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

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*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\textsuperscript{fMet} and a distantly located metY gene encodes a variant, tRNA\textsuperscript{2fMet}. 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\textsuperscript{2fMet} 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\textsuperscript{+} host background. The mutations were observed when the plasmid-borne gene encoded tRNA\textsuperscript{2fMet} (U35A36) with additional changes in the acceptor stem (G72; G72G73) but not in the anticodon stem (U29C30C41). The usefulness of this strain, and a possible role for multiple tRNA\textsuperscript{1fMet} 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\textsuperscript{1Met}) bind directly to EFTu, the initiators (Met-tRNA\textsuperscript{2Met}) are modified to fMet-tRNA\textsuperscript{2Met} 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 eu bacterial 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\textsuperscript{1fMet} and the one at 71:5 min encodes a variant, tRNA\textsuperscript{2fMet}. The two species differ by a single nucleotide at position 46 in the variable loop by which the tRNA\textsuperscript{1fMet} possesses 7mG and the tRNA\textsuperscript{2fMet} an A (RajBhandary & Chow, 1995). tRNA\textsuperscript{1fMet} represents the major form (~ 75–80 %) of the tRNA\textsuperscript{fMet} in the cell. On the other hand, tRNA\textsuperscript{2fMet} 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\textsuperscript{1fMet} 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 34CUA\textsubscript{36} anticodon in the encoded tRNA\textsuperscript{2fMet}. The tRNA\textsuperscript{2fMet}
(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$$^{\text{F}}$(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$$^{\text{F}}$(U35A36/G72) and the non-availability of its formylated form for initiation (Lee & RajBhandary, 1991; Varshney et al., 1991a,b). However, overproduction of methionyl-tRNA synthetase, methionyl-tRNA (fMet) formyltransferase or IF2 (Varshney & RajBhandary, 1992; Mangroo & RajBhandary, 1995), or generation of an intragenic mutation (C to T), resulting in a U1:G72 wobble base pair at the top of the acceptor stem (Thanedar et al., 2000), rescued the initiation defect of tRNA$_2$$^{\text{F}}$(U35A36/G72).

During the course of identifying suppressors of another formylation-defective initiator mutant, tRNA$_2$$^{\text{F}}$(U35A36/G72G73) (Fig. 1B), we have serendipitously isolated a stable strain of E. coli K-12 in which the metY locus encodes tRNA$_2$$^{\text{F}}$(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 (CmR) 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$_2$EDTA), 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 electoblotted onto a PVDF membrane (Amersham). The membrane was blocked with 5 % 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 and the non-available form of p-nitro blue tetrazolium...
chloride and 5-bromo-4-chloro-3-indolyl phosphate in 0-1 M Tris/ 
HCl (pH 9-0) and 4 mM MgCl2 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 

contains 150 μM Cm, 10 μM [32P]Cl (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 CHCl3 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 
proteins) per milligram of total protein.

**P1 transduction assay.** P1 phage lysate preparations and transductions were performed as described by Miller (1972).

**Northern blot analysis.** Total tRNA from various strains was iso-

dicated high risk. Isolated on 5% polyacrylamide 

and 4°C gel at 4 °C, and electroblotted onto a Nitran mem-

brane (Varshney et al., 1991a). A 32P end-labelled oligodeoxy-
ribonucleotide complementary to positions 29-47 of tRNAAsp 
(U35A36) was used as probe. The probe possessed two mismatches 
from the tRNAAsp, and three from the tRNAAsp sequence comple-

tents. To generate the trNAAsp 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 pCATam1metYCUA 

carries the gene for the mutant initiator tRNAAsp 

(U35A36) and the CATam1 reporter. Similarly, the plasmids pCATam1metYCUA/G72 

and pCATam1metYCUA/G72G73 encode mutant tRNAAsp 

(U35A36/G72) and tRNAAsp (U35A36/G72G73), respectively, in addition to the reporter 

CATam1 (Table 1). The initiator tRNAs containing 

the U35A36 mutation (CUA anticodon) are aminoacylated 

with the U35A36/G72 and P72G73 (A72 to G72 and A73 to G73) 


<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/details</th>
<th>Reference/source</th>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA274</td>
<td>E. coli K-12, Hfr, lacZ125amu, trpA49am, relA1, spoT1</td>
<td>Brenner &amp; Beckwith (1965)</td>
</tr>
<tr>
<td>Su15</td>
<td>Derivative of CA274, metYCUA</td>
<td>This work</td>
</tr>
<tr>
<td>Su31</td>
<td>Derivative of CA274, metYCUA</td>
<td>This work</td>
</tr>
<tr>
<td>CAG12072</td>
<td>zha-203::Tn10</td>
<td>Singer et al. (1989)</td>
</tr>
<tr>
<td>CAG12152</td>
<td>zgi-3075::Tn10</td>
<td>Singer et al. (1989)</td>
</tr>
<tr>
<td>CAG12153</td>
<td>zhc-6::Tn10</td>
<td>Singer et al. (1989)</td>
</tr>
<tr>
<td>MA200</td>
<td>E. coli K, F−, ΔlacX74 thi bgIR11(bgIR::SI1) [Bgl−] srl::Tn10 recA56 [Bgl−] (ΔbgIR bgIC lacZ+ lacY+ ψ[bgI–lacI])</td>
<td>Mahadevan et al. (1987)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pCATam1</td>
<td>Renamed from PRSVCATam1.2.5. A pBR322 derivative harbouring the CAT reporter gene with UAG as an initiation codon</td>
<td>Varshney &amp; RajBhandary (1990)</td>
</tr>
<tr>
<td>pCATam1metYCUA</td>
<td>Renamed from PRSVCATam1.2.5G72 from pCATam1.2.5-strp4MUC35A36. A pBR322 derivative harbouring the CAT reporter gene with UAG as an initiation codon and expressing tRNAAsp with a CUA anticodon (U35A36 mutation)</td>
<td>Varshney &amp; RajBhandary (1990)</td>
</tr>
<tr>
<td>pCATam1metYCUA/G72</td>
<td>Derivative of pCATam1metYCUA with additional mutations at positions 72 (A72 to G72)</td>
<td>Varshney et al. (1991b)</td>
</tr>
<tr>
<td>pCATam1metYCUA/G72G73</td>
<td>Derivative of pCATam1metYCUA with additional mutations at positions 72 and 73 (A72 to G72 and A73 to G73)</td>
<td>Varshney et al. (1991b)</td>
</tr>
<tr>
<td>pACQS</td>
<td>Derivative of pAC1 harbouring E. coli GlnRS gene</td>
<td>Varshney &amp; RajBhandary (1990)</td>
</tr>
</tbody>
</table>

**Table 1. E. coli strains and plasmids used in this study**
Isolation and characterization of CmR strains from E. coli CA274

In our earlier studies, we used a tRNA_{2}^{Met} (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 CmR 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 CmS 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}^{Met} genes (Fig. 2). As tRNA_{2}^{Met} (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}^{Met} (U35A36), used here as a positive control, conferred CmR 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}^{Met} (U35A36/G72G73) conferred CmR 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}^{Met} (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 CmR to the host (Fig. 2A, right, sector 1). However, it was surprising to note that it conferred CmR 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 CmR 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_{2}^{Met}, 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 (KanR) plasmid harbouring the GlnRS gene and pCAT_{am1} (AmpR) with or without the mutant tRNA_{2}^{Met} (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.
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 Tn10 (TcR) at 69-3 min, 71-8 min or 72-5 min in the vicinity of metY (71-8 min) encoding tRNA2 fMet (Fig. 3A). As shown in Table 2, replacement of the 71 min region of the suppressors (harbouring the plasmid pCATam1) with that from a CAG12072 (71-8 min) strain resulted in the loss of CmR 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 CmS. These observations suggested that the mutation in Su15 and Su31, which conferred CmR, 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 34CAU36 to 34CUA36 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 pCATam1) using P1 phage raised from the Su15 and Su31 (TcR+CmR, marked at 71-8 min in the above experiment) resulted in an identical level of CmR by the pCATam1 construct.

**Table 2. P1 transductional crosses with various CAG strains as donors and two independent mutants transformed with the pCATam1 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 (\(\sim 50-100\)) is shown as a percentage.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Percentage of Cm-sensitive transductants</th>
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<tbody>
<tr>
<td>CAG12072 (71-8 min)</td>
<td>Su15</td>
<td>77</td>
</tr>
<tr>
<td>CAG12072 (71-8 min)</td>
<td>Su31</td>
<td>72</td>
</tr>
<tr>
<td>CAG12152 (69-5 min)</td>
<td>Su15</td>
<td>0</td>
</tr>
<tr>
<td>CAG12152 (69-5 min)</td>
<td>Su31</td>
<td>0</td>
</tr>
<tr>
<td>CAG12153 (72-5 min)</td>
<td>Su15</td>
<td>0</td>
</tr>
<tr>
<td>CAG12153 (72-5 min)</td>
<td>Su31</td>
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</tr>
</tbody>
</table>

**Abundance and mechanism of appearance of CmR colonies**

Since the mutations in both the suppressors and a large number of other CmR 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

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**Fig. 3.** (A) Genetic map (not to scale) depicting the E. coli chromosomal region at 71 min. The locations of metY, infB and Tn10 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 rRNA2 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.
chromosomal suppressors, it was of interest to further investigate the mechanism of the gene alteration. As depicted in Table 3, when the plasmid pCATam1metYCUA/G72 was introduced into *E. coli* CA274, Cm\textsuperscript{R} colonies arose with an abundance of approximately 10\textsuperscript{-7}. This abundance was similar to the one obtained from the cells harbouring pCATam1metYCUA/G72G73. No spontaneous mutants (Cm\textsuperscript{R}) arose when the plasmid construct without any initiator tRNA (pCATam1) was used. Similarly, no Cm\textsuperscript{R} colonies appeared when the cells harboured pCATam1metYCUA/G72 or pCATam1metYCUA/G72G73 as resident plasmids might have been a consequence of the chromosomal metY encoding a protein involved in homologous pairing and strand exchange. We scored for spontaneously appearing Cm\textsuperscript{R} colonies to check if the event was mediated by RecA (Table 3). It was observed that the MA200 recA\textsuperscript{+} strain produced results that were similar to those obtained with the CA274 strain. However, using the same assay, no Cm\textsuperscript{R} colonies appeared with the recA\textsuperscript{−} strain. These observations suggested that the acquisition of a U35A36 mutation in the chromosomal metY was via a RecA-dependent mechanism.

### Table 3. Abundance of appearance of Cm-resistant colonies in transformants containing pCATam1 with or without various tRNA mutants in *E. coli* CA274, *E. coli* MA200 recA\textsuperscript{+} and *E. coli* MA200 recA\textsuperscript{−} backgrounds

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Abundance of Cm\textsuperscript{R} colonies appearing in strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CA274</td>
</tr>
<tr>
<td>pCATam1</td>
<td>0</td>
</tr>
<tr>
<td>pCATam1metYCUA/G72</td>
<td>3 × 10\textsuperscript{-7}</td>
</tr>
<tr>
<td>pCATam1metYCUA/G72G73</td>
<td>3 × 10\textsuperscript{-7}</td>
</tr>
<tr>
<td>pCATam1metYCUA/G72G73</td>
<td>0</td>
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</tbody>
</table>

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\textsuperscript{R} colonies to check if the event was mediated by RecA (Table 3). It was observed that the MA200 recA\textsuperscript{+} strain produced results that were similar to those obtained with the CA274 strain. However, using the same assay, no Cm\textsuperscript{R} colonies appeared with the recA\textsuperscript{−} 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) with panels (ii)–(v)]. However, in the presence of the pCATam1 plasmid, the single copy of the chromosomally located metYCUA in Su15 and Su31 initiated from the CATam1 reporter and sustained growth not only at a concentration of 50 μg Cm ml\textsuperscript{-1} [panel (ii)] in the medium (which was used to isolate them) but also at 100 and 150 μg Cm ml\textsuperscript{-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\textsuperscript{-1} [panel (v)]. The observation that the single-copy metYCUA gene supported growth of the host up to 200 μg Cm ml\textsuperscript{-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.](http://www.microbiologyresearch.org)
system. As shown in Fig. 4B, in the absence of overproduced GlnRS, Su15 and Su31 produced a CAT activity which converted $\sim 50–60$ nmol Cm min$^{-1}$ (mg total cell protein)$^{-1}$ to acetyl-Cm (lanes 7 and 8). This activity was increased to $\sim 90–100$ 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 $\sim 1100$ 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 ColEI 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
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 Gln-tRNA\(^{\text{fMet}}\) (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\(^{\text{fMet}}\) (U35A36) accumulated as Gln-tRNA\(^{\text{fMet}}\) (U35A36) (lanes 3 and 5). However, overproduction of GlnRS resulted in a slight increase in the steady-state accumulation of Gln-tRNA\(^{\text{fMet}}\) (U35A36) to ~61% (lanes 4 and 6). Such an increase in the steady-state accumulation of the Gln-tRNA\(^{\text{fMet}}\) (U35A36) agrees well with the increase in their initiation efficiency (Varshney et al., 1991a).

The wild-type form of tRNA\(^{\text{fMet}}\) accumulated as fMet-tRNA\(^{\text{fMet}}\) 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\(^{\text{fMet}}\) (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 \textit{in vivo} and \textit{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 \textit{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 remedied 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 \textit{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 \textit{metZ}, \textit{metW} or \textit{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 \textit{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 tRNA 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 tRNA2-Met (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.

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REFERENCES


