The RNA acetyltransferase driven by ATP hydrolysis synthesizes \( N^4 \)-acetylcytidine of tRNA anticodon

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The wobble base of *Escherichia coli* elongator tRNA\(^{\text{Met}}\) is modified to \( N^4 \)-acetylcytidine (ac\( ^4 \)C), which is thought to ensure the precise recognition of the AUG codon by preventing misreading of near-cognate AUA codon. By employing genome-wide screen of uncharacterized genes in *Escherichia coli* (‘ribonucleome analysis’), we found the ypfi gene, which we named *tmcA* (tRNA\(^{\text{Met}}\) cytidine acetyltransferase), to be responsible for ac\( ^4 \)C formation. TmcA is an enzyme that contains a Walker-type ATPase domain in its N-terminal region and an N-acetyltransferase domain in its C-terminal region. Recombinant TmcA specifically acetylated the wobble base of *E. coli* elongator tRNA\(^{\text{Met}}\) by utilizing acetyl-coenzyme A (CoA) and ATP (or GTP). ATP/GTP hydrolysis by TmcA is stimulated in the presence of acetyl-CoA and tRNA\(^{\text{Met}}\). A mutant study revealed that *E. coli* TmcA strictly discriminates elongator tRNA\(^{\text{Met}}\) from the structurally similar tRNA\(^{\text{Ile}}\) by mainly recognizing the C27–G43 pair in the anticodon stem. Our findings reveal an elaborate mechanism embedded in RNA molecules that is involved in the recognition of wobble bases by the respective RNA-modifying enzymes.

**Subject Categories:** RNA

**Keywords:** \( N^4 \)-acetylcytidine (ac\( ^4 \)C); RNA acetyltransferase; TmcA; tRNA; wobble modification

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**Introduction**

RNA molecules are decorated by various post-transcriptional modifications. To date, more than 100 species of modified nucleosides have been identified in RNA molecules from all domains of life (Rozenski et al., 1999; Grosjean, 2005; Dunin-Horkawicz et al., 2006). The majority of these RNA modifications were identified and characterized in tRNA molecules. In particular, RNA modifications at the first (wobble) position of the tRNA anticodon participate in the precise decoding of the genetic code that is mediated by the codon–anticodon interaction (Bjork, 1995; Yokoyama and Nishimura, 1995; Curran, 1998; Suzuki, 2005).

\( N^4 \)-acetylcytidine (ac\( ^4 \)C) (Figure 1A) is a modified nucleoside that was identified at position 34 (the wobble position) of *Escherichia coli* elongator tRNA\(^{\text{Met}}\) in 1972 (Oashi et al., 1972). It is known that ac\( ^4 \)C is widely present in a variety of tRNAs and rRNAs; it is present at the wobble position of bacterial tRNA\(^{\text{Met}}\) and archaeal tRNAs (Gupta, 1984; Sprinzl and Vassilenko, 2005) and is found only at position 12 in euksaryotic tRNAs (Sprinzl and Vassilenko, 2005). In tRNAs, ac\( ^4 \)C was found in 5S rRNA from *Pyrodictium occultum* (Bruenger et al., 1993), in the 3′-terminal helix of 18S rRNAs from *Dictyostelium discoideum* (McCarroll et al., 1983) and rat liver (Thomas et al., 1978). It is known that ac\( ^4 \)C favours the C3′-endo form of its ribose puckering (Kawai et al., 1989), conferring stable codon–anticodon pairing at the wobble position of bacterial tRNA\(^{\text{Met}}\). In fact, a biochemical study using *in vitro* protein synthesis indicated that ac\( ^4 \)C prevents misreading of the AUA codon by *E. coli* tRNA\(^{\text{Met}}\) (Stern and Schulman, 1978). However, the biogenesis and functions of ac\( ^4 \)C in the cell are not fully understood. In *Saccharomyces cerevisiae*, TAN1 was identified as a protein that is required for ac\( ^4 \)C formation at position 12 of tRNA\(^{\text{Ser}}\) (CGA) (Johansson and Byström, 2004). Although TAN1 contains the THUMP domain that presumably binds to tRNA, TAN1 seems to lack a catalytic domain for ac\( ^4 \)C formation. This indicates the requirement of an unknown partner enzyme for this reaction. Thus, it remains unknown what the acetyl donor is and how the enzyme catalyses the acetylation of RNAs.

In the bacterial decoding system for AUR (R = A or G) codons, there are two structurally similar tRNAs with the CAU anticodon: tRNA\(^{\text{Leu}}\) for the AUA codon and the elongator tRNA\(^{\text{Met}}\) for the AUG codon (Figure 1B). Both tRNAs bear specific wobble modifications. The AUA codon-specific tRNA\(^{\text{Leu}}\) contains lysidine (L, k\( ^2 \)C) at the first letter of the anticodon, whereas elongator tRNA\(^{\text{Met}}\) has ac\( ^4 \)C at the same position. It is known that L is an essential modification that determines both the codon and amino-acid specificities of tRNA\(^{\text{Leu}}\) (Muramatsu et al., 1988; Soma et al., 2003). Hence, the wobble modification of each tRNA strictly governs its identity and decoding accuracy. Each of these tRNAs has identity elements embedded in its sequence and tertiary structure that are recognized by an RNA-modifying enzyme for the wobble position and by a cognate aminoaacyl-tRNA synthetase (Ikeuchi et al., 2005). For lysidine formation in tRNA\(^{\text{Leu}}\), we previously identified the tRNA\(^{\text{Leu}}\)-lysidine synthetase (tlS)
that strictly discriminates tRNA\textsuperscript{lie} from tRNA\textsuperscript{Met} by recognizing two consecutive base pairs in the acceptor stem (Soma et al, 2003; Ikeuchi et al, 2005). If ac\textsuperscript{C} is accidentally introduced at the wobble position of tRNA\textsuperscript{lie}, tRNA\textsuperscript{lie} loses its isoacceptancy and AUA-decoding abilities. Thus, it was speculated that tRNA\textsuperscript{lie} also has another set of determinants negatively recognized by a putative enzyme responsible for the ac\textsuperscript{C} formation that occurs in elongator tRNA\textsuperscript{Met}.

To identify genes responsible for RNA modifications from uncharacterized genes, we have frequently employed a reverse genetic approach combined with mass spectrometry (‘ribonucleome’ analysis) (Suzuki, 2005; Ikeuchi et al, 2006; Noma and Suzuki, 2006; Noma et al, 2006; Suzuki et al, 2007). This analysis utilizes a series of gene-deletion strains of \textit{E. coli} or \textit{S. cerevisiae}. The total RNA extracted from each strain is analysed by liquid chromatography/mass spectrometry (LC/MS) to determine whether a particular gene deletion leads to the absence of a specific modified base, thereby permitting us to identify the enzyme or protein responsible for this modification. In the case of essential genes, we analyse either temperature-sensitive mutants cultured at the non-permissive temperature or expression-controlled strains. This ribonucleome analysis enables us to identify not only enzyme genes directly responsible for RNA modifications, but also genes that encode non-enzymatic proteins necessary for the biosynthesis of RNA modifications. These include carriers of the metabolic substrates used for RNA modifications and partner proteins needed for RNA recognition. In fact, using this approach, we previously identified \textit{ttsS}, an essential gene for lysidine formation (Soma et al, 2003), \textit{tusA-E} for 2-thiouridine formation (Ikeuchi et al, 2006) and TYW1-4 for wybutosine synthesis (Noma et al, 2006).

Here, we used ribonucleome analysis to identify and characterize a gene, which we named \textit{tmcA} (tRNA\textsuperscript{Met} cytidine acetyltransferase), responsible for ac\textsuperscript{C} formation in the \textit{E. coli} elongator tRNA\textsuperscript{Met}. Biochemical analyses revealed mechanistic insights into ac\textsuperscript{C} formation and how TmcA discriminates elongator tRNA\textsuperscript{Met} from the structurally similar tRNA\textsuperscript{Met}.

**Results**

**Ribonucleome analysis identified the \textit{ypfI} gene to be required for ac\textsuperscript{C} formation**

To identify a gene responsible for ac\textsuperscript{C} formation in \textit{E. coli}, we employed a genome-wide screen of a series of knockout strains using the ribonucleome analysis (Suzuki, 2005; Ikeuchi et al, 2006; Suzuki et al, 2007). If the strain contains a deleted gene encoding an enzyme or protein involved in RNA modification, the absence of a specific modified nucleoside can be identified by LC/MS analysis. Initially, we analysed 130 genomic-deletion strains covering ~50% of \textit{E. coli} genes, each of which lacked about 20 kbps (~20 genes) (Baba et al, 2006). In the analysis, we found a strain OCL58 that specifically lacks ac\textsuperscript{C} (data not shown). The deleted genomic region of OCL58 spans \textit{ypfI} to \textit{yfgD} (55.84–56.38 min) and contains 24 genes.

To narrow down the target gene, we employed a computational domain search by Pfam (Finn et al, 2006) to characterize these candidates. As a result, the \textit{ypfI} gene was found to have an N-acetyltransferase domain (Acetyltransf\_1) belonging to the GCN5-related histone acetyltransferase family (GNAT family). We constructed a single knockout strain of \textit{ypfI} (\textit{ΔypfI}). Nucleosides analysis by LC/MS of the total RNA obtained from the \textit{ΔypfI} strain revealed the specific absence of ac\textsuperscript{C} (Figure 2A), demonstrating that \textit{ypfI} is an essential gene for ac\textsuperscript{C} formation in the cell. Despite the absence of ac\textsuperscript{C}, the \textit{ΔypfI} strain showed a healthy phenotype without any growth defects compared with wild-type cells (data not shown). When the \textit{ΔypfI} strain was co-cultivated with wild-type cells, no difference in the survival rate could be observed (data not shown).

Non-essential modifications are known to have an important function in tRNA stability in the cell (Alexandrov et al, 2006; Chernyakov et al, 2008). We next sought to observe the synthetic phenotype of \textit{ΔypfI} when it was combined with additional deletions of genes responsible for biogenesis of other modified nucleosides in tRNA\textsuperscript{Met}. We chose nine deletion strains, \textit{Δthil}, \textit{ΔdusA}, \textit{ΔdusB}, \textit{ΔdusC}, \textit{ΔtruH}, \textit{ΔtruA}, \textit{ΔtrmB}, \textit{ΔtrmA} and \textit{ΔtruB}, each of whose deletion was transferred to \textit{ΔypfI} by P1 transduction, to construct a series of double-deletion strains. No significant growth phenotype was seen in any of the double-deletion strains when cultured at 37°C. However, when cultured at 24°C, the \textit{ΔypfI}/\textit{ΔdusC} strain showed a severe growth defect compared with each of the single-deletion strains (Figure 2B). The gene \textit{dusC} encodes an enzyme responsible for dihydrouridine formation in the D-loop of tRNAs (Bishop et al, 2002). The \textit{ΔdusC} mutant originally showed a cold-sensitive phenotype, and the additional deletion of \textit{ypfI} enhanced this phenotype.

**Reconstitution of ac\textsuperscript{C} formation in vitro using recombinant TmcA**

We found apparent homologues of \textit{E. coli} YpfI in \textgamma-proteobacteria, including \textit{Salmonella typhimurium}, \textit{Yersinia pestis}, \textit{Haemophilus influenzae}, \textit{Pasteurella multocida} and \textit{Vibrio cholerae} (Figure 3). Sequence alignment of YpfI showed that these proteins shared many conserved regions. The N-terminal region contains the uncharacterized DUF699

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**Figure 1** Chemical structure of N\textsuperscript{4}-acetylcytidine (ac\textsuperscript{C}) and secondary structure of \textit{E. coli} elongator tRNA\textsuperscript{Met}. (A) Chemical structure of ac\textsuperscript{C}. (B) Secondary structure of \textit{E. coli} elongator tRNA\textsuperscript{Met} with modified nucleosides: 4-thiouridine (s\textsubscript{U}), 2′-O-methylguanosine (Gm), dihydrouridine (D), N\textsuperscript{4}-acetylcytidine (ac\textsuperscript{C}), N\textsuperscript{6}-threonylcarbamoyladenosine (\textit{tA}), pseudouridine (Ψ), 7-methylguanosine (m\textsubscript{G}), 3-(3-amino-3-carboxypropyl) uridine (acp\textsubscript{3}U) and 5-methyluridine (m\textsubscript{5}U).
domain (PF05127). DUF699 purportedly functions as a putative ATPase, bearing the highly conserved ATP/GTP-binding motif (P-loop) known as the Walker A motif (AxRGRGKT/S) and the Walker B motif (hhhhDEAA) (Figure 3). The C-terminal Acetyltransf_1 domain (PF00583) is a member of the GNAT family.

The structural characteristics of YpfI prompted us to speculate that acetyl-CoA and ATP are required for ac4C formation. To characterize the E. coli YpfI protein and to reconstitute ac4C formation in vitro, a hexa-histidine-tagged E. coli YpfI was expressed and purified. We then tried to reconstitute ac4C formation at the wobble position of in vitro-transcribed tRNA^Met^ in the presence or absence of acetyl-CoA and ATP. After the reaction, total nucleosides of the tRNA substrate for each reaction were analysed by LC/MS. As shown in Figure 4A and B, ac4C clearly appeared only when the reaction was performed in the presence of both acetyl-CoA and ATP. No ac4C formation occurred under conditions without acetyl-CoA or ATP. This data demonstrates that YpfI is an acetyltransferase responsible for ac4C formation at the wobble position of tRNA^Met^.

We therefore renamed ypfI as tmcA (tRNA^Met^ cytidine acetyltransferase).

Reconstitution of ac4C formation in vitro was further analysed by a filter assay. The substrate tRNA^Met^ was acetylated by the recombinant TmcA in the presence of [1-14C] acetyl-CoA and ATP. As shown in Figure 4C, ATP-dependent acetylation could be confirmed. To quantify the acetylated tRNA on the filter by liquid scintillation counting, we first had to remove the free [1-14C] acetyl-CoA by phenol extraction and ethanol precipitation, due to its high background signal (see Experimental procedures). Therefore, we did not attempt to measure initial velocity of the acetylation, which is necessary to determine the exact kinetic parameters of ac4C formation. The radioactivity of 14C-labelled acetylated tRNAs was visualized on a gel (Figure 4D). In this experiment, TmcA did not acetylate tRNA^Met^ as well as tRNA^Met^ with a C34G mutation. According to the gel-mobility shift experiment (Figure 4E), TmcA specifically interacts with tRNA^Met^ as TmcA did not acetylate tRNA^Met^ as well as tRNA^Met^ with a C34G mutation. These data suggest that TmcA strictly recognizes the wobble base and discriminates tRNA^Met^ from the structurally similar tRNA^Ile^.

We also found that TmcA can utilize GTP in place of ATP for ac4C formation (Figure 4D). It is known that some enzymes bearing a P-loop motif (Walker A motif) utilize GTP as a substrate instead of ATP (Saraste et al., 1990). Therefore, we considered whether GTP is also a natural substrate for TmcA. We employed ADPCP and GDPCP, which are non-hydrolyzable analogues of ATP and GTP, for ac4C formation, and found that these analogues could not produce ac4C (data not shown). This demonstrated that TmcA requires the hydrolysis of ATP or GTP for ac4C formation.

**Kinetic analysis of ATP and GTP hydrolysis by TmcA**

To gain mechanistic insights into ac4C formation driven by ATP/GTP hydrolysis, the ATPase (or GTPase) activity of TmcA was characterized. We employed [α-32P]-labelled ATP (or [α-32P]-labelled GTP) as a substrate to examine and quantify the products released by the ATP/GTP hydrolysis catalysed by TmcA. TmcA hydrolysed ATP or GTP between the β- and γ-phosphates, as shown by the detection of labelled ADP (or GDP) on the polyethylenimine (PEI)-cellulose thin-layer chromatography (Supplementary Figure S1). The kinetic parameters of ATP (or GTP) hydrolysis were measured in the presence or absence of tRNA^Met^ and acetyl-CoA (Table I). Even in the absence of both acetyl-CoA and tRNA^Met^, TmcA intrinsically hydrolysed ATP and GTP, indicating that ATP/GTP hydrolysis by TmcA is an independent reaction from that of ac4C formation by the acetyltransferase.

The K_m value of ATP hydrolysis (2.93 μM) was markedly lower than that of GTP hydrolysis (117 μM) in the absence of both acetyl-CoA and tRNA^Met^. Addition of acetyl-CoA (200 μM) to the reaction stimulated the k_cat value of ATP (2.8-fold) and GTP (2.5-fold) hydrolyses. In the presence of tRNA^Met^ (2.5 μM), the k_cat value of ATP hydrolysis was doubled, whereas no significant change in the k_cat value of GTP hydrolysis was observed. In ATP hydrolysis, the addition of both acetyl-CoA and tRNA^Met^ increased the k_cat value 3.7-fold and slightly affected the K_m value, resulting in a 2.6-fold increase in k_cat/K_m. In GTP hydrolysis, the addition of both substrates decreased the K_m value 0.57-fold and increased the k_cat value 2.1-fold, which resulted in a 3.8-fold increase in k_cat/K_m. The data reveal that ATP/GTP hydrolysis by TmcA is stimulated by the addition of acetyl-CoA and tRNA^Met^.
TmcA recognizes the anticodon stem of tRNA<sub>Met</sub>

In eubacteria, it is difficult to distinguish primary transcripts of elongator tRNA<sup>Met</sup> and tRNA<sup>Ile2</sup> because of their high sequence similarity, and especially because they have identical anticodon loop sequences. To explore elements embedded in tRNA<sup>Met</sup> that are recognized by TmcA, we next employed mutation studies using in vitro-transcribed tRNAs. Various mutants of <i>E. coli</i> elongator tRNA<sup>Met</sup> and tRNA<sup>Ile2</sup> were constructed by in vitro transcription (Figure 5A). The relative activity of ac<sub>4</sub>C formation, which was visualized on a gel by the imaging analyser, was quantified by measuring the radioactivity of the <sup>14</sup>C-labelled acetyl group in each variant at the end point of the reaction.

As most of the differences between elongator tRNA<sup>Met</sup> and tRNA<sup>Ile2</sup> can be seen in their acceptor and anticodon stems, we first swapped each of these stems in the two tRNAs. When their acceptor stems were exchanged, no change in the specificity was observed in either mutant (EM(AAstemI) and EI(AAstemM)) (Figure 5B). However, when their anticodon stems were exchanged, no acetylation was detected in mutant tRNA<sub>Met</sub> bearing the anticodon stem of tRNA<sub>Ile2</sub> (EM(ACstemI)) (Figure 5B). On the other hand, the mutant tRNA<sub>Ile2</sub> bearing the anticodon stem of tRNA<sup>Met</sup> (EI(ACstemM)) acquired acetylation capability (Figure 5B). The only difference in the D arms between tRNA<sup>Met</sup> and tRNA<sup>Ile2</sup> is the absence of a uridine at position 16 of tRNA<sup>Ile2</sup>. However, U<sub>16</sub> deletion of tRNA<sup>Met</sup> (EM(delU16)) did not affect ac<sub>4</sub>C formation (Figure 5C). Next, we replaced the T arm of tRNA<sup>Met</sup> with that of tRNA<sup>Ile2</sup> (EM(TarmI)), but no significant change in ac<sub>4</sub>C formation was observed (Figure 5C). These results reveal that TmcA specifically recognizes the anticodon stem of tRNA<sup>Met</sup>.

To identify which bases in the anticodon stem are important for ac<sub>4</sub>C formation, base pairs in the anticodon stems of these tRNAs were interchanged. When the top pair (C27-G43) of tRNA<sup>Met</sup> was replaced with G27-U43 of tRNA<sup>Ile2</sup>, the mutant tRNA<sup>Met</sup> (EM(G27U43)) was not acetylated (Figure 5C), suggesting that the C27-G43 pair of tRNA<sup>Met</sup> is critical for ac<sub>4</sub>C formation. However, as mutant tRNA<sup>Ile2</sup> bearing C27-G43 (EI(C27G43)) was not acetylated (Figure 5C), C27-G43 is not sufficient for ac<sub>4</sub>C formation on tRNA<sup>Ile2</sup>. Swapping the second base pair (EM(G28C42) and EI(A28U42)) and the third base pair (EM(C29G41) and EI(U29A41)) in the anticodon stem did not influence acetylation (Figure 5C). When C30-G40 of tRNA<sup>Met</sup> was replaced by G30-C40 of tRNA<sup>Ile2</sup>, we saw a considerable reduction in ac<sub>4</sub>C formation (EM(G30C40)) (Figure 5C). In addition, when G44 of tRNA<sup>Met</sup> was replaced by U44 of tRNA<sup>Ile2</sup>, the mutant tRNA<sup>Met</sup> (EM(U44)) showed a slight reduction in acetylation. However, neither C30-G40 nor G44 of tRNA<sup>Met</sup> acted as positive determinants for ac<sub>4</sub>C formation on tRNA<sup>Ile2</sup> (EI(C30-G40) and EI(G44)) (Figure 5C).

We next constructed tRNA<sup>Ile2</sup> mutants bearing the two positive elements for ac<sub>4</sub>C formation, to determine the minimum necessary elements for the conversion of the specificity of the wobble modification from lysidine to ac<sub>4</sub>C. When C27-G43 and C30-G40 were introduced into tRNA<sup>Ile2</sup> simultaneously, the mutant tRNA<sup>Ile2</sup> (EI(C27G43, C30G40)) was acetylated (Figure 5C). In addition, introduction of C27-G43 and G44 into tRNA<sup>Ile2</sup> (EI(C27G43, G44)) also conferred a specificity for TmcA (Figure 5C). However, when C30-G40 and G44 were simultaneously introduced into tRNA<sup>Ile2</sup> (EI(C30G40, G44)), no ac<sub>4</sub>C formation was observed (Figure 5C). Introduction of C27-G43 is a critical, but
insufficient, element for ac$^4$C formation on tRNA$^{\text{Ile}}$. Additional introduction of C30-G40 or G44 with the C27-G43 mutation is therefore required for the acetylation of tRNA$^{\text{Ile}}$. Finally, when these three elements were introduced simultaneously (EI(C27G43, C30G40, G44)), full acetylation was observed (Figure 5C).

Discussion

Here, we successfully identified the RNA acetyltransferase (TmcA) responsible for ac$^4$C formation at the wobble position of tRNA$^{\text{Met}}$. Despite the purported function of ac$^4$C in preventing misreading of the AUA codon, at least in in vitro translation (Stern and Schulman, 1978), a healthy phenotype for the $\Delta$tmcA strain was unexpectedly observed. Thus, ac$^4$C is a dispensable modification of tRNA$^{\text{Met}}$, at least in the presence of the AUA codon-specific tRNA$^{\text{Ile}}$. The only phenotype we observed in this study was a cold-sensitive phenotype for the double-deletion strain ($\Delta$tmcA/ADusC) (Figure 2B). We have no plausible explanation why the lack of ac$^4$C in tRNA$^{\text{Met}}$ caused a growth defect at low temperatures in the absence of dihydrouridine. In eukaryotic tRNAs, it is known that non-essential modifications are required for tRNA stability in the cell (Alexandrov et al, 2006).
In addition, recent studies have revealed that RNA metabolism is involved in the rapid degradation of hypomodified tRNAs (Chernyakov et al, 2008; Wang et al, 2008). Further study is necessary to reveal whether a mechanism similar to euukaryotic tRNA metabolism is involved in the E. coli system. It is also possible to speculate that a functional defect of the hypomodified tRNA\textsuperscript{Met} in the double-deletion strain causes a functional defect in protein synthesis at low temperature. On the other hand, in some pathogenic microorganisms, it is known that RNA modifications have an important function in virulence expression (Durand et al, 1994, 1997; Takano et al, 2006). From the viewpoint of virulence in γ-proteobacteria, the cellular function of ac\textsuperscript{4}C remains to be investigated.

TmcA belongs to COG1444 in the Clusters of Orthologous Groups gene database (Tatusov et al, 2001). Although homologues of TmcA occur in many Archaea and Eukarya, in bacteria the gene appears to be limited to the γ-proteobacterial subphylum. Consistently, ac\textsuperscript{4}C was not reported at the wobble position in other sequenced tRNAs\textsuperscript{Met} from Bacillus subtilis, Mycoplasma capricolum or Thermus thermophilus, which are not γ-proteobacteria (Sprinzl and Vassilenko, 2005). The limited distribution of TmcA and ac\textsuperscript{4}C at the wobble position of tRNA\textsuperscript{Met} in γ-proteobacteria could be caused by the loss of tmcA in other bacteria. Otherwise, tmcA might have arose in a common ancestor of γ-proteobacteria by horizontal gene transfer from other domains of life. Consistent with this, tmcA homologues are widely distributed in archaea and euukaryotic species (KOG2036 in eukaryotic tRNA\textsuperscript{met} database). It is known that ac\textsuperscript{4}C occurs at the wobble positions of several tRNAs in archaea (Gupta, 1984), and at position 12 in a subset of tRNAs in eukaryotes (Sprinzl and Vassilenko, 2005). In addition, it has been reported that 18S rRNA contains ac\textsuperscript{4}C in its 3\textsuperscript{'}-terminal region in some euukaryotes (Thomas et al, 1978; McCarroll et al, 1983). Further studies should reveal whether euukaryotic homologues of tmcA are actually involved in ac\textsuperscript{4}C formation on tRNA or rRNA.

TmcA contains an ATPase domain in its N-terminal region and an N-acetyltransferase domain related to the histone acetyltransferase family in its C-terminal region. In vitro reconstitution of ac\textsuperscript{4}C formation revealed that TmcA specifically acetylates C34 of elongator tRNA\textsuperscript{Met} by using acetyl-CoA as an acetyl donor, and this reaction requires ATP/GTP hydrolysis. Unlike histone acetyltransferases, TmcA was found to be an energy-consuming acetyltransferase. ATP hydrolysis had a higher \(k_{\text{cat}}/K_m\) value \((7.10 \times 10^{-3} \text{s}^{-1} \text{M}^{-1})\) than that of GTP hydrolysis \((8.07 \times 10^{-3} \text{s}^{-1} \text{M}^{-1})\) (Table I). Considering the cellular concentration of ATP (3 mM) and GTP (0.9 mM) in E. coli (Bochner and Ames, 1982) and the \(K_m\) values of ATP/GTP hydrolysis by TmcA, ATP would be a more favourable substrate for TmcA than GTP under physiological conditions. According to our kinetic study of ATP/GTP hydrolysis, we observed that TmcA hydrolyses ATP/GTP in the absence of acetyl-CoA and tRNA\textsuperscript{Met}, indicating that TmcA can hydrolyse ATP/GTP independently of its RNA acetylation activity. In the presence of acetyl-CoA or tRNA\textsuperscript{Met}, the \(k_{\text{cat}}\) value of ATP hydrolysis increased, suggesting a functional interplay between ATPase and GNAT domains in TmcA. The mechanisms of ATP hydrolysis-driven ac\textsuperscript{4}C formation needs to be elucidated, although it can be speculated that some conformational change of TmcA induced by the binding of acetyl-CoA or tRNA\textsuperscript{Met} might stimulate its ATPase activity.

There are other cases where wobble modifications require ATP hydrolysis for their biogenesis. TilS (Soma et al, 2003; Ikeuchi et al, 2005) and MnmA (Ikeuchi et al, 2006; Numata et al, 2006) are RNA-modifying enzymes responsible for the formation of lysidine (L) and 2-thiouridine \((\text{s}2\text{U})\), respectively. Both enzymes share a common N-type ATP pyrophosphatase catalytic domain (Rizzi et al, 1996; Tesmer et al, 1996; Lemke and Howell, 2001) that contains a different type of P-loop motif \((\text{SSG} \times \text{DS})\). TilS and MnmA recognize tRNAs and activate the C2 position of the pyrimidine base at position 34 by forming an adenylated intermediate. For lysidine formation by TilS (Ikeuchi et al, 2005), the ε-amino group of lysine attacks the activated C2 position of C34 to synthesize lysidine by releasing AMP. Similarly, the persulfide sulphur of MnmA replaces AMP to form 2-thiouridine (Ikeuchi et al, 2006; Numata et al, 2006). In both cases, the α-β phosphate bond of AMP is hydrolysed. As TmcA hydrolyses the β-γ phosphate bond of ATP (or GTP), this indicates that the mechanism of ac\textsuperscript{4}C formation utilizing ATP hydrolysis is completely different from the biogenesis of L or s\textsuperscript{2}U.

The Walker-type ATPase domain is widely found in AAA\textsuperscript{+} superfamily proteins, which are molecular chaperones for nucleic acids and proteins (Ogura and Wilkinson, 2001), including various motor proteins, the ABC transporter, ATP-dependent proteases and RNA helicases (White and Lauring, 2007). The ATPase module in TmcA might be required to accommodate C34 in the catalytic centre of the GNAT domain by twisting tRNA\textsuperscript{Met}, and/or for the efficient turnover of the reaction by promoting product release.

In vitro acetylation of tRNA variants revealed that TmcA discriminates tRNA\textsuperscript{Met} from the structurally similar tRNA\textsuperscript{Glu} by recognizing C27-G43, C30-G40 and G44 in the anticodon stem. In fact, when these three elements were introduced into tRNA\textsuperscript{Glu} simultaneously, the mutant tRNA\textsuperscript{Glu} acquired full acetylation capability (Figure 5C). C27-G43 in tRNA\textsuperscript{Met} is the

### Table I: Kinetic parameters for ATP/GTP hydrolysis

| NTP    | tRNA\textsuperscript{Met} (2.5 μM) | Acetyl-CoA (200 μM) | \(K_m\) (μM) | \(k_{\text{cat}}\) (10\textsuperscript{3} s\textsuperscript{-1}) | \(k_{\text{cat}}/K_m\) (10\textsuperscript{3} s\textsuperscript{-1} M\textsuperscript{-1}) |
|--------|-----------------------------------|---------------------|--------------|----------------|----------------|
| ATP    | –                                 | –                   | 2.93         | 8.07           | 2.75           |
|        | –                                 | +                   | 3.20         | 22.2           | 6.95           |
|        | +                                 | –                   | 2.79         | 16.4           | 5.88           |
|        | +                                 | +                   | 4.16         | 29.5           | 7.10           |
| GTP    | –                                 | –                   | 117          | 25.2           | 0.215          |
|        | –                                 | +                   | 88.7         | 63.3           | 0.714          |
|        | +                                 | –                   | 107          | 28.7           | 0.267          |
|        | +                                 | +                   | 66.6         | 53.7           | 0.807          |
most critical positive determinant for ac\(^4\)C formation, and as a mutant tRNA\(^{\text{Met}}\) having a G27·U43 mutation (EM(G27U43)) completely failed to be acetylated (Figure 5C), G27·U43 of tRNA\(^{\text{Ile2}}\) is a negative determinant for ac\(^4\)C formation. We previously reported that TilS positively recognizes two consecutive base pairs (C4·G69 and C5·G68) in the acceptor stem of tRNA\(^{\text{Ile2}}\) for lysidine formation (Ikeuchi et al., 2005). In contrast, the corresponding base pairs (U4·A69 and A5·U68) in tRNA\(^{\text{Met}}\) exert an effect as negative determinants of lysidine formation (Ikeuchi et al., 2005). As shown in Figure 6, two sets of determinants for wobble modifications embedded in both tRNA\(^{\text{Met}}\) and tRNA\(^{\text{Ile2}}\) create mutual and exclusive recognition sites for two RNA-modifying enzymes. Thus, TmcA and TilS successfully utilize limited differences in these similar tRNAs. In particular, TmcA mainly discriminates differences in bases in the anticodon stem, whereas TilS...
recognizes base pairs in the acceptor stem. There are no overlaps in the determinants of these two enzymes. In summary, this study has elucidated an exquisite mechanism in tRNA\textsuperscript{Met} and tRNA\textsuperscript{Ile} for the accurate decoding of AUA/AUG codons on the basis of the recognition of cognate wobble modifications by two RNA-modifying enzymes.

Materials and methods

Strains and media

Series of \textit{E. coli} genomic-deletion strains (OCL/R-series) derived from MG1655\textsuperscript{sp} (MG1655 \textit{rpsL polA12 Zih::Tn10}, each of which lacks about 20 kbp (~20 genes), were kindly provided by Dr Jun-ichi Kato (Hashimoto et al., 2005); OCL58 (MG1655\textsuperscript{sp} \textit{gfpH-yfgD::kan}) specifically lacked ac\textsuperscript{4C}. The \textit{E. coli} K-12 strain BW25113 \textit{(lac}\textsuperscript{I} \textit{rpsL} \textit{rpsB} \textit{hsdZ\textsubscript{+}R\textsubscript{E} hsdR\textsubscript{+}S\textsubscript{+} D\textsubscript{RA}BAD\textsubscript{K\textsubscript{R}} D\textsubscript{RA}BAD\textsubscript{K\textsubscript{R}})} was used for the ‘one-step inactivation of chromosomal genes’ procedure (Datsonen and Wanner, 2000; Baba et al., 2006). To amplify DNA fragments of the chloramphenicol acetyltransferase \textit{gene} \textit{(Cmr)} with 40-nt extensions at both ends homologous to the amplifying DNA fragments of the chloramphenicol acetyltransferase template of Cmr.

\textbf{Construction and purification of recombinant protein}

Oligonucleotides \textit{YpfI-NdeI-F} (5'\texttt{-gagatatacatagggccagctgagcgccgctc} | \texttt{aca-3'}) and \textit{YpfI-XhoI-R} (5'\texttt{-ggtgctcgaggtgaaataattgccattgcgttatg} | \texttt{-3'}) were used to amplify \textit{ypfI} from the \textit{E. coli} genome by PCR. The PCR products were cloned into the \textit{Ndel} and \textit{Xhol} sites of pET21b (Novagen) to generate pET21-TmcA. \textit{E. coli BL21(DE3)} was used as the host for the expression of recombinant TmcA. The C-terminal 6 \times His-tagged TmcA protein was expressed in soluble form by induction with 0.1 mM IPTG. Recombinant TmcA was purified by Ni-charged HiTrap Chelating HP chromatography (GE Healthcare) according to the manufacturer’s instructions. The protein concentration was determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard. The pooled protein was dialysed against a buffer consisting of 50 mM Tris–HCl (pH 7.5), 1 mM diethylthreitol and 10 mM KCl. Glycerol was added to the protein solution to a final concentration of 30%, and the solution was frozen quickly in liquid nitrogen and stored at −70°C.

\textbf{Preparation of tRNA variants}

A series of tRNA variants were transcribed \textit{in vitro} using T7 RNA polymerase as described (Ikeuchi et al., 2005). Templates for \textit{in vitro} transcription were constructed by PCR using synthetic DNA oligonucleotides carrying the tRNA gene under the T7 promoter sequence (Sampson and Uhlenbeck, 1998). The oligo-DNAs used for the construction of 22 tRNA variants are shown in the Supplementary Table S1. The transcribed \textit{tRNAs} were gel-purified by running them on 10% polyacrylamide gel containing 7 M urea.

\textbf{In vitro ac\textsuperscript{4C} formation}

\textit{ac\textsuperscript{4C} formation} was performed at 37°C in a reaction mixture (50 μl) consisting of 100 mM Tris–HCl (pH 7.8), 10 mM KCl, 10 mM MgCl\textsubscript{2}, 10 mM DTT, 1 mM ATP, 120 μM [\textsuperscript{1-\textsuperscript{14}C}] acetyl-CoA (American Radiolabeled Chemicals, 55 mCi mmol\textsuperscript{-1}), 5.0 μg of recombinant TmcA protein and 0.04 A\textsubscript{260} units (60 pmol) of \textit{in vitro}-transcribed tRNA\textsuperscript{Met}. An aliquot (10 μl) of reaction mixture taken at various time points was phenol-chloroform-extracted and ethanol-purifi-
tated to remove free [1-14C] acetyl-CoA. The recovered tRNA was spotted onto Whatman 3 MM filter discs. The discs were washed three times with 5% trichloroacetic acid and the radioactivity was measured by liquid scintillation counting.

To visualize the radioactivity of ac14C, the reaction mixture was directly analysed by running it on a 10% polyacrylamide gel containing 7 M urea with 1× TBE. The gel was stained by ethidium bromide for digital photo recording, dried and the radioactivity of the acetylated tRNAs was visualized and quantified by an imaging analyser (FLA-7000 system, Fujifilm, Japan).

For LC/MS analysis, ac14C formation was performed at 37 °C in a reaction mixture (10 μl) consisting of 100 mM Tris-HCl (pH 7.8), 10 mM KCl, 10 mM MgCl2, 10 mM DTT, 1 mM ATP, 1 mM acetyl-CoA, 1.4 μg of recombinant TmcA and 0.1 A260 units (150 pmol) of in vitro-transcribed tRNA(Met). After the reaction, the tRNA was digested into nucleosides with nuclease P1 and bacterial alkaline phosphatase as described (Suzuki et al., 2007) and was directly analysed by LC/MS (Ikeuchi et al., 2006; Noma et al., 2006; Suzuki et al., 2007).

**Gel retardation experiment**

The gel retardation experiment was basically performed as described (Soma et al., 2003). Recombinant TmcA (40–160 pmol) and in vitro-transcribed tRNA (80 pmol) were incubated at 37 °C for 15 min in a 10 μl buffer consisting of 50 mM Tris-HCl (pH 8.5), 15 mM MgCl2, 5 mM DTT and 1 mM spermine. The complex was run on a 6% native polyacrylamide gel with 50 mM Tris, 5 mM Mg(OAc)2 and 5 mM DTT (pH 7.1, adjusted with acetic acid). After electrophoresis, the gel was stained with ethidium bromide to visualize the tRNA and then stained with Coomassie brilliant blue to visualize the protein.

**Kinetic analysis of ATP/GTP hydrolysis**

The reaction was visualized by running it on a 10% polyacrylamide gel containing 7 M urea with 1× TBE. The gel was stained by ethidium bromide for digital photo recording, dried and the radioactivity of the tRNAs was visualized and quantified by an imaging analyser (FLA-7000 system, Fujifilm, Japan) (Supplementary Figure S1). The initial velocity of each reaction was determined, and kinetic parameters were calculated by a Hanes–Woolf plot.

**Supplementary data**

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

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