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Peptidyl-tRNA hydrolase is involved in λ inhibition of host protein synthesis

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Escherichia coli rap **mutants do not support vegetative growth of bacteriophage λ and die upon transcription of λ DNA *bar* sites. Bacteria harbouring a *pth*(ts) mutation synthesize thermosensitive peptidyl-tRNA hydrolase (Pth) and die at 42°C from a defect in protein synthesis. We present evidence that both *rap* and *pth*(ts) mutations affect the same gene: (i) peptidyl-tRNA hydrolase activity was found to be defective in *rap* mutants; (ii) at a threshold temperature, *pth* cells, like *rap* mutants, prevented λ growth and were killed by transcription of cloned *bar* sites; (iii) sequencing a 1600 bp DNA fragment comprising both loci revealed an ORF located within the limits set by a complementation analysis and encoding a putative polypeptide of 21 kDa; (iv) cloning and sequencing of *rap* and *pth*(ts) mutant DNAs both revealed single nucleotide transitions from the wild type ORF sequence, resulting in Arg134 to His and Gly101 to Asp changes respectively. Analysis of plasmid-directed proteins identified a polypeptide of ~21 kDa; the N-terminal sequence, amino acid composition and isoelectric point of this protein match those expected from the ORF nucleotide sequence. We propose that Pth activity, directly or indirectly, is the target for λ *bar* RNA leading to *rap* cell death.**

Key words: bacteriophage λ *bar* inhibition/*Escherichia coli* *rap* mutants/peptidyl-tRNA hydrolase/protein synthesis inhibition/sequence of *pth*

Introduction

The enzyme peptidyl-tRNA hydrolase (Pth; EC 3.1.1.29) cleaves peptidyl-tRNA or *N*-acyl-aminoacyl-tRNA to yield free peptides or *N*-acyl-amino acid and tRNA. It has been proposed that the natural substrates for the hydrolase are peptidyl-tRNAs which drop off the ribosome during protein synthesis (Atherly and Menninger, 1972; Menninger, 1976). A mutation which directs a thermosensitive Pth activity has been mapped to the *trp* operon region in the *Escherichia coli* chromosome. Bacterial mutants carrying the *pth*(ts) mutation stop protein synthesis upon shift to 42°C (Atherly and Menninger, 1972) and accumulate peptidyl-tRNAs which somehow block translation (Menninger, 1976).

Bacteriophage λ does not grow on the *rap* mutant of *E.coli* (Henderson and Weil, 1976). Phage mutants that compensate for the host deficiency map to at least three different loci (Guzmán and Guarneros, 1989). Mutations in two of these loci, termed *bar*I and *bar*II, correspond to single base-pair (bp) changes within nearly identical 16 bp DNA segments. Plasmid constructions harbouring a *bar*$^+$ sequence under an active promoter are lethal for *rap* bacteria. Thus, transcription of a DNA segment containing a 21mer oligonucleotide sequence of *bar*I is sufficient to kill *rap* cells (Guzmán *et al.*, 1990). Transcription of *bar* sequences causes inhibition of protein synthesis but not of RNA synthesis in the *rap* cells. In addition, the stability of the wild type *bar* RNA in *rap* bacteria is much greater than that of mutant *bar* RNA (Pérez-Morga and Guarneros, 1990).

The *rap* mutation has been located at 26 min in the *E.coli* map linked to the *pth* gene. Molecular clones containing a fragment of 1.6 kilobase pairs (kb) of bacterial DNA from the 26 min region complement both *rap* and *pth*(ts) mutations (Guarneros *et al.*, 1987). Thus, there was a possibility that both mutations affected the same gene. In an effort to clarify the mechanism of λ *bar* inhibition of *rap* bacteria by λ, we have examined the identity of the sites defined by the *rap* and *pth*(ts) mutations. Our evidence shows that both *rap* and *pth* mutations affect the same gene, coding for Pth. Therefore, we propose that Pth, or an element directly controlled by the enzyme, is the target for the lethal effect by λ.

Results

Phenotypic identity between the rap and pth bacterial mutants

To test our supposition that the *pth*(ts) and *rap* mutations affected one and the same gene, we cross-assayed the phenotypes of the *pth* and *rap* mutants. The results (Table I) showed that the *pth*(ts) cells, defective in Pth activity, were unable to support the growth of λ$^+$ phage at the threshold temperature of 39°C. Conversely, the *rap* mutants, inefficient in supporting λ$^+$ growth, were defective in peptidyl-tRNA hydrolase activity. The hydrolase activity of the *pth*(ts) mutant was thermosensitive, as expected (line 3). Both the *pth*(ts) and the *rap* extracts showed ~10% of the wild type activity at 32°C. The data obtained for the *rap* hydrolase also showed a slight but reproducible thermosensitivity (line 2). Furthermore, *pth*(ts) mutants excluded λ *int*C more stringently than wild type phage, a phenotype shared with the bacterial *rap* mutants (Guzmán and Guarneros, 1989). Thus, both *rap* and *pth*(ts) bacterial mutants are phenotypically similar.

It has been shown that plasmids carrying a λ wild type *bar* DNA segment under the control of an active promoter are lethal to *rap* but not to wild type bacteria (Guzmán *et al.*, 1990). To establish further the phenotypic identity between *rap* and *pth*(ts) mutants, we assayed the *bar*$^+$ induced lethality in *pth*(ts) mutants (Table II). Transformation of the

Table I. Phenotypic identity of *pth* and *rap* mutants

Strain	Peptidyl-tRNA hydrolase activity[a]		Phage vegetative growth[b] at 39°C		
	32°C	42°C	λ+	λ*bar*^{s}	λ *bar*$^-$
C600	5.75(100)[c]	6.51(100)	+[d]	+	+
C600*rap*	0.55(9.6)	0.42(6.4)	−	−	+
C600*pth*	0.53(9.1)	0.08(1.1)	±	−	+

[a]Specific activity as a percentage of hydrolysed diacetyl-[^{14}C]lysyl-tRNA/min/μg protein×10^2, assayed as described in Materials and methods.
[b]The phage were, λ$^+$, λ *imm434*; λ *bar*^{s}, λ *intC226*; λ *bar*$^-$, λ *imm434* Δ*b2*.
[c]In parenthesis, relative activity as a percentage of wild type at the same temperature.
[d]Phage growth was estimated as described in Materials and methods.

Table II. Inviability of *rap* and *pth* mutants transformed by *bar*$^+$ plasmids[a]

Strain	Temperature (°C)	pPG 510[b]	pPG 511[b]
C600	37	+[d]	+
C600 *rap*	37	−	+
C600 *pth*(ts)[c]	37	+	+
C600 *pth*(ts)[c]	40	−	+

[a]Cells transformed with the indicated plasmids were plated onto medium containing ampicillin at the specified temperature.
[b]pPG 510 carries the λ *bar*$^+$ allele and pPG 511 harbours the λ *bar101* site. These alleles are transcribed constitutively from the *E.coli* p*gal* promoters.
[c]The batch of C600 *pth*(ts) transformed cells in lines 3 and 4 was the same.
[d]+ and − designate at least a 100-fold difference in the efficiency of transformant recovery.

strain C600 *pth*(ts) with pPG-510, a clone which transcribes the λ wild type *bar*I sequence constitutively, resulted in viable transformants at 37 but not at 40°C (third column, lines 3 and 4). The clone pPG-511, which transcribes the mutant *bar*101 instead, transformed at both 37 and 40°C (fourth column, lines 3 and 4). Hence, *pth*(ts) and *rap* mutants are phenotypically similar also in this respect.

Functional mapping of Pth and Rap with mutations generated in vitro

We tested further the degree of linkage between the *pth* and *rap* sites by *in vitro* mutagenesis of the 1.6 kb bacterial DNA fragment. The changes introduced and the complementation data for the respective molecular clones are summarized in Figure 1B. Insertion of a few bases by repair of the *Nco*I$_2$ site (pEG-3), or insertion of a translation termination synthetic linker at the unique *Nru*I site in the fragment (pFV-*Nru*) abolished both the Pth and the Rap complementing ability of the clones.

We also tested the complementing ability of deletions entering the 1.6 kb fragment from either end as well as that of an *Nco*I-generated internal deletion. The results (Figure 1B, lines 1−5) defined a limited DNA segment that when encroached upon by deletions lost its complementing ability. Thus, Pth and Rap complementation abilities run parallel in the assay suggesting that a unique gene is responsible for both functions.

Sequence analysis of rap−pth complementing fragments

The nucleotide sequence of the 1.6 kb wild type DNA fragment cloned in M13 was determined by the dideoxy chain termination method. The strategy used is shown in Figure 1C. The sequence obtained (Figure 2) was 1620 bp in length and showed one ORF, long enough to encode a protein of 21 kDa and falling within the limits set by the complementation analysis. The ORF extends from coordinates 703 to 1284, initiates with GUG, terminates in UAA and contains 194 amino acid codons. The calculated molecular weight and isoelectric pH of the unmodified polypeptide were 21 050 Daltons and 9.68 respectively. Immediately upstream of the proposed initiation codon, a putative promoter, a Shine−Dalgarno sequence, a discriminator and an A-rich sequence have been identified at consensus spacings (see Figure 2) (Harley and Reynolds, 1987; Travers, 1984; Stormo *et al.*, 1982). Computer analysis of the 1620 nucleotide sequence predicted two other putative ORFs of high coding probability. The second ORF runs from coordinates 424 to 149, is encoded by the opposite strand to that encoding *pth*, and would encode a protein of ~10 kDa. The third ORF begins approximately at coordinate 1404 and continues to the 3′ end of sequence, suggesting that it may terminate outside the sequenced fragment. Again, just upstream of both ORFs we found possible prokaryotic ribosome binding sites and putative promoters (data not shown). We were unable to find significant protein homologies between recent databases and the products predicted by the three ORFs, using the threshold value proposed by Sander and Schneider (1991).

To determine the molecular nature of *rap* and *pth*(ts) mutations, we cloned each mutant 1620 bp segment into M13 vectors and determined their nucleotide sequences (data not shown). The *pth*(ts) mutation was identified as a G to A transition at coordinate 1004; similarly, the *rap* mutation was a G to A transition at coordinate 1103 (Figure 2). Both changes were located within the putative Pth ORF and should result in amino acid substitutions Arg134 to His and Gly101 to Asp in the respective *rap* and *pth*(ts) mutant proteins.

Identification of the pth gene product

The proteins encoded in some of the clones described above were analysed in maxicells. Several proteins were identified by [^{35}S]methionine labelling followed by one-dimensional PAGE (Figure 3). The wild type 1.6 kb fragment directs the synthesis of three polypeptides of 22, 24 and 30 kDa which were not present in the *Nco*I$_1$−*Nco*I$_2$ deletion clone (Figure 3, lanes 1 and 2). The 22 kDa band was the only one that correlated with the Rap and Pth co-complementing activities (Figure 3, lanes 1 and 4), whereas the other three smaller proteins were a common background. A plasmid construct carrying a fragment with a translation termination linker at the *Nru*I site (see Figure 1B) was also defective in directing the synthesis of the 22 kDa polypeptide (result not presented). These data confirmed our supposition that both Pth and Rap are phenotypes of the same *pth* gene. The 24 kDa polypeptide may be a fusion protein initiated at the ORF beginning at coordinate 1404 in the insert sequence, and continued in the truncated β-lactamase gene of the vector. We have no plausible explanation for the 30 kDa band, which is absent in the pEG2 lane. No smaller polypeptides appeared in bacteria transformed with mutant plasmids expected to direct truncated proteins.

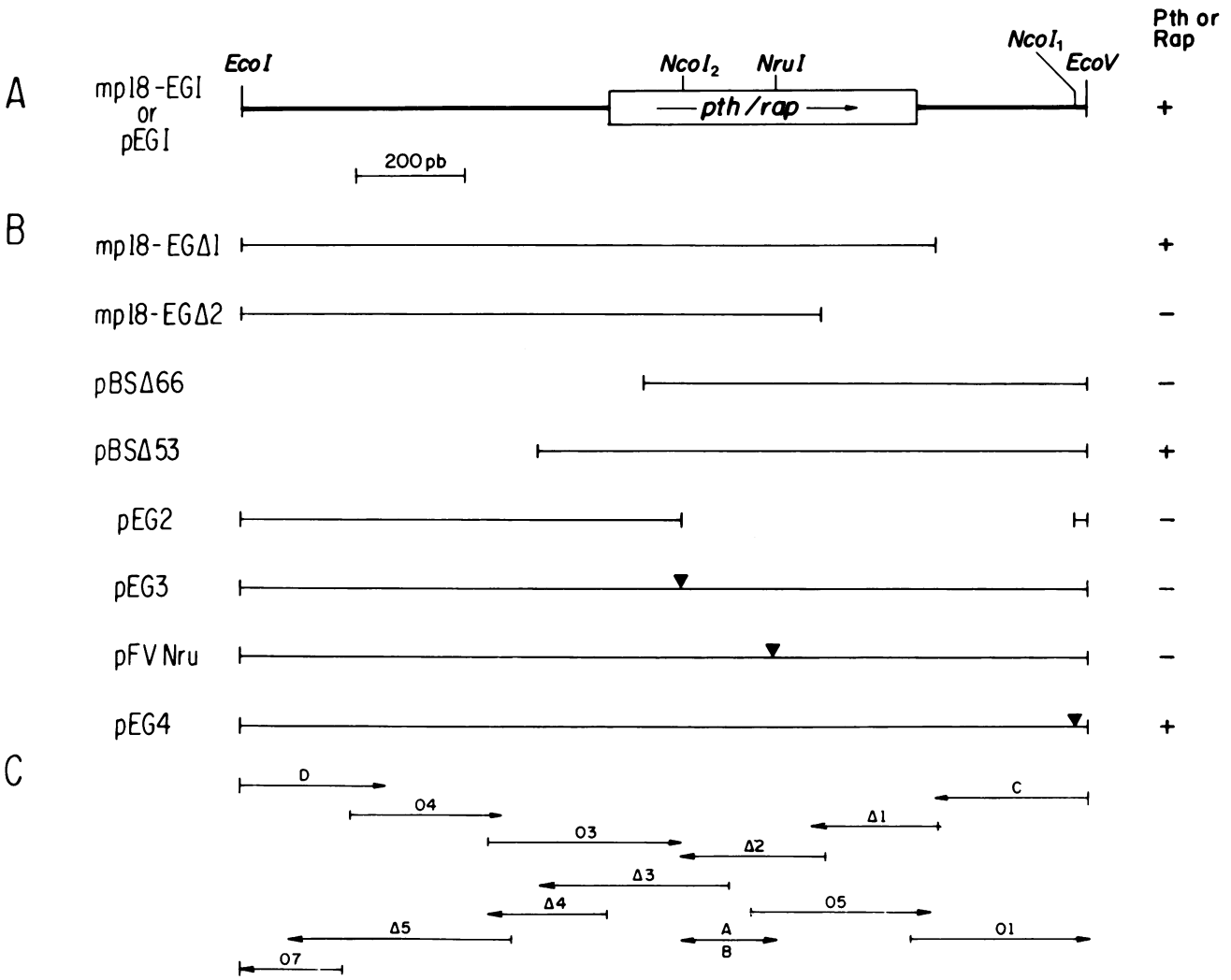

Fig. 1. Functional mapping and sequencing strategy for the *pth*−*rap* complementing fragment. (**A**) Map of the 1.6 kb *Eco*RI−*Eco*RV clone with the relevant restriction sites. The open box represents size, position and direction of the *pth* gene (see Figure 2). (**B**) Sub-clones and their Pth and Rap complementation activities. The M13 or Bluescribe clones contained the indicated segments of bacterial DNA (continuous lines); ▼, marks short insertions (see Materials and methods); + or −, indicate the Rap/Pth phenotype for each mutant. (**C**) Sequencing strategy for both DNA strands. A and B, sequences derived from a pBR322 clone using priming oligonucleotides complementary to vector sequences; C, D and Δ1 to Δ5, sequences obtained with commercial primers specific for mp vector sequences. 01, 03, 04, 05 and 07, sequences determined by priming with synthetic oligonucleotides designed after the sequence obtained for the opposite strand. See Materials and methods for details.

Based on the calculated isoelectric pH for the amino acid composition of the putative Pth protein (pI = 9.68), we set up a two-dimensional electrophoresis system to look for a basic protein of ~22 kDa (Figure 4). Such a protein was identified by Coomassie brilliant blue staining in extracts of cells carrying *pth* complementing plasmids but not in bacteria transformed with vector alone (panels B and A respectively). In addition, this result suggested that the complementing clone overproduced Pth protein. This supposition was proved correct: the more sensitive technique of Western blots applied to two-dimensional gel electrophoresis of cell extracts harbouring the cloning vector without insert, revealed a faint spot of Pth protein (data not shown). Presumably the chromosomal *pth* gene directs the synthesis of this protein.

We tested whether the N-terminal polypeptide sequence and the overall amino acid composition of the protein directed by the clone corresponded to those expected from the nucleotide sequence. Indeed, the amino acid sequence from the second to the seventeenth positions in the protein was exactly as predicted. A relatively reduced methionine signal was detected at the first amino acid position suggesting partial processing of this amino acid from the native protein (data not presented). The total amino acid composition of the protein also agreed fairly well with the predicted composition (Table III). We concluded that the 22 kDa protein is the product of the *pth* gene.

Discussion

In this paper we have shown that the inability of *rap* bacteria to grow λ phage is caused by a mutation in *pth*, the gene for the enzyme Pth. In addition, we have sequenced the *pth* gene in both wild type and mutant forms, identified its protein product and characterized a further mutation in the gene that leads to temperature sensitive growth.

The identity of the gene affected by the *rap* and *pth*(ts) mutations was first established phenotypically. The thermosensitivity of the Pth activity in *pth*(ts) bacteria correlated with the defect in supporting the growth of λ phage and the inability to maintain plasmids which transcribe wild

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cactgcgcactatggctcgcgctggcattcaaccgatcccgggacgcctggcgttcttcccgaatcgtcgcgcggcgatggcggatttat   90
aagtacgagattgaccagtcagcgcaaactgactggtcagcaaactgcattatttgttagctatgacggcgattatcgctacggcggcaa  180
cgtttgtcatagtggcgaacccaccagtaacggtcgcacacctgttcatgcccgccaacacgggcacccgccagccagagaatggcaccg  270
acgaaaatacttagcactgcaccatgtgcgaaatactggggcatattaaactgtggtaactggttgaggattgaataccccacgccgacc  360
accattaccaccagacccaaccccatgagcacgttaccgagtaacgaagcgtttttgcgtttcatatgtcacctccggaactttctgggt  450
tgtaacagggaataccctcttccttatgtgtaaagtatagacaacacccagtgggttatgtgcgggcgtgatcacaattacaaccctta  540
                          Δ53▸
tttcaacaaaactttacaaataaacgcctgaacaccttgactttctaatagtccactcaggcaattattacgttatctgcatttgatacg  630
      -35                 -10                          SD          703
cagtttttttgtcaagcggggccgcaaccagtaaactacgcgccagttatgtacacactcaggacaaaaaaacGTGACGATTAAATTGATT  720
                                                                    METThrIleLysLeuIle
                                                 Δ66▸
GTCGGCCTGGCGAACCCCGGTGCTGAATACGCCGCAACGCGACATAATGCTGGTGCCTGGTTCGTTGACTTACTGGCAGAGCGTTTGCGC  810
ValGlyLeuAlaAsnProGlyAlaGluTyrAlaAlaThrArgHisAsnAlaGlyAlaTrpPheValAspLeuLeuAlaGluArgLeuArg
                                          ↓ NruI
GCTCCGCTGCGCGAAGAGGCTAAATTCTTTGGTTATACTTCGCGAGTCACTCTTGGAGGCGAAGATGTCCGCCTGTTAGTCCCGACTACA  900
AlaProLeuArgGluGluAlaLysPhePheGlyTyrThrSerArgValThrLeuGlyGlyGluAspValArgLeuLeuValProThrThr

TTTATGAATCTCAGCGGCAAAGCCGTTGCGGCGATGGCCAGTTTTTTCCGCATTAATCCGGACGAAATTCTGGTGGCCCACGACGAACTG  990
PheMetAsnLeuSerGlyLysAlaValAlaAlaMetAlaSerPhePheArgIleAsnProAspGluIleLeuValAlaHisAspGluLeu
     pth(Ts):A                            ↓ NcoI
GATCTGCCTCCTGGCGTCGCCAAATTTAAATTGGGCGGTGGCCATGGTGGTCACAATGGACTGAAAGACATCATCAGTAAATTGGGTAAT 1080
AspLeuProProGlyValAlaLysPheLysLeuGlyGlyGlyHisGlyGlyHisAsnGlyLeuLysAspIleIleSerLysLeuGlyAsn
               rap:A                                  ◂Δ2
AACCCTAACTTTCACCGTTTACGCATCGGAATCGGTCATCCGGGCGATAAAAATAAAGTTGTCGGTTTTGTGTTAGGCAAACCGCCTGTT 1170
AsnProAsnPheHisArgLeuArgIleGlyIleGlyHisProGlyAspLysAsnLysValValGlyPheValLeuGlyLysProProVal

AGTGAACAGAAGTTAATTGATGAAGCCATTGACGAAGCGGCGCGTTGTACTGAAATGTGGTTTACAGATGGCTTGACCAAAGCAACGAAC 1260
SerGluGlnLysLeuIleAspGluAlaIleAspGluAlaAlaArgCysThrGluMetTrpPheThrAspGlyLeuThrLysAlaThrAsn

CGATTGCACGCCTTTAAAGCGCAAtaagtcgttgtctgcggcatttttgccgagtgccgtgtataataggcaaagttatttccatttctg 1350
ArgLeuHisAlaPheLysAlaGln
   ◂Δ1
caatctgttagcaataacaggttgattattaagatattaaggtgatttaaatcatgggattcaaatgcggtatcgtcggtttgcccaaac 1440
gtcgggaaatctaccctgttcaacgcgctgaccaaagccggtattgaagcggccaactttccattctgcaccattgagccgaacacaggc 1530
gtcgtaccaatgcctgatcctcgcctggatcaactggctgaaatcgtaaaaccgcagcgtacgcttcccacgaccatggaatttgtcgat 1620
                                                                          ↑ NcoI
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Fig. 2. Nucleotide sequence of the *Eco*RI−*Eco*RV fragment. The predicted amino acid sequence of the *pth* gene product is indicated. Putative Shine−Dalgarno (S-D) and promoter (−10, −35) sequences are underlined. *rap* and *pth*(ts) mutations, restriction sites and deletions are indicated on the sequence. Δ53 and Δ66 eliminated sequences between *Eco*RI and the position marked ▶; Δ1 and Δ2 deleted sequences from the marked nucleotide (◀) to the *Eco*RV end. This sequence has been deposited in the EMBL database under accession number X61941.

type λ *bar* region constitutively (Tables I and II). These abnormal phenotypes in the *pth*(ts) mutant were expressed only at the threshold temperature, 39°C, thus suggesting their dependence on a reduced Pth activity. Consistent with the above results the *rap* cells, isolated through their deficiency in supporting the growth of λ proved to be defective in Pth activity (line 2, Table I). The slight thermosensitivity of the Pth(*rap*) activity reported here correlates with the high stringency in λ exclusion observed with the *rap* strains at 42°C (Henderson and Weil, 1976). This observation further supports the association between limiting Pth activity and the Rap phenotype.

The identification of the *pth* gene was based on the determination of the nucleotide sequence of a DNA fragment which carried the genetic information to complement the *pth*(ts) and *rap* mutations (Figure 2). Analysis of this 1620 bp sequence revealed an ORF of 194 amino acids. Putative promoter, Shine−Dalgarno and discriminator sequences were identified at appropriate positions. Nucleotide sequence data also showed that both *rap* and *pth*(ts) mutations affect the *pth* gene. Both mutations were characterized as G to A transitions, *pth*(ts) at codon 101 and *rap* mutation at codon 134 of the Pth ORF. These changes should result in mutant proteins with substitutions of aspartic acid for glycine and histidine for arginine, respectively. The assignment of the proposed ORF to the *pth* gene from the nucleotide sequence was consistent with the complementation analysis of a set of mutations in the 1.6 kb fragment (Figure 1B). All the clones which co-complemented *pth*(ts) and *rap* mutations left the ORF intact. Conversely, clones defective in complementation for *pth* were also defective for *rap* and affected the ORF.

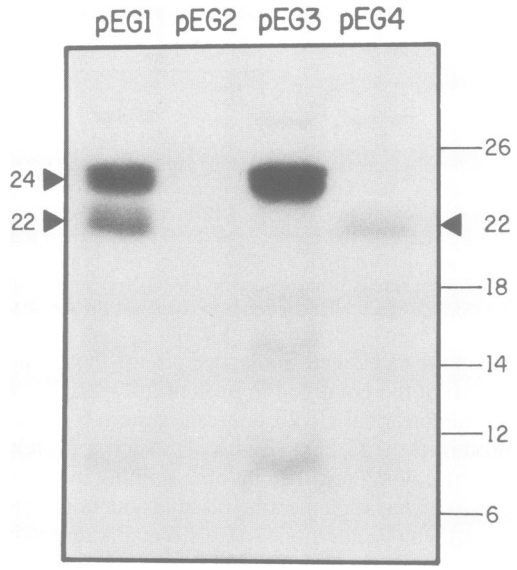

Fig. 3. Analysis of plasmid coded proteins. Maxicells containing the indicated plasmids were prepared, radiolabelled and analysed as described in Materials and methods. The arrowheads indicate the main proteins synthesized and their molecular mass. The scale marks the actual migration position of a mixture of molecular weight standards. Molecular masses are in kDa.

The Pth protein was identified as the product of the *pth* gene by determination of the N-terminal amino acid sequence (data not shown) and by estimation of the overall amino acid composition of the purified polypeptide (Table III). The lower than expected molar proportion of the initial Met found

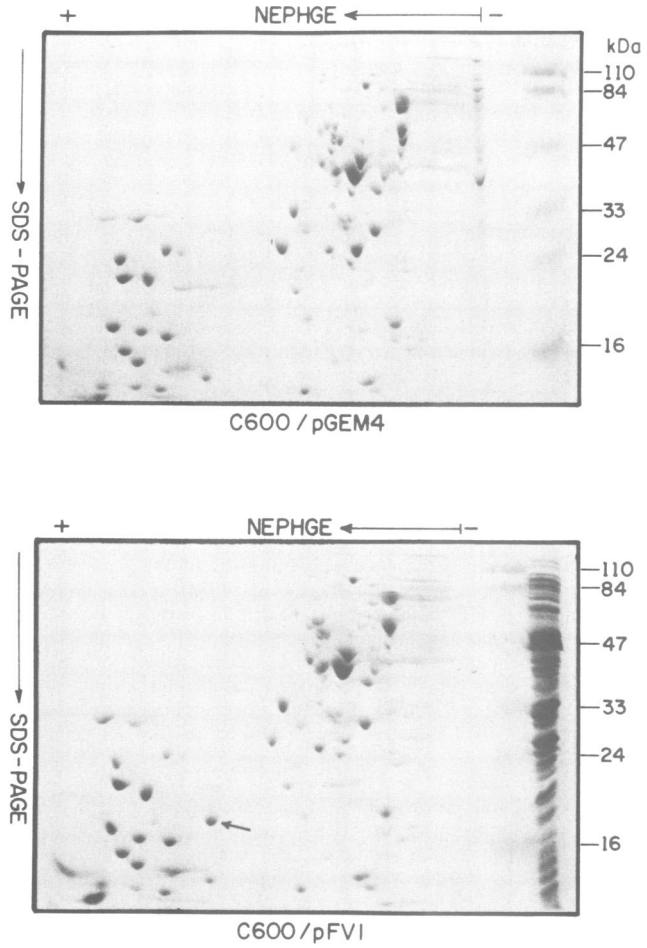

Fig. 4. Two-dimensional gel analysis of extracts from cells transformed with *pth* clones. **(Top)** C600 bacteria carrying the plasmid vehicle pGEM4, **(Bottom)** C600 transformed with pFV1, a clone of *pth* gene. The extracts were prepared as described in Materials and methods. 500 μg of total protein of each extract were submitted, in the first dimension, to non-equilibrium pH gradient electrophoresis (500 V/h, 3 h); and in the second dimension, to SDS−PAGE (12.5%) either with a molecular weight standard mixture (panel A, right) or with crude cell extract (panel B, right). The arrow in the lower panel points to Pth protein. The scales on the right express the apparent molecular weights of the standard proteins in kDa.

in the N-terminal determination probably reflects processing of this amino acid at the N-terminus, a common case in *E.coli*. In addition the apparent molecular weight and isoelectric point of the Pth protein (Figures 3 and 4) also agreed with the values predicted from the nucleotide sequence.

We were unable to detect truncated polypeptides in extracts from transformants for plasmid mutants expected to direct them. Nor were we able to observe a difference between the isoelectric pHs of wild type Pth and Pth(ts) proteins by the techniques used in this work (data not shown). The mutant clones pEG-2, pEG-3, and pFV-Nru (Figure 1B, lines 5−7), as expected, did not encode the 22 kDa protein.

What is the mechanism of λ *bar* RNA inhibition of Pth defective bacteria? Firstly, it must be recognized that the precise role of Pth, and the mechanism by which *pth*(ts) mutations lead to cell death at the non-permissive temperature, are not entirely clear. It has been shown that the reduced Pth activity in the *pth*(ts) mutant allows the accumulation of peptidyl-tRNA molecules in the cells

Table III. Deduced and determined amino acid compositions of purified peptidyl-tRNA hydrolase

Amino acid	Deduced	Determined[c]
Ala	21	20.5
Arg	11	11.3
AsX[a]	20	20.3
Cys	1	n.d.[d]
GlX[b]	13	13.0
Gly	21	21.0
His	7	6.5
Ile	10	10.4
Leu	21	20.0
Lys	13	13.0
Met	4	3.1
Phe	11	10.7
Pro	10	10.5
Ser	5	5.2
Thr	10	10.1
Trp	2	n.d.[d]
Tyr	2	2.3
Val	12	13.0
Total	194	191

[a]AsX = Asp + Asn
[b]GlX = Glu + Gln
[c]Average of three determinations
[d]n.d., not determinable by the method

(Menninger, 1979). These molecules may be toxic to the cell through any of several ways. Since peptidyl-tRNAs chemically resemble fMet-$tRNA_f$, the normal initiator of protein synthesis, they may block protein chain initiation. Alternatively, free ribosomes may bind peptidyl-tRNA and become unable to dissociate into subunits and re-initiate protein synthesis (Atherly and Menninger, 1972). Other possibilities such as binding of peptidyl-tRNA to ribosomes that are synthesizing protein or sequestration of a particular tRNA as peptidyl-tRNA, causing a critically reduced level of that tRNA thus blocking protein synthesis, cannot be ruled out (Menninger *et al.*, 1973; Chapeville *et al.*, 1969).

The results presented here show that defects in Pth activity and wild type λ *bar* transcription act synergistically in the inhibition of cell growth. Different proposals may be made to explain how Pth activity might become limiting in the presence of *bar* transcripts. One explanation assumes that the residual enzyme activity in *pth* mutants is inhibited directly by interaction with *bar* transcripts. This is a refinement of a model proposed previously (Guzmán *et al.*, 1990). It is known that *pth*(ts) mutants at the restrictive temperature stop protein synthesis (Atherly and Menninger, 1972) and die quickly (Menninger, 1979) and similar effects have been observed for *pth(rap)* bacteria following λ *bar* transcription (Pérez-Morga and Guarneros, 1990). However, the prediction of Pth inhibition by *bar* transcription remains to be tested fully. Preliminary results do not support a stable interaction between Pth protein and *bar* RNA (García-Villegas and Guarneros, unpublished results). It is also possible that λ *bar* transcription may prevent hydrolysis of a specific peptidyl-tRNA in cells limited for Pth activity. This mechanism would not require that *bar* inhibition should further reduce Pth activity in the *pth* cells.

An alternative explanation for Pth becoming limiting after λ *bar* transcription is that the RNA interacts with the protein synthetic apparatus in some way that results in an increase in the amount of substrate for the enzyme. The fact that wild

type *bar* RNA, but not the mutant *bar*I101 RNA, is stabilized after induction in *pth(rap)* bacteria (Pérez-Morga and Guarneros, 1990) suggests the formation of a complex with some cellular component that is related to the inhibitory effect of the RNA. This component might be the ribosome. Finally, it is conceivable that the Pth protein has an essential role in cell metabolism that has not been identified so far. Whether or not this is substantiated, the work presented here strongly indicates that it is the cellular function of this protein that is directly or indirectly the target of λ inhibition.

Materials and methods

Bacteria, phage and plasmids

The *E.coli* K-12 strains used were: C600 (*thr1 leuB6 thi1 lacY1 supE44 tonA21*); DH173, a *rap lac* Y14 derivative of C600; C600 *rap* (Guzmán and Guarneros, 1989); CSR 603 (*recA1 uvrA6 phr-1*), a maxicell source (Sancar *et al.*, 1979); C600 *pth*(ts), obtained by P1 cotransduction of *pth*(ts) with *zch*::Tn*10* from strain HO300 (Hove-Jensen, 1985). Phage were: λ *imm434* (*bar*$^+$), λ *imm434* Δ*b2* (*bar*$^-$), λ *intC226* (*bar*^{s}) (Guzmán and Guarneros, 1989), λ HO-1, a D69 derivative harbouring a 5.6 kb chromosomal segment containing *pth* and *prs* genes (Hove-Jensen, 1985). Plasmid pEG-1 is a pBR322 derivative which carries the *pth*−*rap* co-complementing *Pvu*I−*Eco*RI fragment of bacterial DNA; pEG-2 construct is an *Nco*I$_1$−*Nco*I$_2$ deletion of pEG-1 unable to co-complement; pGM-5, a *pth*−*rap* co-complementing derivative of pHO-1 by an internal *Ava*I-generated deletion (Guarneros *et al*, 1987).

Growth conditions and assay of Rap and Pth phenotypes

The media for bacterial growth were: Luria broth (LB) and L agar, and the same media containing ampicillin (50 μg/ml) or tetracycline (12.5 μg/ml) (Maniatis *et al.*, 1982). For [^{35}S]methionine labelling of transformed maxicells, cells were grown to log phase in M9 medium supplemented with 0.4% glucose, 0.2 μg/ml thiamine and 1% casamino acids, then preincubated in M9 medium supplemented with all 20 amino acids except methionine for 1 h at 37°C and labelled for 15 min with 35 μCi/ml of L-[^{35}S]methionine (1000 Ci/mmol, Amersham International, UK) (Neidhardt *et al.*, 1974).

The Rap phenotype of bacteria was assayed by its ability or inability to grow λ *bar*$^+$ phage (Guzmán and Guarneros, 1989). Dilutions of λ *imm434* (*bar*$^+$) or λ Δ*b2 imm434* (*bar*$^-$) stocks were spotted onto lawns of bacteria: Rap$^-$ bacteria plated λ *imm434* at 100- to 10 000-fold less efficiency than wild type (Rap$^+$) bacteria. Thermosensitive *pth* mutants fail to grow above 42°C, therefore the maximal temperature used in this case was 39−40°C. The Pth phenotype of plasmid constructs was determined by transformation of *pth*(ts) strains. Bacteria carrying *pth*$^+$ plasmids grew at 42°C. The Pth phenotype in M13 clones was determined using the same rescue principle. In this case the bacterial strains should also carry an F$^+$ plasmid derivative to provide adsorption receptors for the phage. The *pth*(ts)/F$^+$ bacteria infected with M13 derivatives harbouring a wild type *pth* gene grew at 42°C, whilst bacteria infected with the M13 vector alone, or with M13 clones defective in the *pth* gene, were not viable at 42°C.

Recombinant DNA and genetic manipulations

General procedures for DNA recombinant techniques, plasmid extraction, etc. were performed as described by Maniatis *et al.* (1982). Plasmids pEG-3 and pEG-4 were constructed by partial digestion of plasmid pEG-1 with *Nco*I, repair with the Klenow fragment of DNA polymerase I, religation with T4 DNA ligase and selection of transformants on tetracycline medium. The transformants were screened for loss of either *Nco*I site. The *pth*$^+$ plasmid pFV-1 was constructed by cloning the *Bam*HI−*Ava*I 2 kb fragment from pGM-5 into the pGEM-4 vector (Promega, Madison, WI). The pFV-*Nru* derivative was constructed by cloning a synthetic translation termination linker (*Spe*I site, New England Biolabs, Beverly, MA) at the *Nru*I site of pFV-1. Nested deletions of the 1.6 kb fragment for complementation analysis and for sequencing were generated in clones of Bluescribe M13$^+$ (Stratagene, La Jolla, CA), or of M13 mp18, as recommended by the manufacturer. Rescuing of the *rap* mutation was achieved by cloning in plasmid pGEM4Z (Promega, Madison, WI) *Eco*RI−*Eco*RV restriction fragments of ~1.6 kb from DH173 (*rap*$^-$) genomic DNA, separated by gel electrophoresis and purified by Geneclean (Bio 101 Inc., La Jolla, CA). The *rap* clones were screened for those which complemented C600 *pth*(ts) mutants at 42°C and further verified by restriction mapping. M13 mp18 and M13 mp19 clones for the *rap* segment were also constructed for DNA sequencing. The *pth*(ts) mutation was rescued by homologous excision of λ HO-1 from a C600 *pth*(ts) lysogen. Lysates resulting from mitomycin induction (4 μg/ml) were plated on C600 *pth*(ts), incubated 6 h at 30°C and shifted to 44°C overnight. Two types of plaques appeared on the poor cell lawn, clear and turbid. The former class proved to be phage carrying the *pth*(ts) allele, λ G15. The *Eco*RI−*Eco*RV 1.6 kb fragment containing the *pth*(ts) mutation was purified from λ G15 and cloned into M13mp18 and M13mp19 for sequencing.

DNA sequencing

Both double-stranded and single-stranded DNAs were sequenced by the chain termination method of Sanger *et al.* (1977) with a T7 modified polymerase (Sequenase, US Biochemical Corp., Cleveland, OH) using deoxyadenosine 5′-[α-^{35}S]thiotriphosphate (>600 Ci/mmol; Amersham International, UK). Oligonucleotide primers for template sequencing were synthesized in a Gene Assembler DNA synthesizer (Pharmacia, Uppsala). Several oligonucleotide primers were generously provided by X.Soberón (CEINGEBI, Cuernavaca). The sequencing of *rap* and *pth*(ts) alleles was performed using the following oligonucleotide primers (described 5′ to 3′ using coordinates as in Figure 2): for the upper strand, 504−519; 638−653; 800−816; 967−982; 1197−1212; for the lower strand, 1331−1316; 1127−1113; 939−924.

Computer analyses of DNA sequences

DNA sequences were analysed with University of Wisconsin Genetics Computer Group software (Devereux *et al.*, 1984) running on a VAX computer (Digital Equipment Corp.). Search for potentially homologous proteins was achieved using the computer program TFASTA (Pearson and Lipman, 1988) running on the EMBL and GenBank (release 62.0) sequence databases. Search for ORFs, prokaryotic initiation signals and protein signatures was done with the PC/Gene program package (release 6.2, Intelligenetics, Inc., Mountain View, CA) using an IBM AT computer.

Pth activity assay

Cell cultures were grown at 32°C in supplemented M9 medium and the cell extracts for hydrolase assay were prepared as described by Anderson and Menninger (1987) disrupting the cells in a French press. Diacetyl-[^{14}C]lysyl-tRNA was used as substrate and prepared according to the procedure of Haenni and Chapeville (1966). The assay was performed essentially as described by Anderson and Menninger (1987) using 50 μg of wild type protein per assay in 55 μl and pre-incubating for 10 min at the appropriate temperature. Reactions were initiated by the addition of 0.6 A_{260} units of substrate in 27.5 μl. Duplicate reactions were terminated at 0, 2.5, 5, 7.5 and 10 min by precipitation with 1 ml 10% TCA, filtered through GF/C glass fibre filters, and the radioactivity in the dried filters was determined by liquid scintillation counting.

Analysis of proteins

For the analysis of plasmid coded proteins in maxicells, the procedure of Sancar *et al.* (1979) was essentially followed. The cultures of the maxicell CSR 603 strain transformed with the appropriate plasmids were treated with cycloserine (200 μg/ml) after UV irradiation (Silhavy *et al.*, 1984). The proteins were resolved by SDS−polyacrylamide (12%) gel electrophoresis (SDS−PAGE) by the procedure of Laemmli (1970). Resolution of basic proteins by two-dimensional gel electrophoresis was performed by the non-equilibrium pH gradient electrophoresis (NEPHGE) method of O'Farrell *et al.* (1977). Cells extracts were prepared from log phase LB-Amp cultures of the transformed strains. The cells were sonicated and the lysates centrifuged for 30 min at 30 000 *g*. A pH 3.5−10 ampholyte mixture was used for the first dimension and 12.5% SDS−PAGE for the second dimension. Gels were stained with Coomassie brilliant blue R, dried and autoradiographed in the case of radiolabelled samples. Unlabelled molecular mass standards were included during electrophoresis.

N-terminal amino acid analysis was done from pure Pth protein separated by NEPHGE and electroblotted onto an Immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedlford, MA) (Matsudaira, 1987). Automated gas phase N-terminal sequencing was done in an Applied Biosystems 470A sequencer (Hunkapiller and Hood, 1983). The stepwise liberation of Pth amino acid derivatives was analysed on a Nova Pak column (Millipore Corp., Beldford, MA). For amino acid composition, Pth protein was purified (M.R.García-Villegas, unpublished) and submitted to a final step through reversed phase HPLC on a μ Bondapak C18 column (Waters Assoc., Millipore Corp., Bedford, MA) eluted in 0.075% trifluoroacetic acid with a 7−70% acetonitrile linear gradient. Protein was hydrolysed in 6 M HCl−1% phenol at 110°C for 24 h. A Pico-Tag System (Bidlingmeyer *et al.*, 1984) was used according to the manufacturer's instructions.

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