The *yfiC* gene of *E. coli* encodes an adenine-N6 methyltransferase that specifically modifies A37 of tRNA$_1^{Val}$(cmo^{5}UAC)

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ABSTRACT

Transfer RNA is highly modified. Nucleotide 37 of the anticodon loop is represented by various modified nucleotides. In *Escherichia coli*, the valine-specific tRNA (cmo^{5}UAC) contains a unique modification, N^6-methyladenosine, at position 37; however, the enzyme responsible for this modification is unknown. Here we demonstrate that the *yfiC* gene of *E. coli* encodes an enzyme responsible for the methylation of A37 in tRNA$_1^{Val}$. Inactivation of *yfiC* gene abolishes m^{6}A formation in tRNA$_1^{Val}$, while expression of the *yfiC* gene from a plasmid restores the modification. Additionally, unmodified tRNA$_1^{Val}$ can be methylated by recombinant YfiC protein in vitro. Although the methylation of m^{6}A in tRNA$_1^{Val}$ by YfiC has little influence on the cell growth under standard conditions, the yfiC gene confers a growth advantage under conditions of osmotic and oxidative stress.

Keywords: tRNA; modification; methylation; *yfiC*; N6-methyl adenosine.

INTRODUCTION

Transfer RNA (tRNA) is extremely rich in modified nucleotides, constituting ~10% of the entire tRNA sequence (Auffinger and Westhof 1998) . The role of many modified tRNA nucleotides has been studied; Position 37 is located adjacent to the anticodon triplet and is modified in 34 tRNA species in *Escherichia coli*, whereas unmodified adenosine is found at this position in only 13 tRNA species (Dunin-Horkawicz et al. 2006). The range of nucleotide 37 modifications include hypermodified t^{6}A, m^{6}t^{6}A, i^{6}A, and ms^{2}i^{6}A, as well as the simpler m^{2}A, m^{1}G, and m^{6}A. Curiously, the latter m^{6}A modification is found only in tRNA$_1^{Val}$ (anticodon cmo^{5}UAC) (Dunin-Horkawicz et al. 2006). During decoding, nucleotide 37 of the tRNA stacks over the 5′-codon 3′-anticodon base pair (Weixlbaumer et al. 2007). It has been suggested that some modifications at nucleotide 37 are necessary for stabilization of the anticodon loop structure (Stuart et al. 2003), binding of magnesium ions in the context of ribosomal decoding complex (Konevega et al. 2004), and/or maintenance of the reading frame (Urbonavicius et al. 2001, 2003).

The role of tRNA modifications in the cell can only be tested if the gene encoding the respective modification enzyme is identified and inactivated. However, this is no easy task since the proteins responsible for tRNA modifications are more numerous than the tRNA species themselves. Although the vast majority of genes involved in tRNA modification are known (Dunin-Horkawicz et al. 2006), several are still missing. Among them was the gene responsible for the m^{6}A37 modification in tRNA$_1^{Val}$. In search of the "missing" methylation enzymes, we checked a set of eight open reading frames annotated as putative methyltransferases. Of these, the *yfiC* gene, which belongs to the COG4123 family, had already been suspected to encode a m^{6}A37 methyltransferase in Mollicutes (de Crécy-Lagard et al. 2007). In this report, we demonstrate that the *yfiC* gene encodes the methyltransferase specific for tRNA$_1^{Val}$ m^{6}A37 formation.

RESULTS

YfiC is specific for modification of m^{6}A37 in tRNA$_1^{Val}$

Recombinant protein YfiC, containing a His$_6$ tag on its C-terminus, was expressed and purified from *E. coli* strain AG1 carrying the pCA24YfiC plasmid (Kitagawa et al. 2005). The resulting protein was pure according to the SDS-PAGE and was thus used to modify potential substrates. The substrates were prepared from the strain JW2559 lacking the *yfiC* gene on the chromosome (termed hereafter as Δ*yfiC*) (Baba et al. 2006). Cell lysate was fractionated into ribosome and S100 by ultracentrifugation. Both fractions were subsequently divided in half, and each of the ribosomal and S100 fractions were phenol deproteinized. Thus, four putative substrate groups were generated: Ribosomes, ribosomal RNA (rRNA), S100, and total small RNA. All substrate fractions were mixed with recombinant YfiC protein and radioactive S-adenosyl-L-[methyl-^{3}H]methionine (^{3}H-SAM). The reactions were then analyzed for tritium incorporation. The results showed that rRNA, either as a component of assembled ribosomal subunits or as pure RNA, could not be methylated by YfiC. In contrast, the total small RNA fraction, composed essentially of tRNA, was readily methylated as part of the S100 or in the deproteinized form.

Very few tRNA methylation enzymes are left to be identified in *E. coli*, namely, for modification of Cm34 in tRNA$_5^{Leu}$, m^{6}t^{6}A37 in tRNA$_{3,4}^{Thr}$, and m^{6}A in tRNA$_1^{Val}$. In order to determine if YfiC is responsible for one of those modification, we performed in vitro [^{3}H]-methylation using recombinant YfiC protein of a total tRNA fraction purified from the Δ*yfiC* strain. Biotin-conjugated oligonucleotides, complementary to the divergent sequence regions in T- and acceptor stems of tRNA$_5^{Leu}$, tRNA$_3^{Thr}$, and tRNA$_1^{Val}$, were used to affinity purify the respective tRNA species. In parallel, the same method was used to prepare tRNA$_3^{Arg}$, tRNA$_1^{Glu}$, and tRNA$_2^{Val}$ to serve as a control. As shown in Figure 1A, only the fraction purified using the oligonucleotide complementary to tRNA$_1^{Val}$ was radiolabeled. This result indicates that the YfiC methyltransferase can methylate tRNA$_1^{Val}$, but none of other tRNAs tested.

A similar result could also be obtained using in vitro transcribed tRNAs. Genes encoding tRNA$_5^{Leu}$, tRNA$_3^{Thr}$, tRNA$_1^{Val}$, and tRNA$_2^{Val}$ were PCR-amplified from *E. coli* genomic DNA. The T7 promoter sequence was added to the 5′-end of each gene to allow in vitro transcription of the full-length tRNAs using T7 RNA polymerase. The transcripts were supplied as substrates for in vitro methylation using recombinant YfiC protein and ^{3}H-SAM. Consistently with the in vivo purified tRNAs, methylation was only observed using the in vitro transcript of tRNA$_1^{Val}$ (Fig. 1B).

Since tRNA$_1^{Val}$ contains only one methylated residue for which the respective enzyme has to be identified, it was
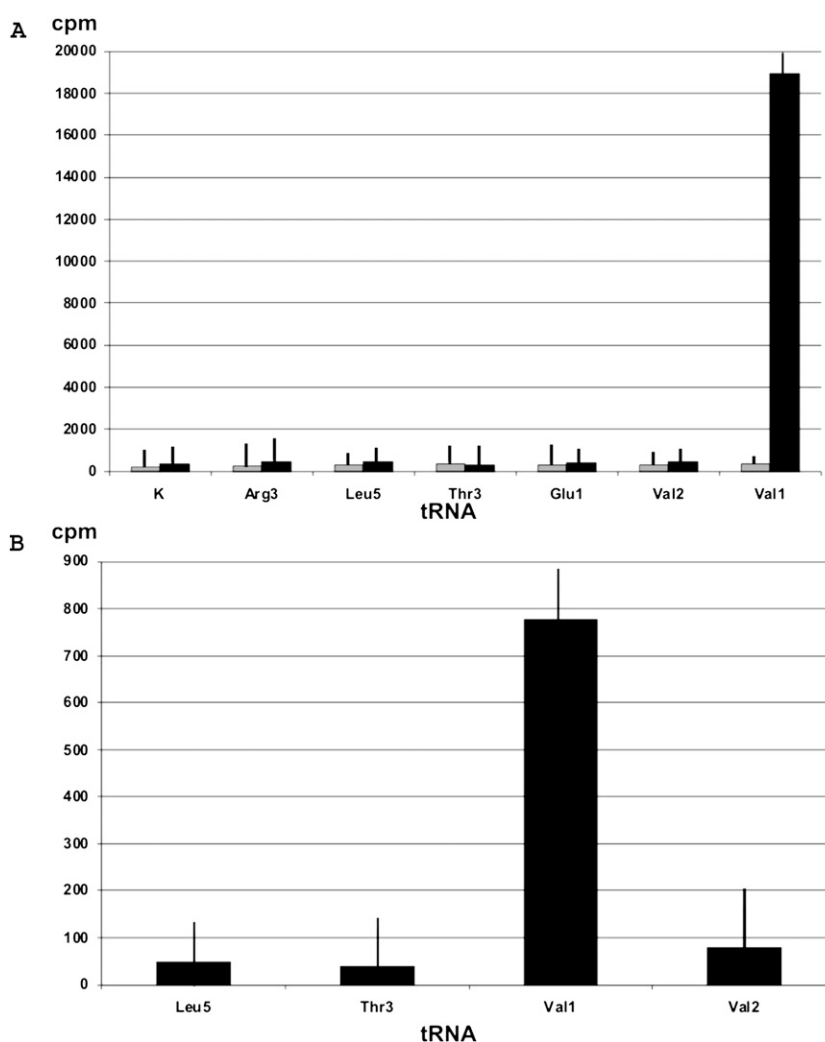

FIGURE 1. Methylation of tRNA species by recombinant YfiC protein in vitro. [^{3}H]-SAM radioactivity introduced into various tRNA species by recombinant YfiC protein is indicated. (*A*) Methylation of tRNA species affinity purified from the cells. Gray bars correspond to methylation of tRNAs extracted from the wild-type strain, i.e., premethylated with nonradioactive SAM. Black bars correspond to methylation of tRNAs extracted from the *yfiC* knockout strain. K corresponds to mock tRNA purification with specific oligodeoxyribonucleotide omitted. (*B*) Methylation of in vitro transcribed tRNA species. Error bars indicate SD borders.

logical to suggest that the function of YfiC is the formation of m^6A37. To confirm this hypothesis, $tRNA_1^{Val}$ from the wild-type and $\Delta yfiC$ strains was treated with RNase T1, and the products of the digestion were analyzed by MALDI-MS (Fig. 2A,B). The fragment of $tRNA_1^{Val}$ carrying m^6A37 from the wild-type strain (Fig. 2B) is 14 Da heavier than the equivalent fragment from the $\Delta yfiC$ strain (Fig. 2A), consistent with an additional methylation being present in this fragment. Indeed, the theoretical masses calculated for the methylated and unmethylated variants of the $tRNA_1^{Val}$ RNase T1 fragments precisely match the masses observed by MALDI MS. Since A37 is the only methylated residue in this RNA fragment, YfiC is indeed responsible for the methylation of m^6A37 in $tRNA_1^{Val}$.

YfiC is sufficient for methylation of m^6A37 of $tRNA_1^{Val}$

To prove that the YfiC protein is directly responsible for modification of A37 in $tRNA_1^{Val}$, rather than a product of another gene that is affected by inactivation of *yfiC* gene, we performed in vivo complementation of the $\Delta yfiC$ strain with the pCA24YfiC plasmid. $tRNA_1^{Val}$ was therefore isolated from the wild-type, $\Delta yfiC$ strain, as well as the $\Delta yfiC$ strain complemented with pCA24YfiC that expresses YfiC exogenously. Following affinity purification, $tRNA_1^{Val}$ was digested by RNase T1, and the resulting fragments were analyzed by MALDI MS (Fig. 2C). As expected, methylation of A37 in the $\Delta yfiC$ strain was restored only when YfiC was expressed from the plasmid. Thus, only the absence of YfiC protein, rather than any changes in the genomic context resulting from the genetic knockout, is the cause of the lack of m^6A37 formation. To demonstrate that YfiC alone is necessary and sufficient for the $tRNA_1^{Val}$ A37 methylation, we used SAM and the recombinant YfiC protein to modify $tRNA_1^{Val}$ purified from the $\Delta yfiC$ strain. The product of modification was treated with RNase T1 and subjected to MALDI MS analysis (Fig. 2D). The results show clearly that the YfiC protein alone is sufficient for $tRNA_1^{Val}$ methylation at A37.

Methylation of m^6A37 of $tRNA_1^{Val}$ is dispensable for growth of *E. coli* cells under standard growth conditions

We next examined how the m^6A37 modification of $tRNA_1^{Val}$ by YfiC influences *E. coli* cell fitness under different growth conditions. *E. coli* doubling time was measured
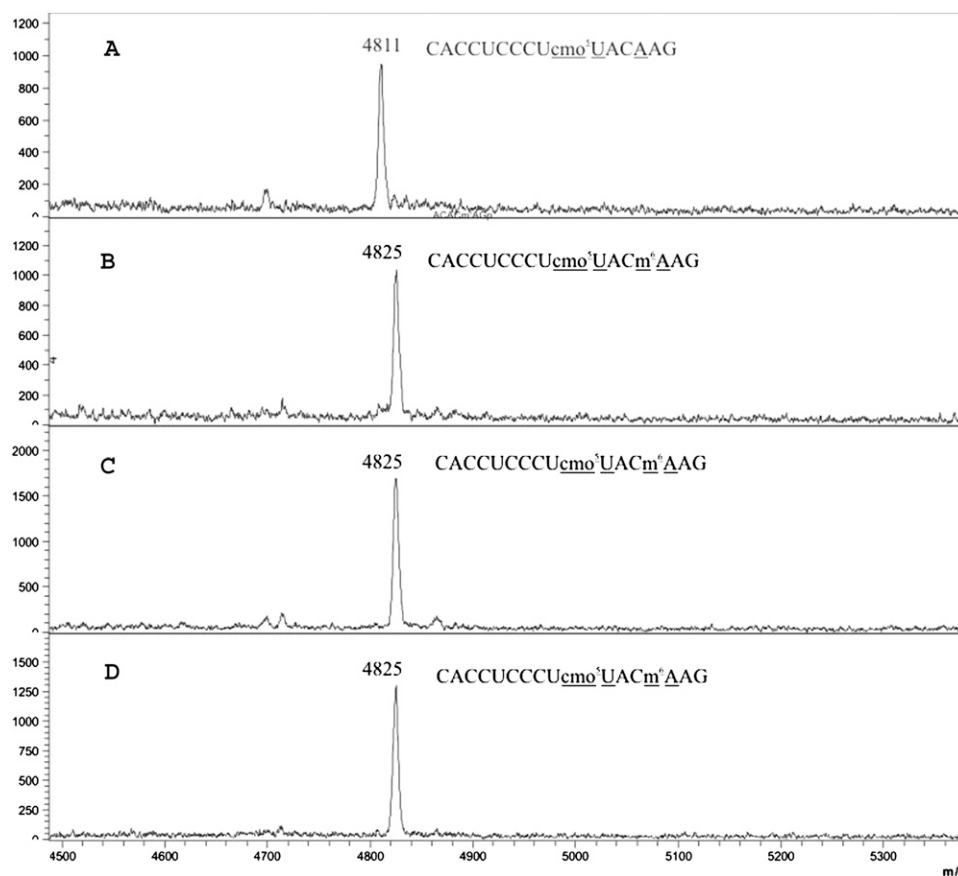

FIGURE 2. MALDI MS analysis of the T1 digest of the $tRNA_1^{Val}$. Heavy part of the spectrum is shown. MALDI spectrum corresponding to fragments of the $tRNA_1^{Val}$ from the (A) $\Delta yfiC$ strain, (B) wild-type strain, (C) $\Delta yfiC$ strain transformed with pCA24YfiC, and (D) $\Delta yfiC$ strain treated with SAM and recombinant YfiC in vitro. Oligonucleotides corresponding to the peak are indicated.

in rich LB and minimal M9 media at 30°C, 37°C, and 42°C (Fig. 3). Comparison of the growth of the wild-type and *ΔyfiC* strains in rich LB media revealed no significant difference at any of the different temperatures tested. In the minimal M9 media, very small growth retardation, resulting from *yfiC* inactivation was observable.

In addition, we checked the ability of the wild-type strain to compete for growth with *ΔyfiC* strain. Both rich LB and minimal M9 media were used for growth competition assays at 30°C, 37°C, and 42°C. Equal numbers of cells of the kanamycin-sensitive parental strain and kanamycin-resistant *ΔyfiC* strain (A$_{600}$ 0.001) were mixed and grown for 24 h. The growth cycles were repeated so that at the end of each cycle 1/1000th of the media containing the mixture of the cells was used to re-inoculate fresh media with 1000-fold dilution. The cells amplify 1000-fold with each growth cycle, which corresponds to ∼10 doublings. After each growth cycle, titers of the wild-type and *ΔyfiC* strains were measured. After 80 generations, no significant (more than one order of magnitude) drop in the ratio of *ΔyfiC* to wild-type cells was detected. We concluded that the modification of A37 in tRNA$_1^{Val}$ by YfiC does not affect growth of *E. coli* cells under the standard growth conditions tested here.

Methylation of m⁶A37 of tRNA$_1^{Val}$ influences survival of *E. coli* cells under conditions of osmotic and oxidative stress

It is generally believed that if no readily detectable phenotype is observed for a knockout strain, the corresponding gene might be helpful for survival under stress conditions. Therefore, we tested if inactivation of *yfiC* gene would cause cell hypersensitivity to a number of different stress conditions, such as high temperature (50°C), hyperosmotic

stress (50% sucrose) (Weber et al. 2006), acetic acid (50 mM at pH 4.5), phosphorus acid (100 mM at pH 1.5) (Blankenhorn et al. 1999, Kannan et al. 2008), and oxidative stress (5 mM hydrogen peroxide) (Almeida et al. 2006). Cells were kept under stress conditions for a period of 2 h, and then the titer of surviving cells was measured by serial dilutions and plating. The conditions were chosen to be at the border of *E. coli* cell survival, and therefore, as expected, all tested conditions led to a drop of several orders of magnitude in the wild-type cell titer (Fig. 4, gray boxes). Absence of *yfiC* gene causes hypersensitivity of *E. coli* to hyperosmotic and, to a lesser extent, oxidative stress (Fig. 4, black boxes). It could also be noted that the presence of modification at A37 decreases cell survival in the presence of acetic acid.

Modifications in tRNA are known to increase stability of its structure (Stuart et al. 2003, Vendeix et al. 2008); however, no decrease in temperature sensitivity was detected with the *ΔyfiC* strain. *E. coli* cells counteract hyperosmotic stress by accumulation of extreme, up to 1 M, concentrations of potassium in the cytoplasm (Dinnbier et al. 1988). At such ionic conditions, subtle advantage of tRNA$_1^{Val}$ modified at m⁶A37 could be essential. Addition of acetic acid to the media, in contrast to addition of phosphorus acid, leads to acidification of internal cytoplasm (Blankenhorn et al. 1999). It might be possible that presence of methyl group at A37 could pose some disadvantage at intracellular pH lower than neutral. Oxidative stress causes the majority of modification mutants to be compromised (P.V. Sergiev, unpubl.), among them the *yfiC* deficiency displays a modest phenotype. The reason for this could be more a stringent requirement for the efficiency of the protein synthesis apparatus, which should replace cellular proteins damaged by oxidation. Increase of the survival of bacteria under such harsh conditions could be one function of the tRNA modifications.

Proteome analysis of the *yfiC* knockout strain

Valine codon distribution among variously expressed genes of *E. coli* was analyzed (Fig. 5A,B): While GUA codons are equally used in highly expressed genes and total proteins (Fig. 5A), GUG codons are underrepresented in highly expressed genes (Karlin et al. 1998, 2001) (Fig. 5B). Obviously, the GUA, but not the GUG, codon forms perfect Watson–Crick base-pairing with the cmo⁵UAC anticodon. To test the significance of A37 methylation for maintaining the correct balance of protein expression in the cell, we utilized a proteomic approach. Total proteins
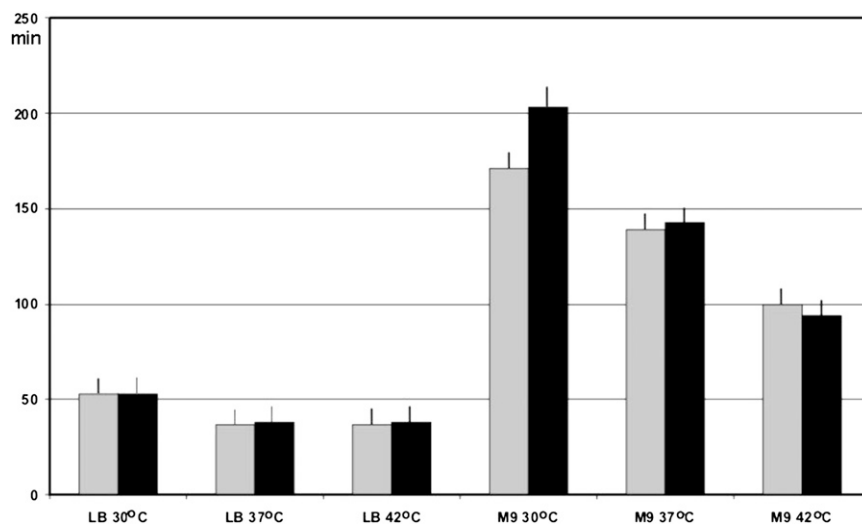

FIGURE 3. Doubling time of the wild-type (gray bars) and *ΔyfiC* (black bars) strains. Growth conditions, such as temperature and medium, are indicated below the bars. Error bars indicate SD borders.
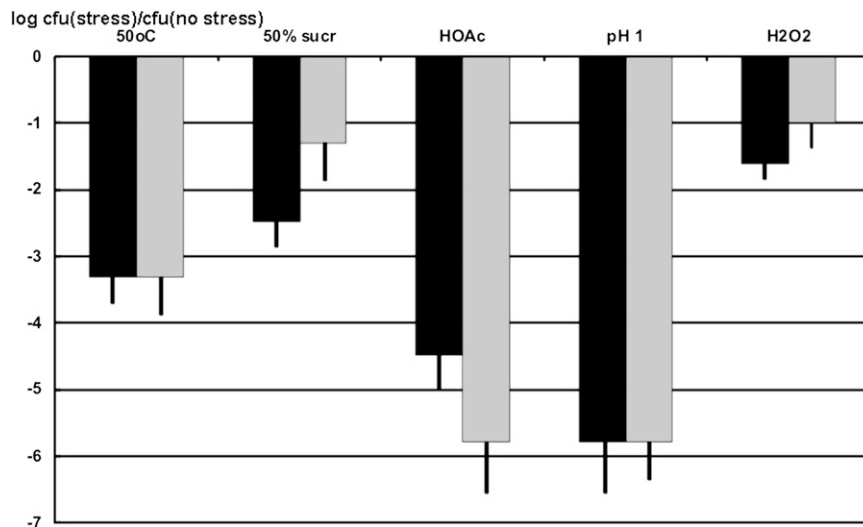
FIGURE 4. Cell titer drop (cfu indicates colony forming units) in the logarithmic scale after exposure of the culture to various stress conditions. Stress type is indicated above the bars. Gray bars correspond to the wild-type strain; black bars correspond to the $\Delta yfiC$ strain. Error bars indicate SD borders.

prepared from the $\Delta yfiC$ strain were compared with those from the parental wild-type strain by two-dimensional protein gel electrophoresis (Fig. 5C). However, no significant differences in protein expression profile were observed.

DISCUSSION

tRNAs are particularly enriched in modified nucleotides (Auffinger and Westhof 1998). They play a role in stabilization of tRNA structure (Stuart et al. 2003, Vendeix et al. 2008) and enhancement of the correct codon–anticodon duplex formation (Konevega et al. 2004, Kothe and Rodnina 2007). Nucleotide 37 is located next to the codon–anticodon duplex, stacking over the base pair between the 5′-nucleotide of a codon and the 3′-nucleotide of anticodon (Weixlbaumer et al. 2007). This position in tRNA is represented exclusively by purines, in particular by modified purines. In *E. coli*, m^1G is located at position 37 of $tRNA_2^{Arg}$ and all species of $tRNA^{Pro}$, while an unknown derivative of guanosine is located at this position in $tRNA_{1-3}^{Leu}$. All other tRNAs contain either unmodified adenosine ($tRNA_{1,2}^{Ala}$, $tRNA_{1-3}^{Gly}$, $tRNA_4^{Ser}$, $tRNA_{1,2}^{Thr}$, $tRNA_{2,3}^{Val}$, and $tRNA_f^{Met}$) or modified adenosine residues. Methylation of the C2 atom of A37 is found for $tRNA_1^{Arg}$, $tRNA_1^{Asp}$, $tRNA_{1,2}^{Gln}$, $tRNA_1^{Glu}$, and $tRNA_1^{His}$. N^6-

threonylcarbamoyladenosine occupies position 37 of $tRNA_{3,4}^{Arg}$, $tRNA_1^{Asn}$, $tRNA_{1-3}^{Ile}$, $tRNA_1^{Lys}$, $tRNA_1^{Met}$, and $tRNA_3^{Ser}$, while N^6-threonylcarbamoyl, N^6-methyl adenosine is located in $tRNA_3^{Thr}$. N^6-isopentenyl adenosine could be found exclusively in $tRNA^{Sec}$, while the more complex 2-methylthio, N^6-isopentenyl adenosine is located in $tRNA_{1,4,5}^{Cys}$, $tRNA_1^{Leu}$, $tRNA_1^{Phe}$, $tRNA_{1,2}^{Ser}$, $tRNA_1^{Trp}$, and $tRNA_{1,2}^{Tyr}$. The monomethylation of the N6 position is found exclusively at nucleotide 37 of $tRNA_1^{Val}$ (cmo^5UAC) (Dunin-Horkawicz et al. 2006).

$tRNA_1^{Val}$ (cmo^5UAC) belongs to a group of tRNA species carrying a uridine 5-oxyacetic acid residue at the 5′-position of the anticodon. These tRNA species are characteristic for reading four-codon families and can pair with all nucleotides at the wobble position of a codon (Nasvall et al. 2004; Kothe and Rodnina 2007). While additional tRNA species, specific for reading of pyrimidines at wobble position, are usually present, their function can be assumed by cmo^5U-containing tRNAs.
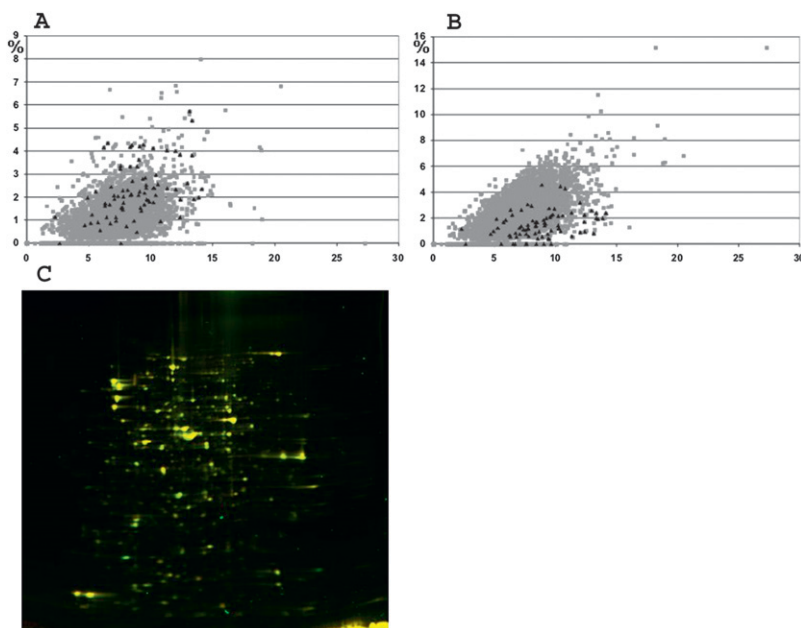

FIGURE 5. Highly expressed gene codon bias for $tRNA_1^{Val}$ GUA (*A*) and GUG (*B*) codons. Plotted is the particular codon frequency (in percent relative to total codons) against total number of valine codons (in percent relative to total codons). Gray boxes correspond to each of *E. coli* genes. Black triangles correspond to highly expressed genes (Karlin et al. 1998, 2001). (*C*) Two-dimensional protein gel, showing a comparison of protein composition of wild-type strain (labeled red) and $\Delta yfiC$ strain (labeled green). Yellow color corresponds to equal amount of protein in both strains.

Evidence for in vivo and in vitro translation of the codons ending with pyrimidines by cmo^{5}U-containing tRNAs was previously reported by the Björk (Nasvall et al. 2004), Agris et al. (2007) and Rodnina (Kothe and Rodnina 2007) laboratories. Although modification of U is dispensable for reading of 3′-adenine codons, it is required for reading codons ending with a pyrimidine (Nasvall et al. 2004). The structural basis for this phenomenon was studied by Ramakrishnan and co-workers (Weixlbaumer et al. 2007), revealing that the modification allows tautomerization of the uridine derivative to an enol form, enabling more efficient pairing with guanosine. The unusual geometry of cmo^{5}U-U and cmo^{5}U-C pairs is also facilitated by 5-oxyacetylation.

While modification of U in tRNA$_1^{Val}$ (cmo^{5}UAC) is important for decoding of some codons, modification of A37 is less so. However, it is known that like other modified bases at position 37, m^{6}A stacks over the codon–anticodon duplex (Weixlbaumer et al. 2007), facilitating codon–anticodon interaction. Specifically, the methyl group causes distortion in the structure of the anticodon loop, preventing intraloop pairing (Vendeix et al. 2008). However, the exact function of the methylated adenosine at position 37 of tRNA$_1^{Val}$(cmo^{5}UAC) in the cell remained unknown. It is also curious that this particular modification is specific for only one tRNA. To test the importance of this modification, it is necessary to identify the enzyme responsible for the modification.

In our study we have identified *yfiC* gene as encoding the methyltransferase responsible for A37 methylation of tRNA$_1^{Val}$. YfiC is a 27-kDa protein, classified as a putative methyltransferase. It contains the conserved N-methyltransferase motifs (Jeltsch 2002) ^{50}DxGxGxG^{56} and ^{122}NPPY^{125}. The protein belongs to the COG4123 (Altschul et al. 1997), which has representatives among several phylum of bacteria, such as *Aquificae*, *Thermotogae*, *Fusobacteria*, *Firmicutes*, and *Proteobacteria*, exemplified by *E. coli*. YfiC has no orthologs in archaea and eukarya. Phylogenetic distribution of the *yfiC* gene is in agreement with the distribution of tRNA^{Val} species, carrying m^{6}A at position 37. The nonuniversal distribution of this particular modification goes in concert with nonessential nature of this modification in *E. coli*.

One possible function for the A37 methylation is that it is necessary for maintaining a balance in terms of the translational decoding efficiencies of GUA and GUG codons. Genes that have different expression levels (Karlin et al. 2001) also display a different preference for the codons (Karlin et al. 1998) recognized by tRNA$_1^{Val}$(cmo^{5}UAC) (Fig. 5A,B), such that highly expressed genes are biased against GUG codons (Fig. 5B). Thus, balancing the intracellular protein levels might be dependent on fine-tuning the decoding efficiency. However, at least under the conditions tested here, we find no evidence for any difference in *E. coli* proteome (Fig. 5C) caused by loss of A37 methylation.

It became a common point to speculate that nonessential modifications in tRNA could increase tRNA stability and favor uniform codon–anticodon recognition, which could be essential at extreme conditions. We have determined how inactivation of the *yfiC* gene affects cell survival under conditions of heat, osmotic, acidic, and oxidative stress. It turned out that methylation of tRNA^{Val} at A37 enhances cell survival during osmotic and oxidative stress. Increasing osmolarity should decrease the value of ionic interactions and increase the value of hydrophobic interactions. Thus, necessity for A37 methylation at high osmotic conditions could be explained by hydrophobic interactions of the methyl group that shields the codon–anticodon minihelix from the solution. The dependence of cell survival on m^{6}A37 formation during oxidative stress is harder to explain. One possible explanation might be the necessity to increase the overall efficiency of protein synthesis, which would be necessary for replacement of damaged protein following oxidative stress. A role for which the nonessential tRNA$_1^{Val}$ modification m^{6}A37 plays in cell fitness under extreme conditions, must encourage a search for the functional value of other nonessential RNA modifications in processes of cell survival, adaptation, and regulation.

MATERIALS AND METHODS

Strains and plasmids

BW25141 parental strain (Datsenko and Wanner 2000) and JW2559 strain, carrying *kanR* cassette, inserted at the place of *yfiC* gene (Baba et al. 2006), as well as pCA24*yfiC* plasmid (Kitagawa et al. 2005) were kindly provided by Dr. H. Mori (Keio University).

Protein preparation

Recombinant N-terminal hexahistidine tagged YfiC protein was prepared from AG1 *E. coli* cells, harboring pCA24N plasmid with *yfiC* gene cloned under control of T5lac promoter (Kitagawa et al. 2005). Cells were grown in LB media at 37°C until A$_{600}$ 0.5 and induced by IPTG, 0.5 mM final concentration. After induction, cells were grown for an additional 3 h and lysed by sonication in a buffer of 20 mM Hepes K at pH 7.5, 1 mM Mg(OAc)$_2$, and 200 mM NH$_4$OAc. Protein purification on Ni-NTA agarose (Qiagen) was done in the same buffer, containing 10 mM imidazole. After washing three times with the same buffer containing 30 mM imidazole, the protein was eluted by increasing imidazol concentration up to 200 mM. Eluted YfiC protein was dialyzed, and its purity was proven by SDS-PAGE.

Substrate preparation

To prepare YfiC putative substrates, wild-type cells and cells of JW2559 strain were grown to A$_{600}$ 0.8 in LB media at 37°C. Cells were harvested by centrifugation and lysed by grinding with 2× cell mass of aluminum oxide at 4°C. Cell debris and aluminum oxide was pelleted by 30-min centrifugation at 16000 rpm in JA20 rotor. Lysates were subjected to 18-h

ultracentrifugation in Ti70 rotor at 35000 rpm to pellet ribosomes. Ribosomes were resuspended in 20 mM Hepes K (pH 7.5), 1 mM $Mg(OAc)_2$, 200 mM NH_4OAc, and 4 mM DTT buffer. Ribosomes and S100 were used to prepare rRNA and total tRNA by phenol extraction in buffer 300 mM NaOAc, 0.5% SDS, and 5 mM EDTA, followed by ethanol precipitation.

In vitro methylation

For in vitro methylation, 100 pmol of ribosomes, rRNA, or 2 A600 units of S100 or total tRNA were mixed with 2 mkCi S-adenosyl-L-[methyl-^{3}H]methionine (GE Healthcare) and 10 pmol of recombinant YfiC protein in 200 μL volume. The ionic conditions for in vitro methylation were kept to 20 mM Hepes K (pH 7.5), 1 mM $Mg(OAc)_2$, 200 mM NH_4OAc, and 4 mM DTT. After 30-min incubation at 37°C, reaction was quenched by equal volume of 300 mM NaOAc, 0.5% SDS, and 5 mM EDTA, and RNA was extracted by phenol. After ethanol precipitation, the pellet was washed five times vigorously with 70% ethanol to remove residual S-adenosyl-L-[methyl-^{3}H]methionine. Radioactivity incorporated into RNA was measured by scintillation counting.

Bulk tRNA, prepared from the wild type, JW2559, JW2559 transformed with pCA24*YfiC*, and those modified with YfiC in vitro was used to affinity purify $tRNA_5^{Leu}$, $tRNA_3^{Thr}$, $tRNA_1^{Glu}$, $tRNA_3^{Arg}$, $tRNA_1^{Val}$, and $tRNA_2^{Val}$. Purification was done by a procedure, resembling one published by Miyauchi et al. (2007). Biotinylated oligonucleotides, complementary to the most divergent part of T and acceptor stems, were annealed to samples of total tRNA by heating to 75°C and slow cooling to 37°C. We used following primers:

BIO-GGGTGATGACGGGAT for $tRNA_1^{Val}$;
BIO-GCGTCCGAGTGGACT for $tRNA_2^{Val}$;
BIO-GCTGATAGGCAGATT for $tRNA_3^{Thr}$;
BIO-GCCGAAGGCCGGACT for $tRNA_5^{Leu}$;
BIO-GTCCCCTAGGGGATT for $tRNA_1^{Glu}$; and
BIO-GCGCCCTGCAGGATT for $tRNA_3^{Arg}$.

After annealing, hybridized tRNA species were separated by streptavidin resin and washed 10 times with the buffer of 20 mM Hepes K (pH 7.5), 1 mM $Mg(OAc)_2$, 200 mM NH_4OAc, and 4 mM DTT. For scintillation counting, the resin was resuspended in scintillation cocktail without tRNA elution.

Genes coding for tRNA species were amplified from the total DNA of *E. coli* strain BW25141 using the following primers:

VAL1_5 TAATACGACTCACTATAGGGGTGATTAGCTCAGCTG;
VAL1_3 T_mG_mGTGGGTGATGACGGGA;
VAL2_5 TAATACGACTCACTATAGGCGTCCGTAGCTCAGTTGG;
VAL2_3 T_mG_mGTGCGTCCGAGTGGAC;
THR3_5 TAATACGACTCACTATAGGCTGATATAGCTCAGTTGG;
THR3_3 T_mG_mGTGCTGATAGGCAGATTC;
LEU5_5 TAATACGACTCACTATAGGCCGAAGTGGCGAAATCG; and
LEU5_3 T_mG_mGTGCCGAAGGCCGGAC.

PCR products were agarose gel-purified and used for in vitro transcription by T7 RNA polymerase. Transcription products were purified by electrophoresis in 10% polyacrylamide gel.

For MALDI MS analysis, tRNA was eluted by heating to 75°C in 6 M Urea, purified further by electrophoresis in denaturing 10% acrylamide gel, eluted from the gel, and dissolved in 50 mM ammonium citrate buffer. RNase T1 digestion and MALDI MS analysis were carried out as described for YbiN methyltransferase (Sergiev et al. 2008).

Examination of cell growth and survival characteristics

Growth rate measurements and growth competition experiments were carried as was previously described for YcbY (Lesnyak et al. 2006) and YgjO (Sergiev et al. 2006) methyltransferases. Survival of wild-type and JW2559 cells at stress conditions was checked by growing cell in LBK media (1% bacto tryptone, 0.5% yeast extract, 0.64% KCl) to A_{600} 0.5 followed by application of a stress factor. A sample of the prestressed culture was used to determine initial cell titer. For heat-shock, the cultures were transferred to a 50°C water bath and incubated for 2 h. Hyperosmotic shock was introduced by addition of equal weight of sucrose. Acetic or phosphorus acid stress was made by addition of 1/10 a volume of 0.5 M KOAc-HOAc buffer (pH 4.5) or 1 M KH_2PO_4-H_3PO_4 buffer (pH 1.5). Oxidative stress was applied by addition of 1/10 of a volume of 50 mM hydrogen peroxide. In all cases, cells were incubated at a particular stress condition for 2 h in a cell culture shaker at 37°C. Following that, the survived cell titer was measured by serial dilutions and plating. A drop in cell titer was calculated, relative to cell titer before the stress.

Proteomics

Two-dimensional gel electrophoresis of total proteins was done as follows. Cell pellets were disrupted by sonication for 5 min at 4°C in ice water and centrifuged (10 min at 15,000*g*) to discard insoluble particles. Samples (10 μL) were dissolved in buffer: 8 M urea, 2 M thiourea, 4% CHAPS, and 30 mM TrisHCl (pH 8.5). Protein concentration was determined using Quick start Bradford dye reagent (Bio-Rad). In 50 μg of the sample, proteins was labeled with 400 pmol of either Cy3 or Cy5 CyDye DIGE Fluor minimal dyes (Amersham Biosciences) according to the manufacturer's instruction. Since protein equalization, Cy3 and Cy5 labeled samples were mixed, and 100 mM DTT (Bio-Rad) and 2% (v/v) Ampholine 3-10 (Bio-Rad) was added. IEF was performed using tube gels (20 cm × 1.5 mm) containing carrier ampholytes and applying a voltage gradient in an IEF-chamber produced in house (Dansenko and Wanner 2000). After IEF, the ejected tube gels were incubated in equilibration buffer (125 mM TrisHCl, 40% [w/v] glycerol, 3% [w/v] SDS, 65 mM DTT at pH 6.8) for 10 min. The tube gels were placed onto the polyacrylamide gels (9%–16%) of 1.5-mm thickness, 20 × 18 cm (Protean II Multi-Cell, Bio-Rad) and fixed using 1.0% (w/v) agarose containing 0.01% (w/v) bromphenol blue. The electrophoresis was carried out for 12–14 h, and then the gels were scanned using a Typhoon Trio Imager at 200-dpi resolution (Amersham Biosciences). Just afterward, the gels were fixed and silver stained according to the method described by Shevchenko et al. (1996). Image analysis was performed using PDQest 8.0 software (Bio-Rad). Two independent experiments were performed for each experimental setup. Spot quantities were normalized to remove nonexpression-related variations in spot intensity. The criterion of differential expression of a certain protein between the two subsets was posed as at least a twofold change in spot optical density between the two matched sets in duplicates.

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