Microbiology (2005), **151**, 1741–1750 DOI 10.1099/mic.0.27915-0

Acquisition of a stable mutation in *metY* allows efficient initiation from an amber codon in *Escherichia coli*

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Received 26 January 2005
Revised 23 February 2005
Accepted 23 February 2005

Escherichia coli strains harbouring elongator tRNAs that insert amino acids in response to a termination codon during elongation have been generated for various applications. Additionally, it was shown that expression of an initiator tRNA containing a CUA anticodon from a multicopy plasmid in *E. coli* resulted in initiation from an amber codon. Even though the initiation-based system remedies toxicity-related drawbacks, its usefulness has remained limited for want of a strain with a chromosomally encoded initiator tRNA 'suppressor'. *E. coli* K strains possess four initiator tRNA genes: the *metZ*, *metW* and *metV* genes, located at a single locus, encode $tRNA_1^{fMet}$, and a distantly located *metY* gene encodes a variant, $tRNA_2^{fMet}$. In this study, a stable strain of *E. coli* K-12 that affords efficient initiation from an amber initiation codon was isolated. Genetic analysis revealed that the *metY* gene in this strain acquired mutations to encode $tRNA_2^{fMet}$ with a CUA anticodon (a U35A36 mutation). The acquisition of the mutations depended on the presence of a plasmid-borne copy of the mutant *metY* and *recA*$^+$ host background. The mutations were observed when the plasmid-borne gene encoded $tRNA_2^{fMet}$ (U35A36) with additional changes in the acceptor stem (G72; G72G73) but not in the anticodon stem (U29C30A31/U35A36/ψ39G40A41). The usefulness of this strain, and a possible role for multiple $tRNA_1^{fMet}$ genes in *E. coli* in safeguarding their intactness, are discussed.

INTRODUCTION

Organisms have evolved with two types of methionyl tRNAs, the initiators and the elongators. The initiators function at initiation, and the elongators decode the subsequent AUG codons in an mRNA. Both tRNAs are aminoacylated by the same methionyl tRNA synthetase. In eubacteria, while the elongator tRNAs (Met-$tRNA^{Met}$) bind directly to EFTu, the initiators (Met-$tRNA^{fMet}$) are modified to fMet-$tRNA^{fMet}$ by methionyl-tRNA (fMet) formyltransferase prior to their interaction with IF2 (Kozak, 1983; Guillon *et al.*, 1996; Wu & RajBhandary, 1997). A striking feature of the eubacterial initiators, the presence of a mismatch at the 1:72 position, is responsible for at least three of their important properties: their recognition by methionyl-tRNA (fMet) formyltransferase; the prevention of their binding to EF-Tu; and their resistance to peptidyl-tRNA hydrolase, an enzyme which hydrolyses N-blocked aminoacyl and peptidyl moieties attached to tRNAs that possess a Watson–Crick base pair at this position (RajBhandary, 1994). Another highly conserved feature of most initiators is the presence of three consecutive G,C base pairs (G29G30G31 : C39C40C41) in the anticodon stem that preferentially direct the initiators to the ribosomal 'P' site (Seong & RajBhandary, 1987).

In *E. coli*, four genes encode initiator tRNAs. Three of these, *metZ*, *metW* and *metV*, are located in a single locus at 63·5 min and the fourth one, *metY*, is located at 71·5 min (Berlyn, 1998). In *E. coli* K-12, the genes located at 63·5 min code for $tRNA_1^{fMet}$ and the one at 71·5 min encodes a variant, $tRNA_2^{fMet}$. The two species differ by a single nucleotide at position 46 in the variable loop by which the $tRNA_1^{fMet}$ possesses ^{7}mG and the $tRNA_2^{fMet}$ an A (RajBhandary & Chow, 1995). $tRNA_1^{fMet}$ represents the major form ($\sim$75–80 %) of the $tRNA^{fMet}$ in the cell. On the other hand, $tRNA_2^{fMet}$ is a minor component (Mandal & RajBhandary, 1992), and a disruption of its gene (*metY*) with a kanamycin resistance gene produces a mutant strain that shows the same growth rate as the wild-type strain (Kenri *et al.*, 1992). However, replacement of the $tRNA_1^{fMet}$ genes (*metZWV*) with a chloramphenicol resistance gene results in a mutant strain with a slow growth phenotype, the extent of which varies with the growth temperature (Kenri *et al.*, 1991).

We generated a T35A36 (termed U35A36) mutation in a plasmid-borne copy of *metY* to introduce a $_{34}CUA_{36}$ anticodon in the encoded $tRNA_2^{fMet}$. The $tRNA_2^{fMet}$

Abbreviations: Amp, ampicillin; Cm, chloramphenicol; CAT, chloramphenicol acetyl transferase; Kan, kanamycin; Tc, tetracycline.

(U35A36) thus produced initiates from a UAG initiation codon (Varshney & RajBhandary, 1990). Both *in vitro* and *in vivo* studies have shown that the initiator tRNA mutants containing U35A36 mutations are aminoacylated with Gln (Schulman & Pelka, 1985; Seong *et al.*, 1989). Further, N-terminal sequence analysis of the translated products using such initiator tRNA mutants has confirmed that Gln is inserted in response to a UAG initiation codon, with no evidence of initiation with Met (O'Connor *et al.*, 2001). However, when MetRS is overproduced in *E. coli*, a limited aminoacylation of $tRNA_2^{fMet}$ (U35A36) by Met does take place, especially when its recognition by GlnRS is compromised by mutations in the acceptor stem (Varshney & RajBhandary, 1992).

Recently, Rothschild and co-workers demonstrated that N-terminal protein labelling efficiency could be drastically improved using such initiator 'suppressors' *in vitro* (Mamaev *et al.*, 2004). Using the *in vivo* amber initiation assays, we have shown that the creation of a strong base pair at the 1 : 72 position (as in the G72 mutation, Fig. 1B) results in a loss of initiation activity, primarily because of a severe defect in formylation of the $tRNA_2^{fMet}$ (U35A36/G72)

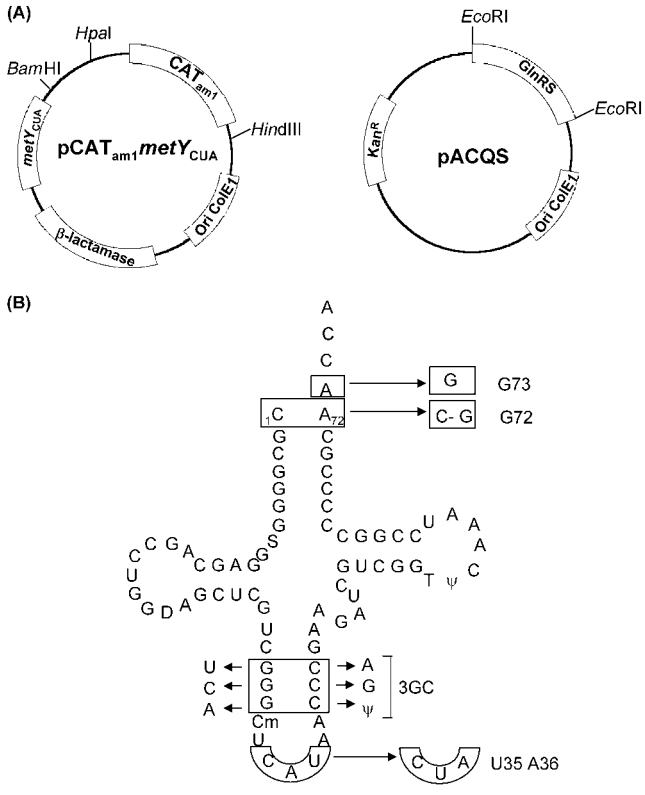


Fig. 1. (A) Diagrammatic sketches of the plasmids. $pCAT_{am1}metY_{CUA}$ harbours the CAT_{am1} and mutant $tRNA_2^{fMet}$ (U35A36) genes. pACQS harbours the *E. coli* glutaminyl-tRNA synthetase (GlnRS) gene. (B) Cloverleaf structure of the *E. coli* initiator $tRNA_2^{fMet}$ indicating the sites of mutations.

and the non-availability of its formylated form for initiation (Lee & RajBhandary, 1991; Varshney *et al.*, 1991a,b). However, overproduction of methionyl-tRNA synthetase, methionyl-tRNA (fMet) formyltransferase or IF2 (Varshney & RajBhandary, 1992; Mangroo & RajBhandary, 1995), or generation of an intragenic mutation (C to T), resulting in a U1 : G72 wobble base pair at the top of the acceptor stem (Thanedar *et al.*, 2000), rescued the initiation defect of $tRNA_2^{fMet}$ (U35A36/G72).

During the course of identifying suppressors of another formylation-defective initiator mutant, $tRNA_2^{fMet}$ (U35A36/G72G73) (Fig. 1B), we have serendipitously isolated a stable strain of *E. coli* K-12 in which the *metY* locus encodes $tRNA_2^{fMet}$ (U35A36). We demonstrate that a single copy of a chromosomally located gene for a minor form of initiator tRNA is sufficient for efficient initiation from a termination codon. The characterization of this strain is of considerable significance for various genetic and biotechnological applications.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids are listed in Table 1. *E. coli* strains were grown in LB broth or solid (1·5 % agar) media (Miller, 1972). Media were supplemented with ampicillin (Amp, 100 μg ml^{-1}), kanamycin (Kan, 25 μg ml^{-1}), tetracycline (Tc, 15 μg ml^{-1}) or chloramphenicol (Cm, 50-200 μg ml^{-1}), as needed.

Isolation of *E. coli* suppressor strains by spontaneous mutagenesis. An overnight culture (0·2 ml) of *E. coli* CA274 harbouring $pCAT_{am1}metY_{CUA/G72G73}$ was spread on LB agar plates containing 100 μg Amp ml^{-1} and 50 μg Cm ml^{-1}. Cm-resistant (Cm^R) colonies that appeared within 18 to 24 h, and which, upon subsequent culturing in antibiotic-free medium, lost resistance to Cm because of the loss of the resident plasmid, were selected for further characterization.

Preparation of cell-free extracts. The $pCAT_{am1}$ derivatives with or without pACQS were introduced into *E. coli* by transformation. The transformants were grown to exponential phase and the cells from 2 ml cultures were harvested. The cell pellet was thoroughly resuspended in 200 μl TME (25 mM Tris/HCl, pH 8·0, 2 mM β-mercaptoethanol, 1 mM Na_2EDTA), lysed by sonication and subjected to centrifugation at 10 000 r.p.m. at 4 °C for 30 min in a microfuge. The supernatant was transferred to a new tube, quantified for total proteins using Bradford's method, mixed with an equal volume of 2 × storage buffer (20 mM Tris/HCl, pH 8·0, 10 mM β-mercaptoethanol, 200 mM NaCl, 80 % glycerol, v/v) and stored at −20 °C.

Immunoblot analysis. Cell-free extracts (15 μg total protein) were resolved by 12 % SDS-PAGE and electroblotted onto a PVDF membrane (Amersham). The membrane was blocked with 1 % BSA in Tris/HCl buffered saline, TBS (20 mM Tris/HCl, pH 7·5, 0·9 % NaCl), overnight and then incubated for 4 h at room temperature with anti-chloramphenicol acetyl transferase and anti-β-lactamase rabbit antibodies (1 : 3000 dilutions). After three washings with TBS/Tween 20 (0·2 %, v/v), the blot was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1 : 3000 dilution) for 4 h at room temperature. After three washings with TBS/Tween 20 (0·2 %, v/v), the blot was developed with *p*-nitro blue tetrazolium

Table 1. *E. coli* strains and plasmids used in this study

Strain/plasmid	Genotype/details	Reference/source
Strains		
CA274	*E. coli* K-12, Hfr, *lacZ125am*, *trpA49am*, *relA*1, *spoT*1	Brenner & Beckwith (1965)
Su15	Derivative of CA274, $metY_{CUA}$	This work
Su31	Derivative of CA274, $metY_{CUA}$	This work
CAG12072	*zha*-203::Tn*10*	Singer *et al.* (1989)
CAG12152	*zgj*-3075::Tn*10*	Singer *et al.* (1989)
CAG12153	*zhc-6*::Tn*10*	Singer *et al.* (1989)
MA200	*E. coli* K, F^-, Δ*lacX74 thi bglR*11(*bglR*::IS1) [Bgl^+] *srl*::Tn*10 recA56* [Bgl^+] (λ*bglR7 bglC' lacZ*$^+$ *lacY*$^+$ φ[*bgl–lacI*])	Mahadevan *et al.* (1987)
Plasmids		
$pCAT_{am1}$	Renamed from pRSVCATam1.2.5. A pBR322 derivative harbouring the CAT reporter gene with UAG as an initiation codon	Varshney & RajBhandary (1990)
$pCAT_{am1}metY_{CUA}$	Renamed from pRSVCATam1.2.5*trnfM*U35A36. A pBR322 derivative harbouring the CAT reporter gene with UAG as an initiation codon and expressing $tRNA^{fMet}$ with a CUA anticodon (U35A36 mutation)	Varshney & RajBhandary (1990)
$pCAT_{am1}metY_{CUA/G72}$	Derivative of $pCAT_{am1}metY_{CUA}$ with additional mutation at position 72 (A72 to G72)	Varshney *et al.* (1991b)
$pCAT_{am1}metY_{CUA/G72G73}$	Derivative of $pCAT_{am1}metY_{CUA}$ with additional mutations at positions 72 and 73 (A72 to G72 and A73 to G73)	Varshney *et al.* (1991b)
$pCAT_{am1}metY_{CUA/3GC}$	Derivative of $pCAT_{am1}metY_{CUA}$ with additional mutations at positions 29:41, 30:40 and 31:39 (from G:C, G:C and G:C to U:A, C:G and A:ψ)	Mandal *et al.* (1996)
pACQS	Derivative of pAC1 harbouring *E. coli* GlnRS gene	Varshney & RajBhandary (1990)

chloride and 5-bromo-4-chloro-3-indolyl phosphate in 0·1 M Tris/HCl (pH 9·0) and 4 mM $MgCl_2$ to visualize the bands (Sambrook *et al.*, 1989).

Assay for chloramphenicol acetyl transferase (CAT) activity. The cell-free extracts (0·2–1 μg total protein) were assayed in 50 μl reaction volumes containing 150 μM Cm, 10 μM [^{14}C]Cm (specific activity 12·5 mCi $mmol^{-1}$, 463 MBq $mmol^{-1}$), 500 mM Tris/HCl (pH 8·0) and 400 μM acetyl-CoA. The reactions were carried out at 37 °C for 20 min, extracted with 500 μl ethyl acetate and processed by TLC on silica gel plates (Merck) using $CHCl_3$ and methanol (95:5, v/v) as the mobile phase (Shaw, 1983). The plates were dried, exposed to a phosphor-imaging screen and quantified using a BioImage analyser (BAS1800, Fuji Films). Enzyme activity was expressed as nanomoles of acetyl chloramphenicol (1 acetyl + 3 acetyl) formed per minute per milligram of total protein.

P1 transductional cross. P1 phage lysate preparations and transductions were performed as described by Miller (1972).

Northern blot analysis. Total tRNA from various strains was isolated under acidic conditions, separated on 6·5% polyacrylamide acid urea (8 M) gels at 4 °C, and electroblotted onto a Nytran membrane (Varshney *et al.*, 1991a). A 5′-^{32}P end-labelled oligodeoxyribonucleotide complementary to positions 29–47 of $tRNA_2^{fMet}$ (U35A36) was used as probe. The probe possessed two mismatches from the $tRNA_2^{fMet}$, and three from the $tRNA_1^{fMet}$ sequence complements. To generate the $tRNA_2^{fMet}$ marker, the tRNA preparation was treated with 100 mM Tris/HCl (pH 9·0) (Sarin & Zamecnik, 1964).

RESULTS

In vivo assay system and initiator tRNA mutants

A plasmid system used to carry out *in vivo* initiation assays is shown in Fig. 1A. The plasmid $pCAT_{am1}metY_{CUA}$ carries the genes for the mutant initiator $tRNA_2^{fMet}$ (U35A36) and the CATam1 reporter. Similarly, the plasmids $pCAT_{am1}metY_{CUA/G72}$ and $pCAT_{am1}metY_{CUA/G72G73}$ encode mutant $tRNA_2^{fMet}$ (U35A36/G72) and $tRNA_2^{fMet}$ (U35A36/G72G73), respectively, in addition to the reporter CATam1 (Table 1). The initiator tRNAs containing the U35A36 mutation (CUA anticodon) are aminoacylated with glutamine (Schulman & Pelka, 1985). To ensure efficient aminoacylation, a plasmid-borne copy of glutaminyl-tRNA synthetase (GlnRS) was also provided in some experiments from a compatible plasmid, pACQS. We have earlier shown that the $tRNA_2^{fMet}$ (U35A36) initiates from the UAG initiation codon of a reporter CATam1 mRNA and confers chloramphenicol resistance (Cm^R) to the host. Mutations in the acceptor stem (A72 to G72, or A72A73 to G72G73) in $tRNA^{fMet}$ result in a strong base pair at the top of the acceptor stem, severely affecting their formylation. In addition, they become substrates for peptidyl-tRNA hydrolase. Consequently, they are rendered inept in initiation from CATam1, leading to a Cm^S phenotype in the host (Thanedar *et al.*, 2000; Mayer *et al.*, 2001).

Isolation and characterization of Cm^R strains from *E. coli* CA274

In our earlier studies, we used a $tRNA_2^{fMet}$ (U35A36/G72) mutant to characterize suppressors that resulted in initiation from CATam1 mRNA (Varshney & RajBhandary, 1992; Thanedar *et al.*, 2000). In the present study, we used overnight cultures of *E. coli* CA274 harbouring $pCAT_{am1}metY_{CUA/G72G73}$ and screened for a Cm^R phenotype. Of the several colonies that grew on the antibiotic plate, two, named Su15 and Su31, were selected for further studies. Multiple rounds of growth in antibiotic-free medium resulted in curing of the resident plasmid from these strains, and the cured strains showed a Cm^S phenotype (e.g. Fig. 4A, sectors 4 and 8).

In order to further characterize the suppressors, we reintroduced into these (Su15 and Su31) and the parent (CA274) strain, the $pCAT_{am1}$ plasmids harbouring various mutant $tRNA_2^{fMet}$ genes (Fig. 2). As $tRNA_2^{fMet}$ (U35A36/G72) is a poor substrate for glutaminyl-tRNA synthetase (GlnRS), in this experiment, the pACQS plasmid was co-introduced into the strains to ensure efficient aminoacylation of the encoded tRNAs. The $pCAT_{am1}metY_{CUA}$ which encodes the formylation-proficient $tRNA_2^{fMet}$ (U35A36), used here as a positive control, conferred Cm^R to the parent and Su15 and Su31 strains (Fig. 2A, right, sectors 10–12). As expected, the $pCAT_{am1}metY_{CUA/G72G73}$ encoding $tRNA_2^{fMet}$ (U35A36/G72G73) conferred Cm^R to Su15 and Su31 (sectors 8 and 9) but not to the parent CA274 strain (sector 7). Exactly the same results were obtained for the other formylation-defective mutant $tRNA_2^{fMet}$ (U35A36/G72) (sectors 4–6). All transformants grew on Kan and Amp plates (Fig. 2A, left).

As a negative control, when the $pCAT_{am1}$ plasmid lacking the tRNA gene was introduced into the parent strain CA274, as expected it did not confer Cm^R to the host (Fig. 2A, right, sector 1). However, it was surprising to note that it conferred Cm^R to the Su15 and Su31 strains (Fig. 2A, right, sectors 2 and 3). Immunoblot analysis (Fig. 2B) using anti-CAT antibodies revealed the presence of CAT protein in the transformants that grew on the Cm plates but not in the ones that did not (Fig. 2A, right, Fig. 2B) suggesting that Cm^R resulted from the presence of CAT protein. As an internal control, the blot was also probed for the presence of β-lactamase (Fig. 2B) to show the intactness and equivalence of amounts of total protein used in the analysis.

Mapping of mutation(s) in Su15 and Su31

The observation that the suppressors (Su15 and Su31) were able to grow on Cm in the presence of a CATam1 reporter, even in the absence of any plasmid-encoded $tRNA^{fMet}$, led us to believe that one of the initiator tRNA genes (genomic

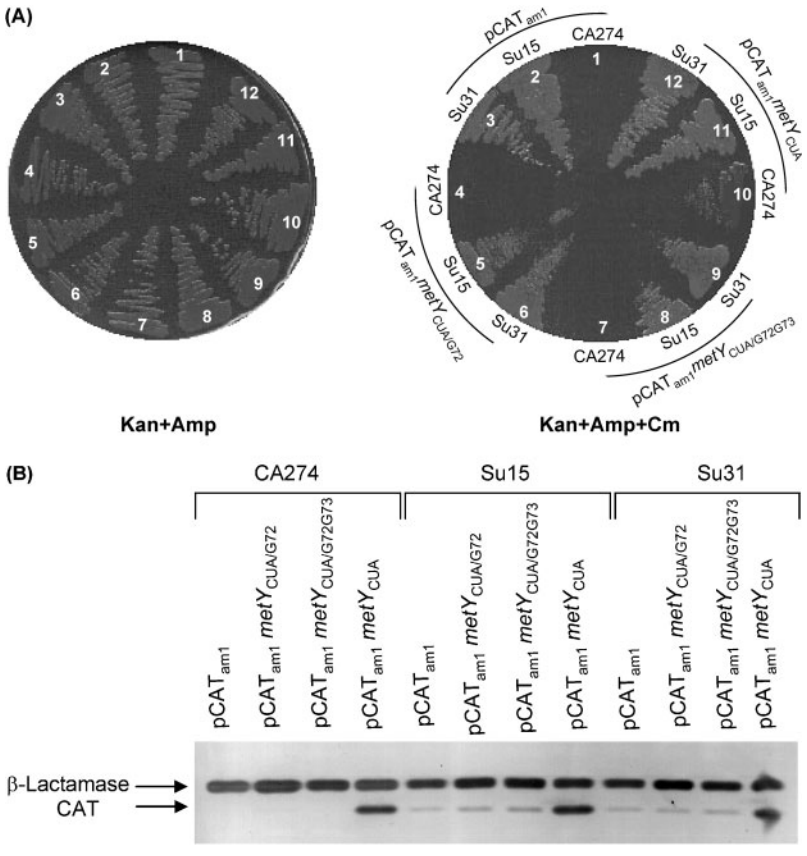


Fig. 2. (A) Growth of *E. coli* transformants derived from the parent (CA274) and the Su15 and Su31 strains on LB agar plates containing 25 μg Kan ml^{-1} and 100 μg Amp ml^{-1} (left), and 25 μg Kan ml^{-1}, 100 μg Amp ml^{-1} and 50 μg Cm ml^{-1} (right). All transformants contained the pACQS (Kan^R) plasmid harbouring the GlnRS gene and $pCAT_{am1}$ (Amp^R) with or without the mutant $tRNA_2^{fMet}$ (*metY*) genes, as indicated on the right. Plates were incubated at 37 °C for approximately 15 h. (B) Immunoblot analysis of cell-free extracts (15 μg protein) using rabbit anti-CAT and anti-β-lactamase antibodies. Bands corresponding to CAT and β-lactamase are indicated by arrows.

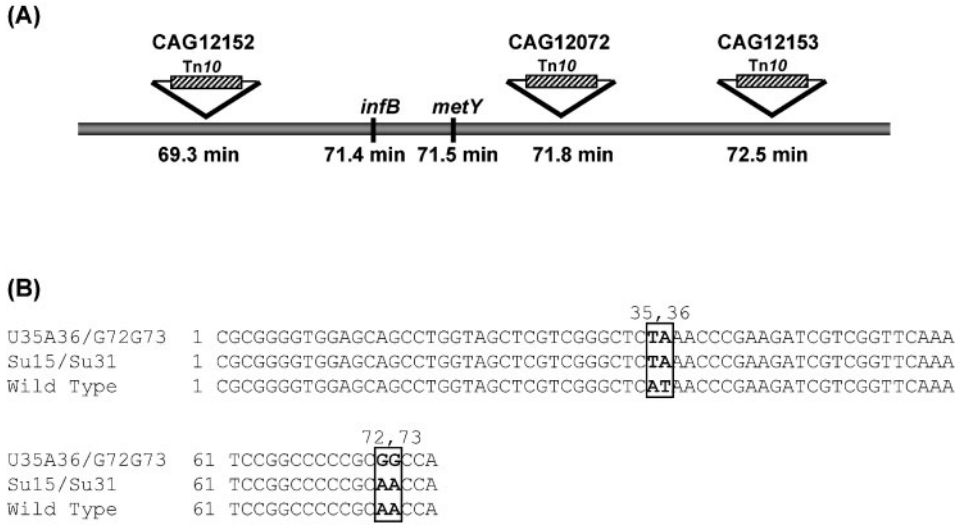


Fig. 3. (A) Genetic map (not to scale) depicting the *E. coli* chromosomal region at 71 min. The locations of *metY*, *infB* and Tn*10* in CAG strains are as shown. (B) Comparison of the tDNA sequences of the *metY* genes from the wild-type, the suppressors (Su15/Su31) and the plasmid-borne copy of the mutant *metY* gene (U35A36/G72G73). The locations corresponding to positions 35, 36 and 72, 73 are boxed. While the wild-type $tRNA_2^{fMet}$ possesses nucleotides A35T36 resulting in a CAU anticodon, the Su15/Su31 and the U35A36/G72G73 mutants possess T35A36 (resulting in a CUA anticodon). Positions 72 and 73 are represented by A72A73 in the wild-type- and Su15/Su31-encoded tRNAs, and by G72G73 in the U35A36/G72G73 mutant tRNA. The alignment was obtained by using the PILEUP program.

copy) in these strains might have been mutated to effect initiation from the CATam1 reporter. In order to genetically map the mutation by a candidate gene approach, we carried out P1 transductional crosses using various CAG strains marked with Tn*10* (Tc^R) at 69·3 min, 71·8 min or 72·5 min in the vicinity of *metY* (71·5 min) encoding $tRNA_2^{fMet}$ (Fig. 3A). As shown in Table 2, replacement of the 71 min region of the suppressors (harbouring the plasmid $pCAT_{am1}$) with that from a CAG12072 (71·8 min) strain resulted in the loss of Cm^R by a co-transduction frequency [$(Tc^R + Cm^S)/Tc^R$] of about 70 %. The crosses with CAG12152 and CAG12153 strains (69·3 and 72·5 min, respectively) did not result in any transductants that were Cm^S. These observations suggested that the mutation in Su15 and Su31, which conferred Cm^R, must be in the vicinity of 71·8 min. As *metY* is located at 71·5 min, to further narrow down the mutation, we amplified by PCR and sequenced the *metY* genes from both the suppressors. Each of them revealed two mutations corresponding to positions 35 and 36 of the anticodon resulting in a $_{34}CAU_{36}$ to $_{34}CUA_{36}$ change (Fig. 3B). Analysis of several other genes from this locus, including *infB* (which encodes IF2) at 71·4 min, revealed no mutations (data not shown). Further, back crosses onto the parent strain (CA274, harbouring $pCAT_{am1}$) using P1 phage raised from the Su15 and Su31 ($Tc^R + Cm^R$, marked at 71·8 min in the above experiment) resulted in an identical level of Cm^R in the presence of $pCAT_{am1}$; it was concluded that mutations in the *metY* gene alone resulted in the suppressor phenotype of Su15 and Su31 (data not shown).

Table 2. P1 transductional crosses with various CAG strains as donors and two independent mutants transformed with the $pCAT_{am1}$ construct

Transductants were selected on 15 μg Tc ml^{-1} and patched on 15 μg Tc ml^{-1} and 50 μg Cm ml^{-1}. The number of Cm-sensitive transductants as a proportion of the total number of transductants patched (~50–100) is shown as a percentage.

Donor	Recipient	Percentage of Cm-sensitive transductants
CAG12072 (71·8 min)	Su15	77
CAG12072 (71·8 min)	Su31	72
CAG12152 (69·5 min)	Su15	0
CAG12152 (69·5 min)	Su31	0
CAG12153 (72·5 min)	Su15	0
CAG12153 (72·5 min)	Su31	0

Abundance and mechanism of appearance of Cm^R colonies

Since the mutations in both the suppressors and a large number of other Cm^R colonies that we obtained in the screen mapped to the *metY* gene, we reasoned that this mutation could have arisen via a mechanism that utilizes the plasmid-borne copy of the *metY* gene bearing a U35A36 mutation. Since the plasmid-borne copy of the *metY* gene contained additional mutations that were absent from the

Table 3. Abundance of appearance of Cm-resistant colonies in transformants containing $pCAT_{am1}$ with or without various tRNA mutants in *E. coli* CA274, *E. coli* MA200 *recA*$^+$ and *E. coli* MA200 *recA*$^-$ backgrounds

ND, Not determined.

Plasmid	Abundance of Cm^R colonies appearing in strain:		
	CA274	MA200 (*recA*$^+$)	MA200 (*recA*$^-$)
$pCAT_{am1}$	0	ND	ND
$pCAT_{am1}metY_{CUA/G72}$	3×10^{-7}	5×10^{-7}	0
$pCAT_{am1}metY_{CUA/G72G73}$	3×10^{-7}	ND	ND
$pCAT_{am1}metY_{CUA/3GC}$	0	0	0

chromosomal suppressors, it was of interest to further investigate the mechanism of the gene alteration. As depicted in Table 3, when the plasmid $pCAT_{am1}metY_{CUA/G72}$ was introduced into *E. coli* CA274, Cm^R colonies arose with an abundance of approximately 10^{-7}. This abundance was similar to the one obtained from the cells harbouring $pCAT_{am1}metY_{CUA/G72G73}$. No spontaneous mutants (Cm^R) arose when the plasmid construct without any initiator tRNA ($pCAT_{am1}$) was used. Similarly, no Cm^R colonies appeared when the cells harboured $pCAT_{am1}metY_{CUA/3GC}$ containing CATam1 and other initiation-defective $tRNA_2^{fMet}$ (U29C30A31/U35A36/U39G40A41, abbreviated as U35A36/3GC mutations) genes. Interestingly, in the latter instance, the mutations in the anticodon stem limited the sequence homology of the plasmid-borne copy with the chromosomal *metY* to only 2–3 nucleotides in the regions immediately upstream and downstream of the U35A36 mutation (Fig. 1B). These observations indicated that the Cm^R colonies that appeared with $pCAT_{am1}metY_{CUA/G72}$ or $pCAT_{am1}metY_{CUA/G72G73}$ as resident plasmids might have been a consequence of the chromosomal *metY* acquiring the U35A36 sequence from the plasmid-borne copy by homologous recombination, so as to encode a tRNA active in initiation from a CATam1 reporter. Any recombinants arising from recombination in the distal sequences (flanking both the anticodon and the other mutation sites in the tRNA genes) would encode a tRNA inactive in initiation and would not be selected in the screen.

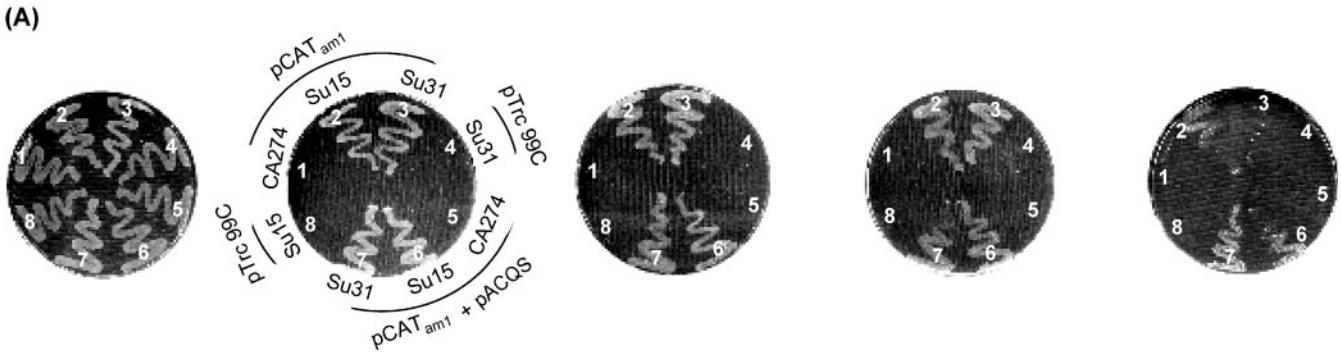
To further probe the role of homologous recombination, we performed similar experiments using two strains of *E. coli* MA200 which are isogenic except for the *recA* allele, which encodes a protein involved in homologous pairing and strand exchange. We scored for spontaneously appearing Cm^R colonies to check if the event was mediated by RecA (Table 3). It was observed that the MA200 *recA*$^+$ strain produced results that were similar to those obtained with the CA274 strain. However, using the same assay, no Cm^R colonies appeared with the *recA*$^-$ strain. These observations suggested that the acquisition of a U35A36 mutation in the chromosomal *metY* was via a RecA-dependent mechanism.

Efficiency of initiation

To determine the utility of the Su15 and Su31 strains for various applications, we assessed the efficiency of these strains in initiation from a CATam1 reporter by phenotypic and biochemical assays for CAT activity. As shown in Fig. 4A, the Su15 and Su31 strains themselves were sensitive to growth on Cm plates [sectors 8 and 4, respectively; compare panel I(i) with panels (ii)–(v)]. However, in the presence of the $pCAT_{am1}$ plasmid, the single copy of the chromosomally located $metY_{CUA}$ in Su15 and Su31 initiated from the CATam1 reporter and sustained growth not only at a concentration of 50 μg Cm ml^{-1} [panel (ii)] in the medium (which was used to isolate them) but also at 100 and 150 μg Cm ml^{-1} [sectors 2 and 3, panels (iii) and (iv), respectively]. In fact, at longer incubation times of about 24 h, growth was detectable even at 200 μg Cm ml^{-1} [panel (v)]. The observation that the single-copy $metY_{CUA}$ gene supported growth of the host up to 200 μg Cm ml^{-1} clearly supports the view that it affords efficient initiation from an amber initiation codon. However, to analyse the effect of GlnRS overproduction on initiation activities of Su15/Su31, we carried out CAT activity assays. These assays also allowed a quantitative comparison between the initiation efficiencies of Su15 and Su31 with that of the multicopy plasmid-based

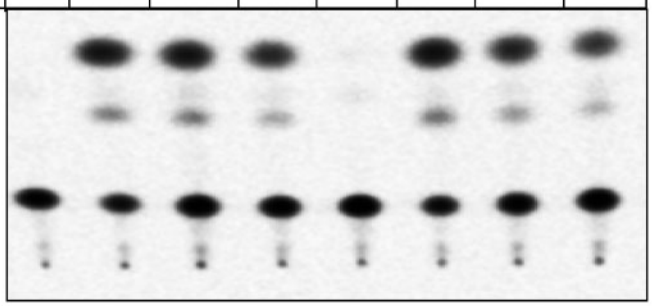
Fig. 4. (A) Growth of *E. coli* parent (CA274) and Su15 and Su31 strains on LB agar plates containing Amp (i) or Amp and Cm (ii–v) at 37 °C for 18 h (i–iv) or 24 h (v). Panels: (i) 100 μg Amp ml^{-1}, (ii) 100 μg Amp ml^{-1} and 50 μg Cm ml^{-1}, (iii) 100 μg Amp ml^{-1} and 100 μg Cm ml^{-1}, (iv) 100 μg Amp ml^{-1} and 150 μg Cm ml^{-1}, (v) 100 μg Amp ml^{-1} and 200 μg Cm ml^{-1}. Strains harboured pTrc99C (sectors 4 and 8), $pCAT_{am1}$ (sectors 1–3) or $pCAT_{am1}$ and pACQS (sectors 5–7), as indicated in (ii). (B) Autoradiogram of the TLC plate showing CAT assays of cellular extracts prepared from various transformants of the parent (CA274) and the Su15 and Su31 strains. The amounts of acetyl-chloramphenicol (Ac-Cm) produced [nmol min^{-1} (mg total protein)$^{-1}$] in the cell-free extracts are shown below the lanes. The various strains and the presence within them of $pCAT_{am1}$, $pCAT_{am1}metY_{CUA}$ and pACQS (GlnRS) plasmids, and the total cell-free extracts used in assays are as indicated. (C) Northern blot analysis of tRNAs from the *E. coli* parent (CA274) and Su15 and Su31 strains. Total tRNA was isolated under acidic conditions (Methods), separated on acid urea gels, Northern-blotted using a DNA oligomer probe specific to the U35A36 mutation containing tRNA, imaged and quantified (shown below the lanes) using a Bioimage analyser (BAS1800, Fuji Films). The positions of formylated and deacylated forms of $tRNA_2^{fMet}$ are indicated. The presence of the pACQS plasmid (GlnRS) is indicated above the lanes.

system. As shown in Fig. 4B, in the absence of overproduced GlnRS, Su15 and Su31 produced a CAT activity which converted ~50–60 nmol Cm min^{-1} (mg total cell protein)$^{-1}$ to acetyl-Cm (lanes 7 and 8). This activity was increased to ~90–100 nmol Cm min^{-1} (mg total cell protein)$^{-1}$ in the presence of overproduced GlnRS (lanes 3 and 4). Under the same assay conditions, the CAT activity from the multicopy plasmid-based amber initiation system was ~1100 nmol Cm min^{-1} (mg total cell protein)$^{-1}$ (lanes 2 and 6). Interestingly, the difference between the initiation efficiencies of Su15/Su31 and the multicopy plasmid system correlated well with the copy number of the ColE1 origin of replication (pBR322-derived $pCAT_{am1}$-based plasmids) (Sambrook *et al.*, 1989; Atlung *et al.*, 1999). More importantly, these observations now allow us to choose the desired level of initiation from a UAG initiation



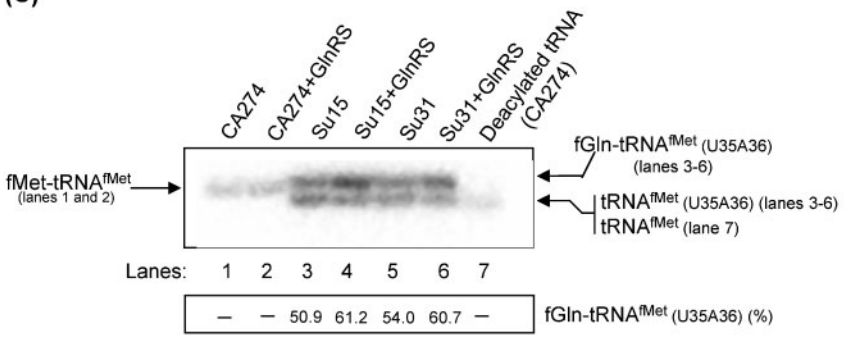
(B)

pACQS	+				–			
Strain	CA274		Su15	Su 31	CA274		Su15	Su 31
$pCAT_{am1}$	+		+	+	+		+	+
$pCAT_{am1}metY_{CUA}$		+				+		
Total cell extract (μg)	1	0.2	1	1	1	0.2	1	1



Lanes:	1	2	3	4	5	6	7	8
CAT activity (Ac-Cm nmol min^{-1} mg^{-1})	5.4	1122.5	100.7	91.6	3	1090.4	61.7	54.4

(C)



codon for regulated expression of genes, especially those that encode proteins toxic to the host.

To determine if the initiation activities correlated well with the availability of fGln-$tRNA_2^{fMet}$ (U35A36) in the cell, we performed Northern blot analysis of the total tRNA prepared under acidic conditions. As shown in Fig. 4C, in the Su15 and Su31 strains, approximately 52 % of the $tRNA_2^{fMet}$ (U35A36) accumulated as fGln-$tRNA_2^{fMet}$ (U35A36) (lanes 3 and 5). However, overproduction of GlnRS resulted in a slight increase in the steady-state accumulation of fGln-$tRNA_2^{fMet}$ (U35A36) to ~61 % (lanes 4 and 6). Such an increase in the steady-state accumulation of the fGln-$tRNA_2^{fMet}$ (U35A36) agrees well with the increase in their initiation efficiency (Varshney *et al.*, 1991a). The wild-type form of $tRNA_2^{fMet}$ accumulated as fMet-$tRNA_2^{fMet}$ quantitatively (lanes 1 and 2). It may be noted that the signals arising from the CA274 samples (lanes 1, 2 and 7) are less intense. This is merely because the hybridization probe [complementary to the $tRNA_2^{fMet}$ (U35A36)] contains two mismatches from the wild-type sequence.

DISCUSSION

Both chromosomally and extra-chromosomally encoded elongator tRNA suppressors are important tools in various genetic applications (Steege & Soll, 1979). Because of their usefulness, *E. coli* strains (*supD*, *supE*, *supF* and *supP*) inserting serine, glutamine, tyrosine and leucine, respectively, in response to UAG codons have been generated. Creation of many more mutant tRNAs by *in vivo* and *in vitro* manipulations (Murgola *et al.*, 1984; Normanly *et al.*, 1990; Kleina *et al.*, 1990) has resulted in the collection of a variety of useful suppressors. The availability of elongator tRNA-based suppressors has greatly facilitated the application of genetics to protein engineering (reviewed by Normanly & Abelson, 1989). Importantly, these systems have allowed the incorporation of modified amino acids into a polypeptide chain for use in various biophysical studies (Ellman *et al.*, 1991; Bain *et al.*, 1989; Cornish *et al.*, 1994). The suppressors have also been instrumental in the propagation of lambda phages engineered to contain nonsense mutations in essential genes for their specialized use in molecular genetics as vehicles for transposon delivery.

Although the efficiency of termination at nonsense codons within the coding region can be minimized to some extent by placing them in an appropriate sequence context (Tate & Mannering, 1996), a drawback of the elongator tRNA-based suppressors is that suppression is not complete and results in the release of incomplete polypeptides due to competition from release factors. In another scenario, the suppressor tRNAs compete with the release factors for binding to the natural termination codons in the A site of the ribosome, resulting in translation of the cellular mRNAs beyond the termination codon. Both of these situations can be toxic to cells (Snyder & Champness, 1997). Furthermore, although not studied systematically, some of the elongator tRNA-based suppressors may also interfere with the autoregulatory mechanisms involving termination codons within ORFs as well as the mechanism that leads to the insertion of selenocysteine in proteins (Craigen & Caskey, 1986; Mansell *et al.*, 2001). Notably, these drawbacks are essentially a consequence of the requirement of the elongator tRNA-based suppressors for recognition of the nonsense codons in the ribosomal A site.

The initiation of protein synthesis from an mRNA occurs from an initiation codon located in a specialized region, the translation initiation region (TIR). While the presence of the Shine–Dalgarno sequence (SD sequence) within the TIR is one of the most important elements that determines the efficiency of initiation, several other features, such as the sequence context within which an initiation codon is located and its spacing from the SD sequence, contribute to efficient utilization of the prokaryotic mRNA in translation (reviewed by Gold, 1988). The observation that the base pairing between the initiating codon and the anticodon of the initiator tRNA, and not the AUG sequence *per se*, is responsible for initiation, allowed us to design a plasmid-based system for initiation from a UAG termination codon (Varshney & RajBhandary, 1990), and by virtue of their (initiator tRNA) binding at the ribosomal 'P' site, they remedy the limitations of the elongator tRNA-based suppression system. In fact, in the plasmid-based system that we described, the initiator tRNA (with CUA anticodon) was overproduced from a multicopy plasmid without any detectable toxic consequences to bacterial growth. In the initiator tRNA-based systems, the issue of production of the incomplete peptide does not arise. Further, as the natural termination codons are generally not located in a sequence context (TIR) required to foster initiation, the chances of inappropriate initiation are also minimized.

Although the plasmid-based system using such initiator 'suppressor' tRNA was described some time ago (Varshney & RajBhandary, 1990), the fact that plasmid vectors are needed to introduce various test genes into the bacteria means that it was desirable to isolate/generate *E. coli* strains in which the initiator tRNA was altered in the chromosomal background. The characterization of the Su15 and Su31 strains in this report bridges this gap.

A question that the present study raises is whether or not such strains could also be generated by introducing mutations in any of the initiator tRNA genes located at 63·5 min. It is possible that the mechanism that led to the generation of Su15/Su31 or a directed approach (Datsenko & Wanner, 2000) could result in the isolation of a strain in which initiation from an amber codon would occur because of mutation in the *metZ*, *metW* or *metV* genes at 63·5 min. However, it may be noted that the tRNA sequences in this cluster have not diverged over the evolutionary time-scale. On the other hand, the tRNA sequence encoded by the *metY* locus (at 71·5 min), which is distantly located, has diverged, at least at position 46, from those located at

63·5 min. Recently, it was suggested that the multiple copies of rRNA gene sequences are prevented from diverging from each other by a gene-conversion process. In addition, genes located near to each other are corrected more efficiently than those located distantly (Hashimoto *et al.*, 2003). Therefore, it is possible that the initiator tRNA genes located at 63·5 min are under selective pressure to maintain the intactness of their sequences. Also, the distantly located *metY* may be the only locus that can be allowed to accumulate mutations. Thus, at a first approximation, it would seem that the *metY*-based system for initiation from a termination codon would be a stable one. Hence, the characterization in this study of the *E. coli* strains that we isolated by serendipity is of notable significance. Interestingly, the Su15 and Su31 strains were isolated more than six years ago in the laboratory, and continue to be stable even in the absence of any selective pressure and, consistent with the studies of Kenri *et al.* (1992), have no apparent growth defect. However, we have not yet studied the natural fitness of these strains *vis-à-vis* their parent strain (*E. coli* CA274). Such studies in future may lead to an understanding of the possible selective advantage of the presence of multiple copies of the initiator tRNA genes in *E. coli*.

The strains Su15 and Su31 that utilize $tRNA_2^{fMet}$ (U35A36) initiate with formyl-glutamine, which is also the most efficient non-methionine amino acid to initiate protein synthesis (Mayer *et al.*, 2001). Therefore, this strain should be the most useful for general purposes to obtain initiation from an amber initiation codon. Recently, it was shown that initiation from a UAG codon is highly comparable to initiation from AUG (Mayer *et al.*, 2003). Interestingly, as we have shown (Fig. 4), the efficiency of initiation from an amber initiation codon in these strains can be enhanced by simultaneous expression from a plasmid-borne copy of the GlnRS gene. Also, given that plasmid-based systems are available for initiation with formylated forms of valine, isoleucine and phenylalanine, etc. (Chattapadhyay *et al.*, 1990; Pallanck & Schulman, 1991), it should be feasible to generate more strains for initiation with other non-methionine amino acids by using the general approach described in this study. We believe that the generation of such a library of strains for the expression of transcriptionally controlled genes with non-AUG initiation codons would be a valuable addition to the field of nonsense suppression genetics and open up further opportunities for its application.

ACKNOWLEDGEMENTS

We thank Dr Mary Berlyn, *E. coli* Genetic Stock Centre, Yale, and Dr S. Mahadevan, IISc, Bangalore, for kindly providing us with the *E. coli* CAG strains and the *E. coli* MA200 strain, respectively, and our laboratory colleagues for their suggestions. This work was supported by grants from the Department of Science and Technology and the Indian Council of Medical Research, Government of India, New Delhi. G. D. is supported by a fellowship from the Council of Scientific and Industrial Research, New Delhi. A postdoctoral fellowship from the Department of Biotechnology, New Delhi, and a K. S. Krishnan Fellowship supported T. K. D. and S. T., respectively.

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