performed as described by Miller (1992).

Screening for suppression of thermosensitivity in pth(ts) strains

Cloned suppressors of thermosensitive *pth*(ts) strains were selected after transformation of strain VH20 with recombinant plasmids by plating directly on LB ampicillin at 42°C. The initial plasmids were constructed by cloning a complete *Pst*I digest of chromosomal DNA from strain VH271. Further steps of subcloning and transformation were performed at 30°C and suppression was screened for by replica plating at 42 and 43°C onto LB ampicillin plates. Deletions in plasmid pVH119 containing the *Cla*I–*Pst*I insert were obtained by exonuclease III and VII treatment after digestion with *Bam*HI and *Sst*I (Sambrook *et al.*, 1989).

DNA sequencing

Plasmid DNA was extracted using the alkaline-SDS procedure (Birnboim and Doly, 1979), treated with DNase-free RNase A at 37°C for 30 min followed by phenol/chloroform extraction. The DNA was isolated by PEG precipitation (Mierendorf and Pfeffer, 1987) and sequenced by the chain termination method of Sanger *et al.* (1977) using T7 DNA polymerase (Pharmacia) and M13 universal and reverse primers.

Southern hybridization

Chromosomal DNA from a K12 strain was digested to completion with the eight enzymes used to establish the *E.coli* physical map (Kohara *et al.*, 1987) and separated by gel electrophoresis on a 0.7% agarose gel. Phage λ DNA digested with *Bst*EII and labelled with [γ-^{32}P]ATP was used as a molecular weight marker. A probe was prepared from pVH86 by thermal cycling using an M13 universal primer and [α-^{32}P]dCTP and the lengths of the hybridizing fragments were used to determine the chromosomal location as described by Heurgué-Hamard *et al.* (1995)

Determination of relative tRNA concentrations

tRNA was extracted from cells in exponential growth, separated by electrophoresis on polyacrylamide gel (12%) in one dimension and transferred to Hybond-N hybridization filter (Amersham) as described by Emilsson and Kurland (1990). The relative quantities of $tRNA^{Leu}_1$ and $tRNA^{Lys}$ were determined by hybridization with 5′-labelled tRNA-specific oligonucleotides probes, 5′- CCCCCACGTCCGTAAGGACA-3′ for Leu and 5′- CCTGCGACCAATTGATTAAA-3′ for Lys (Emilsson and Kurland, 1990). Radioactivity was detected and quantified on a Phosphorimager (Molecular Dynamics). Dot blots were performed in a similar way, using ~2 μg of RNA per spot.

Pth activity

Cell-free extracts were prepared by sonication (Anderson and Menninger, 1987) after growth of bacteria in LB medium at 30°C. Diacetyl-[^{14}C]Lys-tRNA (Haenni and Chapeville, 1966) was used as substrate under conditions previously described (Anderson and Menninger, 1987); radioactive material soluble or insoluble in 10% trichloroacetic acid was determined by filtration through GF/C filters (Whatmann).

tRNA amino acid acceptor capacity

tRNA was extracted with 70% phenol (Cox and Littauer, 1962) from cells grown to an optical density of 1.2 at 600 nm in LB medium and washed once with 0.9% NaCl. After ethanol precipitation, the tRNA was purified by chromatography on DE52 (Whatmann) and deacylated by incubation for 30 min at 37°C in 1 M Tris–HCl, pH 9. Amino acid acceptance was determined as described by Delamarche *et al.* (1987).

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