Role of 16S ribosomal RNA methylations in translation initiation in *Escherichia coli*

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Translation initiation from the ribosomal P-site is the specialty of the initiator tRNAs (tRNA^{fMet}). Presence of the three consecutive G-C base pairs (G29-C41, G30-C40 and G31-C39) in their anticodon stems, a highly conserved feature of the initiator tRNAs across the three kingdoms of life, has been implicated in their preferential binding to the P-site. How this feature is exploited by ribosomes has remained unclear. Using a genetic screen, we have isolated an *Escherichia coli* strain, carrying a G122D mutation in *folD*, which allows initiation with the tRNA^{fMet} containing mutations in one, two or all the three G-C base pairs. The strain shows a severe deficiency of methionine and S-adenosylmethionine, and lacks nucleoside methylations in rRNA. Targeted mutations in the methyltransferase genes have revealed a connection between the rRNA modifications and the fundamental process of the initiator tRNA selection by the ribosome.

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Introduction

Organisms have evolved two classes of tRNAs, the initiators and the elongators. The initiator functions at the step of initiation to decipher the start codon, whereas elongators decode the remaining codons in the open reading frame of an mRNA. The initiator tRNAs (tRNA^{fMet}) are special in that they bind directly to the ribosomal P-site. All other tRNAs first bind to the A-site and are then translocated to the P-site. The accuracy with which the initiators are selected on the P-site of the small subunit of the ribosomes is central to the fidelity of protein synthesis. Studies on the structure–function analyses of *Escherichia coli* tRNA^{fMet} have revealed that the most

important features of tRNA^{fMet}, pertinent to its *in vivo* function as an initiator, are located in the acceptor stem and the anticodon arm regions (Varshney *et al*, 1993; RajBhandary and Chow, 1995). The distinctive features in the acceptor stem are responsible for formylation of the amino acid attached to tRNA^{fMet}, a crucial determinant for its binding to initiation factor 2 (IF2). In the anticodon stem, the three consecutive G-C base pairs (G29-C41, G30-C40 and G31-C39, referred to as 3G-C base pairs hereafter) are essential for translation initiation *in vivo* (Mandal *et al*, 1996). In contrast to formylation, which is absent in eukaryotes and archaea, the 3G-C base pairs are virtually conserved in the initiator tRNAs from all the three kingdoms of life emphasizing their evolutionary importance (Marck and Grosjean, 2002). How the 3G-C base pairs are exploited by ribosomes in selection of the initiator tRNA has been a long-standing question.

Biochemical studies suggested that IF3 facilitates tRNA^{fMet} binding to the 30S ribosomes by recognizing the 3G-C base pairs (Hartz *et al*, 1989, 1990). Further, the genetic studies suggested that the loss in binding of the tRNA^{fMet} containing mutations in the 3G-C base pairs was due, in part, to its discrimination by IF3 (O'Connor *et al*, 2001). However, structural (McCutcheon *et al*, 1999) and foot-printing studies (Dallas and Noller, 2001) suggested that in the ribosome, the binding site of IF3 is located at a distance from the anticodon stem of the tRNA^{fMet}. More recently, it was shown that a major role of IF3 is to increase the rate of dissociation of all tRNAs from the 30S ribosomes. Because the presence of IF2 (along with IF1) strongly favors binding of fMet-tRNA^{fMet} to the 30S subunit and docking of the 50S subunit, such a role of IF3, in essence, allows for higher accuracy of fMet-tRNA^{fMet} selection on the ribosome (Antoun *et al*, 2006). These observations support the view that the role of IF3 in promoting tRNA discrimination in the P-site is an indirect one. In studies on the role of ribosomal proteins (Hoang *et al*, 2004), it was observed that even after deletion of the C-terminal tails of the S9 and S13, which penetrate into the P-site and are within the contacting distance with the anticodon stem-loop (Wimberly *et al*, 2000; Yusupov *et al*, 2001), the ribosomal selectivity towards tRNA^{fMet} was retained. Therefore, it is not clear whether ribosomal proteins play a direct role in initiator–elongator tRNA discrimination.

Recently, the co-crystal structure of the 70S ribosome in complex with tRNA^{fMet} revealed that the two universally conserved nucleotides G1338 and A1339 in the 16S rRNA in the head region of the 30S subunit are in juxtaposition to the minor groove of the 3G-C base pairs (Yusupov *et al*, 2001; Selmer *et al*, 2006). Mutational analysis of the G1338 and A1339 in 16S rRNA (Hui and de Boer, 1987) supported a role of these nucleotides in stabilizing tRNA^{fMet} binding through ‘A-minor’ interactions with the 3G-C base pairs (Lancaster and Noller, 2005). However, it remains to be seen whether the ribosomes carrying mutations at G1338 and A1339 positions of the 16S rRNA would allow efficient initiation with tRNA^{fMet}-containing mutations in the 3G-C base pairs.

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Classical genetic approaches have been routinely used to isolate second site suppressors of mutational defects in the RNA or protein molecules to allow better understanding of the biological processes, especially when the mutations in the participating molecules exhibit a recessive phenotype. Here, we used a mutant tRNA^{fMet}, inactive in initiation because the 3G-C base pairs in it were changed to those found in the elongator tRNA^{Met} (3G-C mutant), to isolate second site compensatory mutations. We show that a mutation (G122D) in the *folD* gene encoding 5,10 methylene tetrahydrofolate dehydrogenase/cyclohydrolase in *E. coli* results in a severe deficiency of S-adenosylmethionine (SAM) and rRNA methylations and allows efficient initiation with the 3G-C mutant of tRNA^{fMet}.

Results

In vivo initiation assay system and the 3G-C mutant initiator tRNA

The plasmid pCAT_{am1}metY_{CUA} (Amp^R) used for the *in vivo* initiation assay is shown in Figure 1A (left). It carries CAT_{am1} reporter gene, and an initiator tRNA gene metY_{CUA} that

encodes tRNA₂^{fMet} (referred to as tRNA^{fMet} hereafter) containing a CAU to CUA change in its anticodon (U35A36 mutation). The tRNA^{fMet} containing CUA anticodon is aminoacylated with glutamine, formylated and initiates from the UAG initiation codon of a CAT_{am1} reporter mRNA (Figure 1A, right). Initiation with fGln-tRNA^{fMet} is efficient albeit slightly less than that with fMet-tRNA^{fMet} (Schulman and Pelka, 1985; Varshney and RajBhandary, 1990; Varshney *et al*, 1991; Mayer *et al*, 2001).

The plasmid pCAT_{am1}metY_{CUA/3GC}, derived from pCAT_{am1}metY_{CUA}, encodes the mutant tRNA^{fMet} wherein the U35A36 mutation is coupled to a set of mutations called the '3G-C' mutations which result in G29G30G31/C39C40C41 to U29C30A31/ψ39G40A41 change in the anticodon stem (Figure 1A, right). Although the 3G-C mutations in tRNA^{fMet} do not affect its aminoacylation, formylation or the post-transcriptional modifications, they render it incompetent in initiation from the CAT_{am1} mRNA leading to Cm^S phenotype of the host (Mandal *et al*, 1996).

Expectedly, introduction of the plasmid pCAT_{am1}metY_{CUA} into *E. coli* KL16 (also referred to as KL16 or the parent strain) confers Cm^R to the host (Figure 1B, right, sector 2), whereas

Figure 1 (A) Left: diagrammatic sketch of the plasmid pCAT_{am1}metY_{CUA} harboring the CAT_{am1} reporter gene, and the metY_{CUA} gene that encodes the mutant tRNA₂^{fMet} (tRNA^{fMet}) containing CUA anticodon. The plasmid pCAT_{am1}metY_{CUA/3GC} (not shown) is the same as pCAT_{am1}metY_{CUA} except that the metY gene in it contains additional mutations in the anticodon stem (as indicated in the tRNA clover leaf structure); and the plasmid pCAT_{am1} (not shown) lacks the metY gene altogether. Right: cloverleaf structure of the *E. coli* tRNA₂^{fMet} indicating the sites of mutations. The positive control tRNA contains only the anticodon mutation from CAU to CUA whereas the '3G-C' mutant contains mutations in the three consecutive G-C pairs in the anticodon stem along with the anticodon change. The CAT_{am1} reporter mRNA with UAG initiation codon, and its pairing with the tRNA anticodon are also shown. (B) Growth of *E. coli* KL16 transformants on LB agar plates containing ampicillin and, ampicillin plus chloramphenicol. Sectors: 1, *E. coli* KL16 + pCAT_{am1}metY_{CUA/3GC}; 2, *E. coli* KL16 + pCAT_{am1}metY_{CUA} and 3, *E. coli* KL16 + pCAT_{am1}. Plates were streaked with overnight cultures and incubated at 37°C for ~15 h. (C) Growth of transformants derived from *E. coli* A48 (sectors 1 and 2) and *E. coli* KL16 (sectors 3 and 4) on LB agar containing ampicillin (left) and, ampicillin plus chloramphenicol (right). Transformants in sectors 1 and 3 harbored pCAT_{am1}, whereas those in sectors 2 and 4 harbored pCAT_{am1}metY_{CUA/3GC}. Plates were streaked with overnight cultures and incubated at 37°C for ~15 h.

the plasmid pCAT_{am1} which lacks the tRNA gene does not (sector 3). Also, as observed earlier (Mandal *et al*, 1996), the pCAT_{am1}metY_{CUA/3GC} does not confer Cm^R to the host to any detectable level (sector 1). As a control, all transformants grow on ampicillin (Amp) plate (Figure 1B, left).

A single mutation (G122D) in the chromosomal copy of the *folD* gene in *E. coli* allows initiation with the 3G-C mutant initiator tRNA

Using a multistep genetic screen (Supplementary data Figure S1), we isolated *E. coli* strains where initiation from the CAT_{am1} reporter (Cm^R) was strictly dependent on the presence of the 3G-C mutant tRNA^{fMet}. Of the isolated suppressors, one called *E. coli* A48 (also referred to as A48 or the suppressor strain) was further characterized. Figure 1C (right) shows that the presence of pCAT_{am1}metY_{CUA/3GC} conferred Cm^R to the suppressor (sector 2) but not to the parent (KL16) strain (sector 4). Relative CAT activity assays showed that A48 afforded ~13.1% activity of initiation in the presence of pCAT_{am1}metY_{CUA/3GC} with respect to a 100% reference value of initiation by pCAT_{am1}metY_{CUA} in KL16. As a control, neither strain showed Cm^R when the pCAT_{am1} plasmid (lacking the tRNA gene) was introduced into them (sectors 1 and 3), and all strains grew on Amp plate (Figure 1C, left). After this initial characterization, we used standard genetic methods to map the suppressor mutation in A48 to a single site in *folD* gene where a C to T mutation resulted in G122D change (termed *folD*₁₂₂ hereafter). An extensive characterization (Supplementary data Figures S2–S7) established that this single mutation was responsible for the phenotype of *E. coli* A48.

The *folD*₁₂₂ mutation results in deficiency of methionine and SAM in *E. coli* A48

Fold is a bi-functional enzyme 5,10 methylene tetrahydrofolate dehydrogenase/cyclohydrolase, central to one-carbon metabolism. As outlined in Figure 2, the cyclohydrolase activity of Fold effects a reversible conversion between 5,10 methenyl-THF and 10 formyl-THF (f¹⁰-THF), whereas its dehydrogenase activity catalyses the reversible reaction between 5,10 methenyl-THF and 5,10 methylene-THF. THF feeds into this pathway through serine hydroxymethyltransferase (*glyA*) to generate 5,10 methylene-THF. These components of the pathway are highly conserved. Besides these, the 5,10 methenyl-THF and f¹⁰-THF are also synthesized through folinic acid (f⁵-THF) and 5-formimino-THF. Additionally, formate is also known to give rise to f¹⁰-THF (Matthews, 1996). Of these, folinic acid, which is readily converted to 5,10 methenyl-THF is also known to be synthesized in *E. coli* through a different pathway (Alimchandani and Sreenivasan, 1957).

Flux balances of various metabolites in this pathway are not known. However, to begin our analysis, we decided to check for f¹⁰-THF (for the lower part of the pathway) and methionine and SAM for the upper part of the pathway (Figure 2). The metabolite f¹⁰-THF is required as a cofactor for formylation of tRNA^{fMet}. Analysis of the formylation status of tRNA^{fMet} (Varshney *et al*, 1991) revealed that tRNA^{fMet} was fully formylated in both the parent and the suppressor strains (data not shown). Considering that alternate pathways feed to accumulation of 5,10 methenyl-THF and f¹⁰-THF, it was not unexpected that consequences of the

Figure 2 One-carbon metabolism pathway. Various intermediates and involvement of various genes at different steps in the pathway are shown (adapted from Matthews, 1996).

mutation in *folD* are not visible for reactions utilizing f¹⁰-THF. This observation suggested that reactions downstream of the biosynthesis of f¹⁰-THF did not contribute to the Cm^R phenotype of A48.

We then focused on the possibility of *folD* mutation exerting its effect through the deficiency of 5,10 methylene-THF that is utilized to produce 5 methyl tetrahydrofolate (5 methyl-THF), which in turn gives rise to methionine and SAM. We analyzed the cell-free extracts of the parent and the A48 strains by LC/MS (Figure 3). A peak (*m/z* 149.9) corresponding to methionine (MW: 149.21) was seen in the extract of the wild-type strain (Figure 3A, panel i). The intensity of this peak increased upon spiking the extract with methionine (panel ii). Moreover, the free methionine marker and the *m/z* 149.9 peak in the spectra eluted with a similar retention time on LC. The *m/z* 149.9 peak was undetectable in the extract of the A48 strain (panel iii) but seen if the extracts were spiked with methionine (panel iv). Similar analyses showed that a peak of *m/z* 399.0–399.2 corresponding to SAM (MW: 399.44) was present in the parent strain but undetectable in A48 (Figure 3B, panels i–iv). These analyses revealed a severe deficiency of steady-state accumulation of methionine and SAM in the A48 strain.

Supplementation of the growth medium with methionine diminishes the suppressor phenotype of *folD*₁₂₂

Results from Figure 3 suggested that enrichment of the growth medium with methionine might weaken the phenotype of A48. As shown in Figure 4, although the A48 strain harboring pCAT_{am1}metY_{CUA/3GC} grows both at Cm³⁰ and Cm¹⁰⁰ in Luria–Bertani (LB), its growth becomes sensitive

Figure 3 (A) LC/ESI-MS analysis of cell-free extracts of *E. coli* KL16 and the *E. coli* A48 harboring pCAT_{am1}metY_{CUA/3GC} for detection of methionine. Panels: (i) *E. coli* KL16; (ii) *E. coli* KL16 spiked with 100 μ M methionine; (iii) *E. coli* A48 and (iv) *E. coli* A48 spiked with 100 μ M methionine. The retention time and the mass corresponding to methionine are indicated. Inset: expansion of m/z 148–153 Da. (B) LC/ESI-MS analysis of cell-free extracts of *E. coli* KL16 and the *E. coli* A48 harboring pCAT_{am1}metY_{CUA/3GC} for detection of S-adenosylmethionine (SAM). Panels: (i) *E. coli* KL16; (ii) *E. coli* KL16 spiked with 100 μ M SAM; (iii) *E. coli* A48 and (iv) *E. coli* A48 spiked with 100 μ M SAM. The retention time and the mass corresponding to SAM are indicated. Inset: expansion of m/z 395–402 Da.

to Cm¹⁰⁰ when the medium was supplemented with 1% methionine (compare sector 1, panels i and ii). Such a consequence of methionine supplementation in the growth medium confirms that the phenotype (Cm^R) of the A48 strain is due to a deficiency of methionine, which in turn would have a bearing on the SAM flux in the cell (Figure 2; Wang *et al.*, 2001). As control, *E. coli* A48 harboring pCAT_{am1}metY_{CUA} showed no visible difference in growth under any of the conditions (sectors 3). Expectedly, the KL16 parent harboring pCAT_{am1}metY_{CUA/3GC} does not grow at any concentration of Cm (sector 2), and all transformants grow well on Amp plate.

The 16S rRNA in *E. coli* A48 lacks nucleoside methylations

SAM is used as a methyl donor by a variety of methyltransferases methylating lipids, proteins, DNA and RNA (Loenen, 2006). As the important events in initiation of protein synthesis occur on the 30S ribosome, as a first step to understand the mechanism of initiation with the 3G-C mutant tRNA^{fMet}, we analyzed 16S rRNA from the parent and the A48 strains

for its methylation status. The *in vitro* methylation assays showed that the 16S rRNA from *E. coli* A48 resulted in an ~ 3.5 -fold more incorporation of ³H from [methyl-³H]SAM than that of the parent strain (2512 versus 718 c.p.m., respectively, per microgram 16S rRNA), suggesting a deficiency of nucleoside methylations in 16S rRNA in A48.

MALDI-MS analysis of 16S rRNA reveals deficiency of methylations at specific positions in *E. coli* A48

The 16S rRNA contains a total of 11 modified nucleosides. Of these, 10 are contributed by the methylated nucleosides (^{m7}G at 527, ^{m2}G at 966, ^{m5}C at 967, ^{m2}G at 1207, ^{m4}Cm at 1402, ^{m5}C at 1407, ^{m3}U at 1498, ^{m2}G at 1516, ^{m6}A at 1518 and ^{m6}A at 1519). We analyzed the methylation status of the 16S rRNAs from the parent and the suppressor strains by MALDI-MS (Figure 5). Theoretical digestions of the wild-type 16S rRNA with RNase A and RNase T1 produced 667 and 487 fragments, respectively. Review of the representative spectra in the regions containing no modified nucleosides showed that they are very much comparable between the parent and the A48 strains for both the RNase A and T1 digests (data not

Figure 4 Growth of transformants derived from *E. coli* KL16 or *E. coli* A48 on LB agar containing ampicillin, and ampicillin plus chloramphenicol with or without 1% methionine (panels i and ii, respectively). Sectors: 1, *E. coli* A48 harboring pCAT_{am1}metY^{CUA/3GC}; 2, *E. coli* KL16 harboring pCAT_{am1}metY^{CUA/3GC} and 3, *E. coli* A48 harboring pCAT_{am1}metY^{CUA}. Plates were streaked with overnight cultures and incubated at 37°C for approximately 15 h.

shown). Spectra (Figure 5) in the regions containing the methylated oligonucleotides show that whereas the peaks of the expected m/z 697.6 corresponding to modifications at positions 966 and 967 (in fragments 966–967, panel i); m/z of 1028.8 corresponding to position 1207 (in fragments 1206–1208, panel ii) and m/z of 684.5 corresponding to position 1498 (in fragments 1497–1498, panel iv) are seen in the RNase A digests of 16S rRNA from the wild-type parent, they are below detection in the corresponding spectra of the A48 strain (panels i, ii and iv, respectively). In the case of the position 1407, it is known that its methylation blocks accessibility of RNase A in its immediate vicinity; so the cleavage occurs at the next available pyrimidine (Kirpekar *et al*, 2000). A peak of m/z 971.3 corresponding to methylation at position 1407 (in fragments 1407–1409) is seen in the sample from the wild-type parent but not from the A48 suppressor (panel iii). Similarly, in the RNase T1 digests, the peaks of m/z 988.6 corresponding to modifications at position 527 (in fragments 525–527, panel v); m/z 1307.1 corresponding to position 1402 (in fragments 1402–1405, panel vi) and m/z 1995.0 corresponding to positions 1518 and 1519 (in fragments 1518–1523, panel vii) are seen in the sample from the wild-type strain but not from the A48 suppressor. In addition, in the case of the fragments 966–967 (m/z 669.5; panel i), 1206–1208 (m/z 1014.1; panel ii) and 1402–1405 (m/z 1279.6; panel vi), intensities of the peaks (with a deficit of m/z of ~14 per methylation) corresponding to the unmethylated fragment sizes were relatively more in the spectra of A48 than the wild-type parent. Taken together, the MALDI-MS analyses revealed that the 16S rRNA methylations present in the wild-type parent are below the limit of detection in A48.

The fold122 allele supports initiation with the derivatives of the 3G-C mutant tRNA^{fMet} lacking one or two G-C base pairs in the anticodon stem

Recently, genome sequencing of several species of mycoplasma has been completed (www.ncbi.nlm.nih.gov). We analyzed these for the initiator tRNA sequences (Figure 6A). This analysis shows that whereas the middle G-C base pair (30:40) is conserved in the initiator tRNAs from all mycoplasma, the presence of all the three consecutive G-C base pairs in the anticodon stem is limited to only three of the eight genomes. Thus, to further understand the implications of rRNA methylations in the initiator tRNA selection on the ribosome, we introduced changes in metY^{CUA} to have 1G-C mutations (A29:U41 or G31:U39) or 2G-C mutations (A29:U41/G31:U39) in the anticodon stem of tRNA^{fMet} (having CUA anticodon) to resemble those found in mycoplasma (Figure 6B, top panel) and checked them for their participation in initiation by the plate (Figure 6B, lower panels) and CAT activity assays (with reference to pCAT_{am1}metY^{CUA} in the parent strain KL16 as 100%). Whereas the 2G-C mutant did not initiate in the parent strain (*E. coli* KL16, sector 3), it did so in the suppressor strain (*E. coli* A48, sector 3; ~4%). And, although both of the 1G-C mutants (A29:U41 and G31:U39) initiated in the parent strain (*E. coli* KL16, sectors 1 and 2; ~25 and ~18%, respectively), their activities increased in the suppressor strain (*E. coli* A48, sectors 1 and 2; ~28 and ~31%, respectively). Observations with the pCAT_{am1}metY^{CUA/3GC}, pCAT_{am1} and pCAT_{am1}metY^{CUA} used as controls (sectors 4, 5 and 6), were as expected. It may be noted that even though the 2G-C (A29:U41/G31:U39) mutant retains the middle G-C pair, it initiated less efficiently than the 3G-C mutant

Figure 5 MALDI-TOF/MS of oligonucleotides from the 16S rRNA from the *E. coli* KL16 and A48 harboring pCAT_{am1}metY_{CUA/3GC} after digestion with RNase A (panels i-iv) and RNase T1 (panels v-vii). Empirical masses of the expected oligonucleotides are shown within insets. Matches with the empirical masses of the relevant peaks have been emphasized.

(compare sectors 3 and 4). This is most likely because of a weaker stability of the anticodon stem in the 2G-C mutant (Figure 6B, top). More importantly, these observations suggest that the role of the *fold122* mutation is not limited to initiation with the 3G-C mutant tRNA^{fMet} used to isolate it, but it contributes to a general phenomenon that allows initiation with tRNAs lacking one, two or all three G-C base pairs in the anticodon stem.

Null mutations in methyltransferases in *E. coli* KL16 reveal a role of rRNA methylations in initiation with the 3G-C mutant tRNA^{fMet}

As mentioned above, SAM is used as cofactor by a number of methyltransferases. Therefore, to support a specific role of the lack of nucleoside methylations in 16S rRNA in the initiator tRNA selection, it becomes important to show initiation with the 3G-C mutant of tRNA^{fMet}, independent of SAM deficiency in the cell. Fortunately, many of the methyltransferases which modify 16S rRNA have now been identified, among these RsmD, RsmB, RsmC, RsmF, RsmE and RsmA methylate positions 966, 967, 1207, 1407, 1498 and 1518+1519, respectively. Hence, to further understand the importance of the methylated nucleosides in the 16S rRNA in initiation, we generated *E. coli* strains where the

methyltransferase genes were knocked out, and used them to analyze initiation by the 3G-C mutant tRNA^{fMet}, both by CAT activity assay (with reference to the A48 strain which was taken as 100%) and the plate assays.

As shown in Figure 7A, knockout of RsmA in KL16 (lane 3) resulted in a notable increase over the parent background (lane 2) in initiation with the 3G-C mutant tRNA^{fMet}. The increase correlated well with the growth of the knockout strain on plates containing chloramphenicol (Figure 7B, panel ii, sector 2). On the other hand, knockouts of RsmB, RsmC, RsmD, RsmE and RsmF resulted in weaker effects (Figure 7A, lanes 4–8). However, among these, a small increase in the initiation activity of RsmF knockout could be validated by its weak growth on chloramphenicol plate (Figure 7B, panel ii, sector 7).

Null mutations in methyltransferases in *E. coli* A48 reveal combinatorial effects of rRNA methylations on initiation with the 3G-C mutant tRNA^{fMet}

The A48 strain does not show a detectable growth defect in LB medium (Supplementary Figure S8, left panel), suggesting that SAM must be produced inside the cell. The analysis in Figure 3 only shows that unlike the parent strain, the steady-state accumulation of SAM in A48 is undetectable.

Figure 6 (A) Multiple sequence alignment of initiator tRNA sequences using Clustal W. The nucleotides at positions 29–31 and 39–41 (corresponding to the 3G-C base pairs) are boxed. (B) Anticodon stem-loop sequences of the *metY*_{CUA} derivatives. Top panel: (i) 1G-C (A29:U41); (ii) 1G-C (G31:U39); (iii) 2G-C (A29:U41/G31:U39) and (iv) 3G-C mutant. The tRNA three-dimensional image was adapted from Hoang *et al*, 2004. Middle and bottom panels: Growth of transformants derived from *E. coli* KL16 and *E. coli* A48 on LB-agar containing ampicillin; ampicillin plus chloramphenicol (30 µg/ml); ampicillin plus chloramphenicol (100 µg/ml); ampicillin plus chloramphenicol (150 µg/ml) and ampicillin plus chloramphenicol (200 µg/ml) at 37°C for 36 h. Sectors: 1, pCAT_{am1}*metY*_{CUA/A29:U41}; 2, pCAT_{am1}*metY*_{CUA/G31:U39}; 3, pCAT_{am1}*metY*_{CUA/2GC}; 4, pCAT_{am1}*metY*_{CUA/3GC}; 5, pCAT_{am1}; and 6, pCAT_{am1}*metY*_{CUA}.

Thus, depending on the abundance and the biochemical properties of the methyltransferase involved, a low frequency of rRNA methylations at various positions (which remains undetectable in our analysis in Figure 5) presumably occurs in A48, which would render the ribosome population heterogeneous with respect to rRNA methylations. Hence, it was of interest to exploit the A48 background further by generating knockouts of the various rRNA methyltransferases. As shown in Figure 7A, the effects of these knockouts turned out to be quite revealing in our understanding of initiation with the 3G-C mutant tRNA^{fMet}. The knockouts of RsmC or RsmE in A48 resulted in an increase in initiation (Figure 7A, compare lanes 11 and 13 with lane 1) and a corresponding increase in resistance to chloramphenicol (Figure 7C, compare sectors 4 and 6 with 1). Although the effect of RsmF knockout (lane 14) in initiation with the 3G-C mutant tRNA^{fMet} was small (compare with lane 1), the increase was supported by its increased resistance to

chloramphenicol (Figure 7C, panel iv, compare sector 7 with sector 1). Initiation with the 3G-C mutant tRNA^{fMet} in the RsmA knockout in A48 was marginally lower (Figure 7A, compare lanes 1 and 9; Figure 7C panel iii, compare sectors 1 and 2). Interestingly, the knockouts of RsmB or RsmD in A48 (Figure 7A lanes 10 and 12, respectively) resulted in decreased initiation with the 3G-C mutant tRNA^{fMet} (see Discussion). Importantly, the observations with the targeted knockouts of methyltransferases in *E. coli* KL16 and A48 backgrounds provide a direct evidence for the contribution of the nucleoside methylations in the 16S rRNA in the process of initiator tRNA selection. However, it should also be said that knockouts of various rRNA methyltransferases in the KL16 and A48 backgrounds result in differential effects, suggesting that the consequences of deficiencies of methylations at multiple positions ('combinatorial effects') are complex and context dependent for initiation with the 3G-C mutant tRNA^{fMet}.

Figure 7 (A) CAT assays with the cell-free extracts of strains derived from *E. coli* KL16 and *E. coli* A48 harboring pCAT_{am1}metY_{CUA/3GC}. CAT activities (% product formed) of the cell-free extracts (1.2 μ g total protein) from three independent cultures were represented with reference to the values obtained for *E. coli* A48 harboring pCAT_{am1}metY_{CUA/3GC} (taken as 100%) in each set. Averages ($\pm$ s.d.) of the values from the three sets are shown as histograms (lanes 2–14). The 100% activity of *E. coli* A48 harboring pCAT_{am1}metY_{CUA/3GC} (lane 1) corresponded to $\sim$ 307 pmol of chloramphenicol converted to 1 acetyl-, and 3-acetyl-, chloramphenicol per 1.2 μ g of the total protein in the cell-free extract used in a 50 μ l reaction containing 0.2 μ Ci of 14 C-chloramphenicol (8000 pmol) and 20 000 pmol acetyl-CoA at 37°C in 30 min. (B) Growth of *E. coli* KL16 and its derivatives ($\Delta rsmA::kan$, $rsmB::kan$, $\Delta rsmC::kan$, $\Delta rsmD::kan$, $\Delta rsmE::kan$ and $\Delta rsmF::kan$) harboring pCAT_{am1}metY_{CUA/3GC} (Amp^R), on LB agar containing ampicillin (panel i) and ampicillin plus chloramphenicol (30 μ g/ml) (panel ii). Plates were streaked with logarithmic phase cultures and incubated at 37°C for $\sim$ 30 h. (C) Growth of *E. coli* A48 and its derivatives ($\Delta rsmA::kan$, $rsmB::kan$, $\Delta rsmC::kan$, $\Delta rsmD::kan$, $\Delta rsmE::kan$ and $\Delta rsmF::kan$) harboring pCAT_{am1}metY_{CUA/3GC}; and A48 harboring pCAT_{am1} (Amp^R) on LB-agar containing ampicillin (panel i); ampicillin plus chloramphenicol (30 μ g/ml; panel ii), ampicillin plus chloramphenicol (150 μ g/ml; panel iii) and ampicillin plus chloramphenicol (200 μ g/ml, panel iv). Plates were streaked with logarithmic phase cultures and incubated at 37°C for $\sim$ 30 h. Streakings for the plate shown in panel iv were carried out with a smaller loop.

Discussion

Starting with a mutant tRNA^{fMet} in which the three consecutive G-C base pairs in the anticodon stem (a feature of the initiator tRNAs highly conserved in all kingdoms of life) had been changed to those found in the elongator tRNA^{Met}, we have isolated and characterized an *E. coli* strain (A48) that initiates with the 3G-C mutant tRNA^{fMet}. The strain harbors a G122D mutation in its *folD* gene product resulting in a deficiency of methionine and SAM. One of the consequences of this deficiency is the lack of methylations in 16S rRNA. The directed knockouts of methyltransferases in the KL16 and the A48 backgrounds on initiation with the 3G-C mutant tRNA^{fMet} suggest that methylations in 16S rRNA make an important contribution to the process of the initiator tRNA selection on the P-site. Although the specific effects of the methylations in different contexts may be distinct, the general notion of the role of rRNA methylations is further corroborated by the observed correlation in mycoplasma species, between the lack of the three G-C base pairs (in the anticodon stem of the initiator tRNA) and the lack of some of the 16S rRNA methyltransferases (Table I). Further, considering that the A48 strain allows efficient initiation with metY_{CUA} (which possesses the 3G-C base pair) in the reporter assay (Figure 6B), it seems that the role of rRNA methylations in

initiator tRNA selection on ribosomes is indirect (also discussed below). Essentially, specific binding of the initiator tRNA (which possesses the 3G-C base pairs) on the ribosome is facilitated by preferential exclusion of tRNAs that lack them.

The three-dimensional structure of ribosomes (Wimberly et al, 2000; Yusupov et al, 2001; Schuwirth et al, 2005; Selmer et al, 2006) has revealed that the modified nucleosides are clustered within several conserved regions associated with essential ribosomal functions (Brimacombe et al, 1993; Decatur and Fournier, 2002). In *Thermus thermophilus* ribosomal P-site, the codon and tRNA anticodon stem-loop are fixed in position by interactions with 10 nucleotides of 16S rRNA, which include four of the eleven post-transcriptionally modified 16S rRNA nucleotides (Guymon et al, 2006; Korostelev et al, 2006). Despite such strong structural correlations, there has been very little evidence of the functional importance of the modified nucleosides in rRNA. However, it may be noted that the lack of modifications in rRNA has been observed to modify the stability of the adjacent stem structure (Heus et al, 1983), control host resistance to antibiotics, for example, kasugamycin (Helser et al, 1972), capreomycin and viomycin (Johansen et al, 2006), etc. and affect translational fidelity (van Buul et al, 1984; O'Connor et al, 1997). Here, we have uncovered yet another crucial role of the rRNA

Table I Status of the 3G-C base pairs in the anticodon stem of the initiator tRNAs and the 16S rRNA methyltransferases in mycoplasma

Species	Anticodon stem	16S rRNA methyltransferases % similarity with <i>E. coli</i> homologs when present					
		RsmB	RsmF	RsmC	RsmE	RsmA	RsmD
<i>M. capricolum</i>	3GC	47	54	48	Absent	52	49
<i>M. mycoides</i>	3GC	47	59	48	Absent	51	49
<i>M. synoviae</i>	3GC	47	41	50	Absent	48	54
<i>M. mobile</i>	2GC	Absent	Absent	41	Absent	51	50
<i>M. genitalium</i>	1GC	Absent	Absent	48	Absent	48	Absent
<i>M. penetrans</i>	1GC	Absent	Absent	Absent	Absent	48	Absent
<i>M. pneumoniae</i>	1GC	Absent	Absent	46	Absent	47	44
<i>M. gallisepticum</i>	1GC	Absent	Absent	51	Absent	51	52

Figure 8 (A) An overview of 16S rRNA harboring tRNA^{fMet} (in metallic brown with anticodon in light blue) and mRNA (in blue). Methylated nucleosides are shown red, and the nucleotides involved in IF3 interaction (Dallas and Noller, 2001) are shown in green. Nucleotides are numbered according to their positions in *E. coli* 16S rRNA. (B) A detailed view of the same in the vicinity of the anticodon stem-loop of tRNA^{fMet}. Nucleotides 1338 and 1339 interacting in the minor groove of G30-C40 and G29-C41, respectively, in the anticodon stem of tRNA^{fMet} are shown in teal color on top left. The initiator tRNA^{fMet} anticodon is shown in light blue and the 3G-C base pairs in the anticodon stem are shown in magenta. Methylation sites 966 (closest to the anticodon), 967 (behind 966), 1519 (partially seen on right), 1498 on top right and 1402 bottom right, visible in this view, are shown in red. Two of the IF3 interaction sites, 791 and 793 (partially seen on right) are shown in green. The C1400 which stacks with the first codon-anticodon base pair and is located against the position 966 is also shown (orange) in the image. Models were prepared in PyMol (DeLano, 2002) using the coordinates (2j00) of the three-dimensional structure of the co-crystal of the 70S ribosome of *Thermus thermophilus* containing tRNA^{fMet} in the P-site and the tRNA^{Phe} in the A and the E sites (Selmer *et al*, 2006).

modifications in the fundamental step of initiator tRNA selection on ribosome.

The three-dimensional structural analyses have shown that G1338 and A1339 are in juxtaposition with the minor groove of the 29-41 and the 30-40 G-C base pairs, respectively, and it has been proposed that these minor groove interactions could offer a mechanism for IF3-mediated steric check of the three consecutive G-C base pairs of the initiator tRNA (Dallas and Noller, 2001; Lancaster and Noller, 2005). It

is possible that IF3-mediated conformational changes which orient the G1338 and A1339 to interact with the 3G-C base pairs are mediated with contributions from the methylated nucleosides. Such a role of the 16S rRNA methylations in IF3-mediated mechanism is borne from the observation that lack of methylations at positions modified by RsmA (1518 and 1519), RsmE (1498) and Rsm F (1407), which result in a gain or enhancement of initiation by the 3G-C mutant tRNA^{fMet} (Figure 7), are located in the vicinity of the IF3-binding region

(Figure 8A; see also the movie in Supplementary data). Also, it has been observed that a compromise in IF3 function in the cell results in a gain of initiation activity with the 3G-C mutant tRNA^{fMet} (O'Connor *et al*, 2001). Thus, these observations espouse for a possible connection between the role of IF3 and the 16S rRNA methylations in the 3G-C base pair-mediated tRNA^{fMet} selection on the P-site.

However, it must be noted that evolution of the initiator tRNA selection on the P-site is a combination of several coordinated events. For example, formylation of initiator tRNA, which facilitates its binding to IF2 and decreases its off-rate upon IF3 binding (Antoun *et al*, 2006), is perhaps one of the most crucial contributors to the initiator tRNA selectivity on the ribosome (Guillon *et al*, 1992). The lack of formylation of elongator tRNAs such as tRNA^{Phe} and tRNA^{Cys} possessing two G-C base pairs (at 29–41 and 30–40 positions) in the anticodon stem may be a major reason for their lack of binding in the P-site of the 30S ribosomal subunit. However, the formylation property alone is neither sufficient (the 3G-C mutant tRNA^{fMet} is efficiently formylated) nor essential (Newton *et al*, 1999; Nilsson *et al*, 2006) for the selection of the initiator tRNAs in the P-site. It was shown that in *Salmonella*, inhibition of formylation activity could be compensated for by initiator tRNA gene amplification. Thus, the cellular abundance of the initiator tRNA is another factor that

determines its favorable binding to the P-site. Additionally, it is clear (Antoun *et al*, 2006) that the rates with which the 50S and 30S subunits interact with each other would also affect retention of a tRNA on the P-site. Hence, it would be important to develop *in vivo* assay systems to understand the biological significance of the modified nucleosides in the interaction of 30S and 50S subunits as well as in various other ribosome function(s).

How the loss of methylation at position 1207 (RsmC) results in an increase, and its loss at positions 967 (RsmB) or 966 (RsmD) in a decrease in initiation (in A48) with the 3G-C mutant tRNA^{fMet}, remains unclear. Interestingly, however, whereas the lack of methylations at 966 or 967 in the total ribosome population results in decreased initiation with the 3G-C mutant tRNA^{fMet} (as shown by their hypersensitive growth on chloramphenicol, compare sectors 3 and 5 with 1 in panels ii and iii, Figure 7C), they utilize the wild-type tRNA^{fMet} apparently normally (their growth in ampicillin alone medium is normal, Figure 7C, panel i). It may well be that methylations at positions 966 and 967 play a role in stabilizing the initiation codon (mRNA) and the tRNA^{fMet} anticodon interaction (Figure 8B; see also the movie in Supplementary data). Hence, unlike for the wild-type tRNA^{fMet}, for the 3G-C mutant tRNA^{fMet} (which would be missing the 3G-C base pair-specific contacts in the P-site),

Table II Description of *E. coli* strains and plasmids used in this study

Strains/plasmids	Genotype/details	Reference/source
<i>E. coli</i> strains		
KL16	<i>E. coli</i> K-12, <i>thi1</i> , <i>relA1</i> , <i>spoT1</i>	Low (1968)
A48	<i>folD122</i> derivative of KL16	This study
CAG12171	<i>purK79::Tn10</i>	Singer <i>et al</i> (1989)
DY330	W3110 Δ <i>lacU169 gal490</i> [<i>lc1857</i> Δ (<i>cro-bioA</i>)]	Lee <i>et al</i> (2001)
KL16 Δ <i>rsmA</i> ::Kan	<i>E. coli</i> KL16 strain carrying a deletion in <i>rsmA</i> gene	This study
KL16 Δ <i>rsmB</i> ::Kan	<i>E. coli</i> KL16 strain carrying an insertion in <i>rsmB</i> gene	This study
KL16 Δ <i>rsmC</i> ::Kan	<i>E. coli</i> KL16 strain carrying a deletion in <i>rsmC</i> gene	This study
KL16 Δ <i>rsmD</i> ::Kan	<i>E. coli</i> KL16 strain carrying a deletion in <i>rsmD</i> gene	This study
KL16 Δ <i>rsmE</i> ::Kan	<i>E. coli</i> KL16 strain carrying a deletion in <i>rsmE</i> gene	Basturea <i>et al</i> (2006); and this study
KL16 Δ <i>rsmF</i> ::Kan	<i>E. coli</i> KL16 strain carrying a deletion in <i>rsmF</i> gene	Andersen and Douthwaite, 2006; and this study
A48 Δ <i>rsmA</i> ::Kan	<i>E. coli</i> A48 strain carrying a deletion in <i>rsmA</i> gene	This study
A48 Δ <i>rsmB</i> ::Kan	<i>E. coli</i> A48 strain carrying an insertion in <i>rsmB</i> gene	This study
A48 Δ <i>rsmC</i> ::Kan	<i>E. coli</i> A48 strain carrying a deletion in <i>rsmC</i> gene	This study
A48 Δ <i>rsmD</i> ::Kan	<i>E. coli</i> A48 strain carrying a deletion in <i>rsmD</i> gene	This study
A48 Δ <i>rsmE</i> ::Kan	<i>E. coli</i> A48 strain carrying a deletion in <i>rsmE</i> gene	This study
A48 Δ <i>rsmF</i> ::Kan	<i>E. coli</i> A48 strain carrying a deletion in <i>rsmF</i> gene	This study
Plasmids		
pCAT _{am1}	Renamed from pRSVCAT _{am1.2.5} . A pBR322 derivative harboring CAT reporter gene with UAG initiation codon	Varshney and RajBhandary (1990)
pCAT _{am1} <i>metY</i> _{CUA}	pCAT _{am1} harboring <i>metY</i> gene which encodes tRNA ^{fMet} with CUA anticodon (U35A36 mutation)	Varshney and RajBhandary (1990)
pCAT _{am1} <i>metY</i> _{CUA/3GC}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with additional mutations in <i>metY</i> 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 <i>et al</i> (1996)
pCAT _{am1} <i>metY</i> _{CUA/2GC}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with additional mutations in <i>metY</i> at positions 29:41 and 31:39 (from G:C base pairs to A:U and G:U base pairs, respectively)	This study
pCAT _{am1} <i>metY</i> _{CUA/A29:U41}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with additional mutations in <i>metY</i> at positions 29:41 from G:C to A:U	This study
pCAT _{am1} <i>metY</i> _{CUA/G31:U39}	Derivative of pCAT _{am1} <i>metY</i> _{CUA} with additional mutation at positions 39 from C to U	This study
pACDH	Plasmid with ACYC ori of replication, which is compatible with Cole1 ori of replication plasmids	Rao and Varshney (2002)
pACDH <i>folD</i>	<i>E. coli folD</i> cloned into pACDH between <i>EcoRI</i> sites	This study
pACDH <i>folD122</i>	<i>E. coli folD122</i> cloned into pACDH between <i>EcoRI</i> sites	This study
pTZ <i>folD</i>	<i>E. coli folD</i> cloned into pTZ19R between <i>EcoRI</i> sites	This study
pTZ <i>folD122</i>	<i>E. coli folD122</i> cloned into pTZ19R between <i>EcoRI</i> sites	This study

any decrease in the stability of codon–anticodon interaction could have severe consequences on its binding to the ribosome.

In conclusion, we have shown that a decrease in the intracellular levels of SAM results in deficiency of rRNA methylations and influences the initiator tRNA selection on the ribosome. These observations reveal a link between one-carbon metabolism and mRNA translation.

Materials and methods

Bacterial strains, plasmids and growth conditions

E. coli strains and plasmids are listed in Table II. Unless mentioned otherwise, bacterial cultures were grown in LB medium and supplemented with kanamycin (Kan, 25 µg/ml), chloramphenicol (Cm, 30 µg/ml), ampicillin (Amp, 100 µg/ml), tetracycline (Tet, 7.5 µg/ml) as required. For growth on solid support, 1.5% bacto-agar (Difco) was included in the medium.

Mutagenesis, genetic screening and mapping of the extragenic suppressor (*E. coli* A48)

The extragenic suppressors of the 3G-C mutations in tRNA^{fMet} were generated by chemical mutagenesis, subjected to a multistep genetic screening and mapped using classical genetic methods. Further details are provided in the Supplementary data.

Preparation of cell-free extracts and biochemical assays for chloramphenicol acetyltransferase

See Supplementary data.

Molecular biology and genetic engineering techniques

See Supplementary data.

In vitro methylation

The assay mixture (15 µl) contained R buffer (100 mM Tris–HCl pH 8.0, 100 mM NaCl, 2 mM dithiothreitol, 0.5 mM Na₂EDTA), 40 µM [methyl-³H]AdoMet (74.7 Ci/mmol, NEN Life Science Products Inc.) and 16S rRNA (1 µg). The reaction was initiated by addition of 10 µg of cell-free extract prepared from *E. coli* MRE600 (RajBhandary and Ghosh, 1969) and incubated at 37°C. Aliquots were taken and rRNA was precipitated on GF/C filters pre-wetted with 5% trichloroacetic acid (TCA) and 2% peptone. The filters were washed three times in 5% TCA and once in ethanol, dried and subjected to liquid scintillation counting (Varshney *et al*, 2004).

Analysis of methionine and SAM in the cellular extracts

Cultures of *E. coli* A48 and KL16 harboring pCAT_{am1}metY_{CUA/3GC} were grown to saturation at 37°C, diluted 10⁶-fold into LB broth (50 ml) containing ampicillin (100 µg/ml), grown for 18 h and chilled on ice. Cells were harvested by centrifugation, resuspended

in 2 ml chilled minimal A salts on ice (Miller, 1972). Cells from 1 ml aliquots were harvested in 1.5 ml tubes by spinning in a microfuge, frozen in liquid nitrogen and stored at –80°C until their use. Frozen pellets were resuspended in chilled 10 mM HCl, sonicated on ice and spun to recover cell-free extracts for LC/MS analysis. The ultraviolet and total ion chromatograms of the cell-free extracts were recorded using an Esquire 3000-plus mass spectrometer (Bruker Daltonics) consisting of two octapoles followed by an ion trap in positive-ion mode. The spectra were recorded with and without spiking of methionine/SAM into the samples. The extract was dissolved in acetonitrile and 15 µl stock solution was run through Phenomenex C₁₈ column (4.6 mm × 250 mm, 10 µm particle size) using acetonitrile/water/0.1% formic acid (MeOH/H₂O/0.1% HCOOH). The flow rate was maintained at 0.2 ml/min and detected at 226 nm. Helium was used as the collision gas for CID experiments. The data were analyzed using Esquire data analysis software, version 3.1. Various peaks obtained were analyzed at different time intervals in the solvent mixtures—acetonitrile/water.

Analysis of 16S rRNA by MALDI-TOF mass spectrometry

Purified 16S rRNA (1 µg) was digested in a buffer containing 50 mM Tris–HCl (pH 7.5), 2 mM Na₂EDTA and 30 U of RNase T1 (MBI Fermentas) or RNaseA (Sigma-Aldrich Chemie GmbH), at 37°C for 12 h. The digests were mixed on the MALDI sample support with 2,5 dihydroxybenzoic acid and the preparation was air dried. MALDI mass spectra were recorded on a Bruker Ultraflex TOF/TOF spectrometer (Bruker-Daltonics) in reflector mode using delayed ion extraction. The spectra were normally recorded in the positive-ion mode. All matrix compounds were obtained from Sigma-Aldrich Chemie GmbH.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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