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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 $tRNA^{Met}$ is modified to N^4-acetylcytidine (ac^4C), 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* ($tRNA^{Met}$ cytidine acetyltransferase), to be responsible for ac^4C 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 $tRNA^{Met}$ by utilizing acetyl-coenzyme A (CoA) and ATP (or GTP). ATP/GTP hydrolysis by TmcA is stimulated in the presence of acetyl-CoA and $tRNA^{Met}$. A mutation study revealed that *E. coli* TmcA strictly discriminates elongator $tRNA^{Met}$ from the structurally similar $tRNA^{Ile}$ by mainly recognizing the C27–G43 pair in the anticodon stem. Our findings reveal an elaborate mechanism embedded in $tRNA^{Met}$ and $tRNA^{Ile}$ for the accurate decoding of AUA/AUG codons on the basis of the recognition of wobble bases by the respective RNA-modifying enzymes.

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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^4C) (Figure 1A) is a modified nucleoside that was identified at position 34 (the wobble position) of *Escherichia coli* elongator $tRNA^{Met}$ in 1972 (Oashi *et al*, 1972). It is known that ac^4C is widely present in a variety of tRNAs and rRNAs; it is present at the wobble position of bacterial $tRNA^{Met}$ and archaeal tRNAs (Gupta, 1984; Sprinzl and Vassilenko, 2005) and is found only at position 12 in eukaryotic tRNAs (Sprinzl and Vassilenko, 2005). In rRNAs, ac^4C 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^4C favours the C3′-*endo* form of its ribose puckering (Kawai *et al*, 1989), conferring stable codon–anticodon pairing at the wobble position of bacterial $tRNA^{Met}$. In fact, a biochemical study using *in vitro* protein synthesis indicated that ac^4C prevents misreading of the AUA codon by *E. coli* $tRNA^{Met}$ (Stern and Schulman, 1978). However, the biogenesis and functions of ac^4C in the cell are not fully understood. In *Saccharomyces cerevisiae*, TAN1 was identified as a protein that is required for ac^4C formation at position 12 of $tRNA^{Ser}$ (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^4C 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: $tRNA^{Ile}$ for the AUA codon and the elongator $tRNA^{Met}$ for the AUG codon (Figure 1B). Both tRNAs bear specific wobble modifications. The AUA codon-specific $tRNA^{Ile}$ contains lysidine (L, k^2C) at the first letter of the anticodon, whereas elongator $tRNA^{Met}$ has ac^4C at the same position. It is known that L is an essential modification that determines both the codon and amino-acid specificities of $tRNA^{Ile}$ (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 aminoacyl-tRNA synthetase (Ikeuchi *et al*, 2005). For lysidine formation in $tRNA^{Ile}$, we previously identified the $tRNA^{Ile}$-lysidine synthetase (*tilS*)

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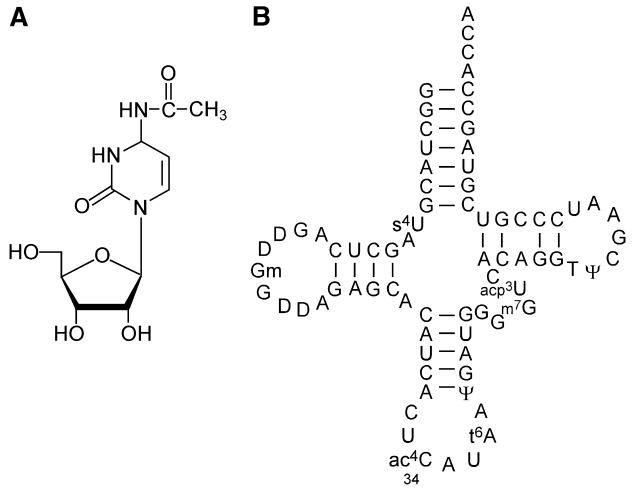

Figure 1 Chemical structure of N^4-acetylcytidine (ac^4C) and secondary structure of *E. coli* elongator $tRNA^{Met}$. (**A**) Chemical structure of ac^4C. (**B**) Secondary structure of *E. coli* elongator $tRNA^{Met}$ with modified nucleosides: 4-thiouridine (s^4U), 2′-*O*-methylguanosine (Gm), dihydrouridine (D), N^4-acetylcytidine (ac^4C), N^6-threonylcarbamoyladenosine (t^6A), pseudouridine (Ψ), 7-methylguanosine (m^7G), 3-(3-amino-3-carboxypropyl) uridine (acp^3U) and 5-methyluridine (m^5U).

that strictly discriminates $tRNA^{Ile}$ from $tRNA^{Met}$ by recognizing two consecutive base pairs in the acceptor stem (Soma *et al*, 2003; Ikeuchi *et al*, 2005). If ac^4C is accidentally introduced at the wobble position of $tRNA^{Ile}$, $tRNA^{Ile}$ loses its isoleucylation and AUA-decoding abilities. Thus, it was speculated that $tRNA^{Ile}$ also has another set of determinants negatively recognized by a putative enzyme responsible for the ac^4C formation that occurs in elongator $tRNA^{Met}$.

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 *E. coli* or *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 *tilS*, an essential gene for lysidine formation (Soma *et al*, 2003), *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 *tmcA* ($tRNA^{Met}$ cytidine acetyltransferase), responsible for ac^4C formation in the *E. coli* elongator $tRNA^{Met}$. Biochemical analyses revealed mechanistic insights into ac^4C formation and how TmcA discriminates elongator $tRNA^{Met}$ from the structurally similar $tRNA^{Ile}$.

Results

Ribonucleome analysis identified the ypfI gene to be required for ac^4C formation

To identify a gene responsible for ac^4C formation in *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 *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^4C (data not shown). The deleted genomic region of OCL58 spans *ypfN* to *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 *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 *ypfI* (Δ*ypfI*). Nucleosides analysis by LC/MS of the total RNA obtained from the Δ*ypfI* strain revealed the specific absence of ac^4C (Figure 2A), demonstrating that *ypfI* is an essential gene for ac^4C formation in the cell. Despite the absence of ac^4C, the Δ*ypfI* strain showed a healthy phenotype without any growth defects compared with wild-type cells (data not shown). When the Δ*ypfI* strain was cocultivated 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 Δ*ypfI* when it was combined with additional deletions of genes responsible for biogenesis of other modified nucleosides in $tRNA^{Met}$. We chose nine deletion strains, Δ*thiI*, Δ*dusA*, Δ*dusB*, Δ*dusC*, Δ*trmH*, Δ*truA*, Δ*trmB*, Δ*trmA* and Δ*truB*, each of whose deletion was transferred to Δ*ypfI* 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 Δ*ypfI*/Δ*dusC* strain showed a severe growth defect compared with each of the single-deletion strains (Figure 2B). The gene *dusC* encodes an enzyme responsible for dihydrouridine formation in the D-loop of tRNAs (Bishop *et al*, 2002). The Δ*dusC* mutant originally showed a cold-sensitive phenotype, and the additional deletion of *ypfI* enhanced this phenotype.

Reconstitution of ac^4C formation in vitro using recombinant TmcA

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

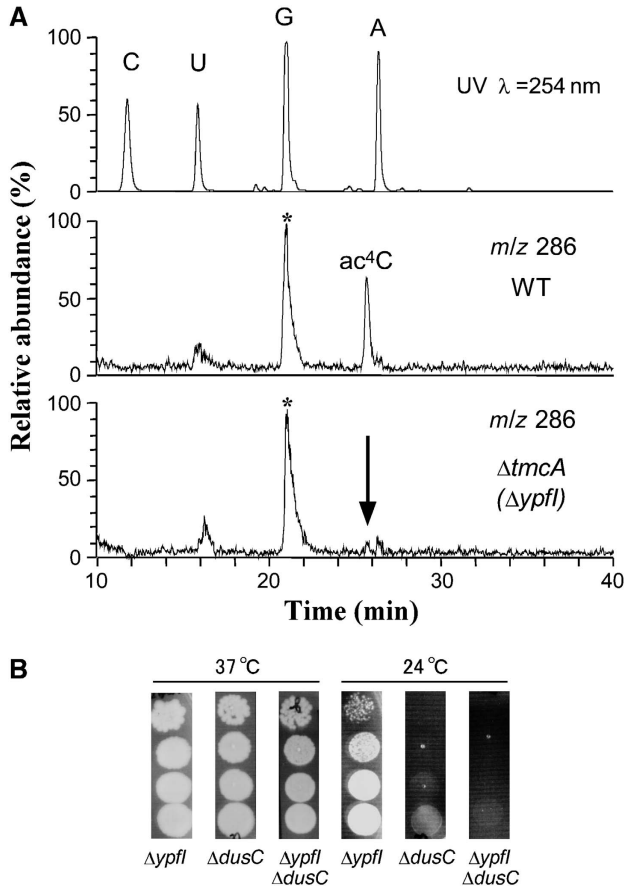

Figure 2 Mass spectrometric analysis of total nucleosides from the Δ*ypfI* strain and growth phenotype of the Δ*ypfI*/Δ*dusC* strain. (**A**) LC/MS analysis of total nucleosides in the wild-type (WT) and Δ*ypfI* strains. The top panel is the UV trace at 254 nm of the WT strain. The middle (WT) and bottom (Δ*ypfI*) panels are mass chromatograms at *m/z* 286 for detecting a proton adduct (MH^+) of ac^4C. Absence of ac^4C in Δ*ypfI* strain is indicated by the arrow. Asterisks indicate isotopic ions of guanosine. (**B**) Growth property of the Δ*ypfI*/Δ*dusC* strain. Each deletion strain was serially diluted (1:10 dilutions) and then spotted onto LB plates and incubated at 37 and 24 °C for 2 days.

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 ac^4C formation. To characterize the *E. coli* YpfI protein and to reconstitute ac^4C formation *in vitro*, a hexa-histidine-tagged *E. coli* YpfI was expressed and purified. We then tried to reconstitute ac^4C 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, ac^4C clearly appeared only when the reaction was performed in the presence of both acetyl-CoA and ATP. No ac^4C formation occurred under conditions without acetyl-CoA or ATP. This data demonstrates that YpfI is an acetyltransferase responsible for ac^4C formation at the wobble position of $tRNA^{Met}$. We therefore renamed *ypfI* as *tmcA* ($tRNA^{Met}$ cytidine acetyltransferase).

Reconstitution of ac^4C 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-^{14}C] 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-^{14}C] 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 ac^4C formation. The radioactivity of ^{14}C-labelled acetylated tRNAs was visualized on a gel (Figure 4D). In this experiment, TmcA did not acetylate $tRNA^{Ile2}$ as well as $tRNA^{Met}$ with a C34G mutation. According to the gel-mobility shift experiment (Figure 4E), TmcA specifically interacts with $tRNA^{Met}$. These data suggest that TmcA strictly recognizes the wobble base and discriminates $tRNA^{Met}$ from the structurally similar $tRNA^{Ile2}$.

We also found that TmcA can utilize GTP in place of ATP for ac^4C 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 ac^4C formation, and found that these analogues could not produce ac^4C (data not shown). This demonstrated that TmcA requires the hydrolysis of ATP or GTP for ac^4C formation.

Kinetic analysis of ATP and GTP hydrolysis by TmcA

To gain mechanistic insights into ac^4C formation driven by ATP/GTP hydrolysis, the ATPase (or GTPase) activity of TmcA was characterized. We employed [α-^{32}P]-labelled ATP (or [α-^{32}P]-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 polyethtyleneimine (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 ac^4C 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 $tRNA^{Met}$.

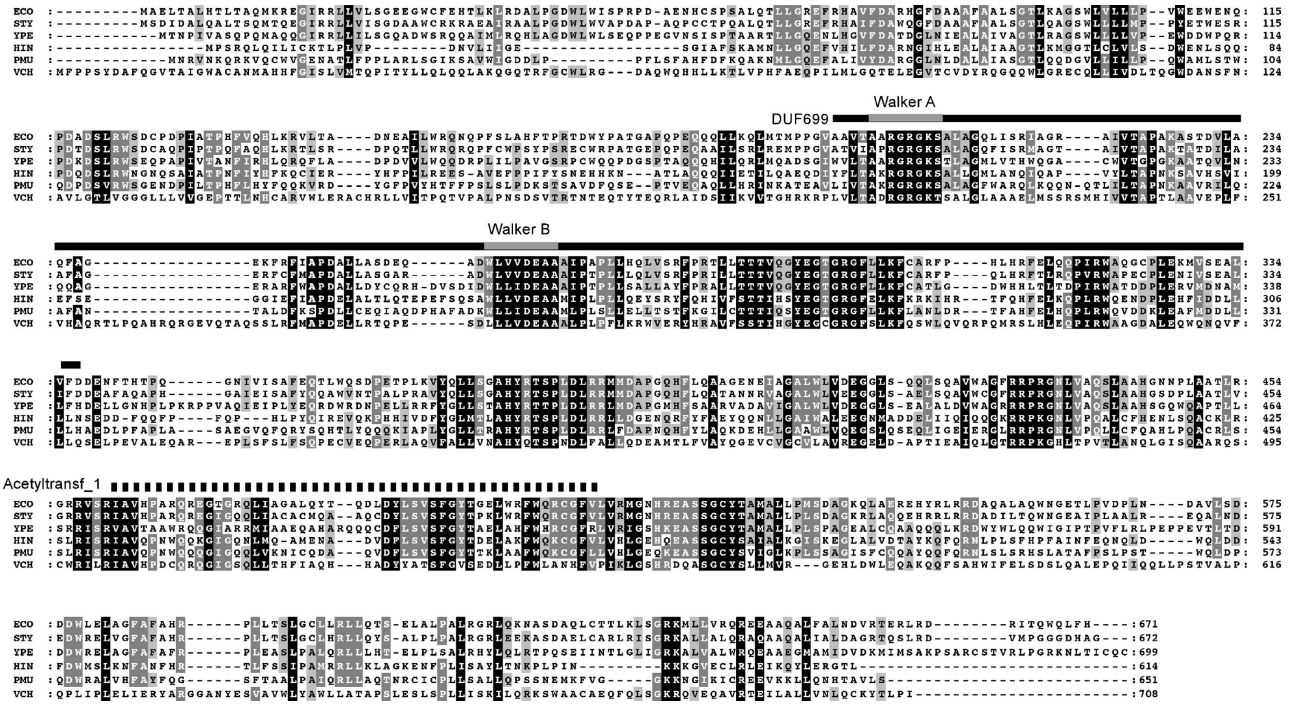

Figure 3 Sequence alignment of TmcA from γ-proteobacteria. Amino-acid sequence alignment of YpfI in *E. coli* (ECO) and homologues in other bacteria (STY, *S. typhimurium* LT2; YPE, *Y. pestis*; HIN, *H. influenzae* R3021; PMU, *P. multocida*; and VCH, *V. cholerae*) were aligned. Two domains (DUF699 and Acetyltransf_1) predicted by Pfam are indicated by black solid and dotted lines, respectively. The Walker A motif is indicated by grey line. The black and grey boxes indicate a degree of sequence similarity.

TmcA recognizes the anticodon stem of tRNA^{Met}

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

As most of the differences between elongator tRNA^{Met} and tRNA^{Ile2} 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^{Met} bearing the anticodon stem of tRNA^{Ile2} (EM(ACstemI)) (Figure 5B). On the other hand, the mutant tRNA^{Ile2} bearing the anticodon stem of tRNA^{Met} (EI(ACstemM)) acquired acetylation capability (Figure 5B). The only difference in the D arms between tRNA^{Met} and tRNA^{Ile2} is the absence of a uridine at position 16 of tRNA^{Ile2}. However, U16 deletion of tRNA^{Met} (EM(delU16)) did not affect ac^{4}C formation (Figure 5C). Next, we replaced the T arm of tRNA^{Met} with that of tRNA^{Ile2} (EM(TarmI)), but no significant change in ac^{4}C formation was observed (Figure 5C). These results reveal that TmcA specifically recognizes the anticodon stem of tRNA^{Met}.

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

We next constructed tRNA^{Ile2} mutants bearing the two positive elements for ac^{4}C formation, to determine the minimum necessary elements for the conversion of the specificity of the wobble modification from lysidine to ac^{4}C. When C27-G43 and C30-G40 were introduced into tRNA^{Ile2} simultaneously, the mutant tRNA^{Ile2} (EI(C27G43, C30G40)) was acetylated (Figure 5C). In addition, introduction of C27-G43 and G44 into tRNA^{Ile2} (EI(C27G43, G44)) also conferred a specificity for TmcA (Figure 5C). However, when C30-G40 and G44 were simultaneously introduced into tRNA^{Ile2} (EI(C30G40, G44)), no ac^{4}C formation was observed (Figure 5C). Introduction of C27-G43 is a critical, but

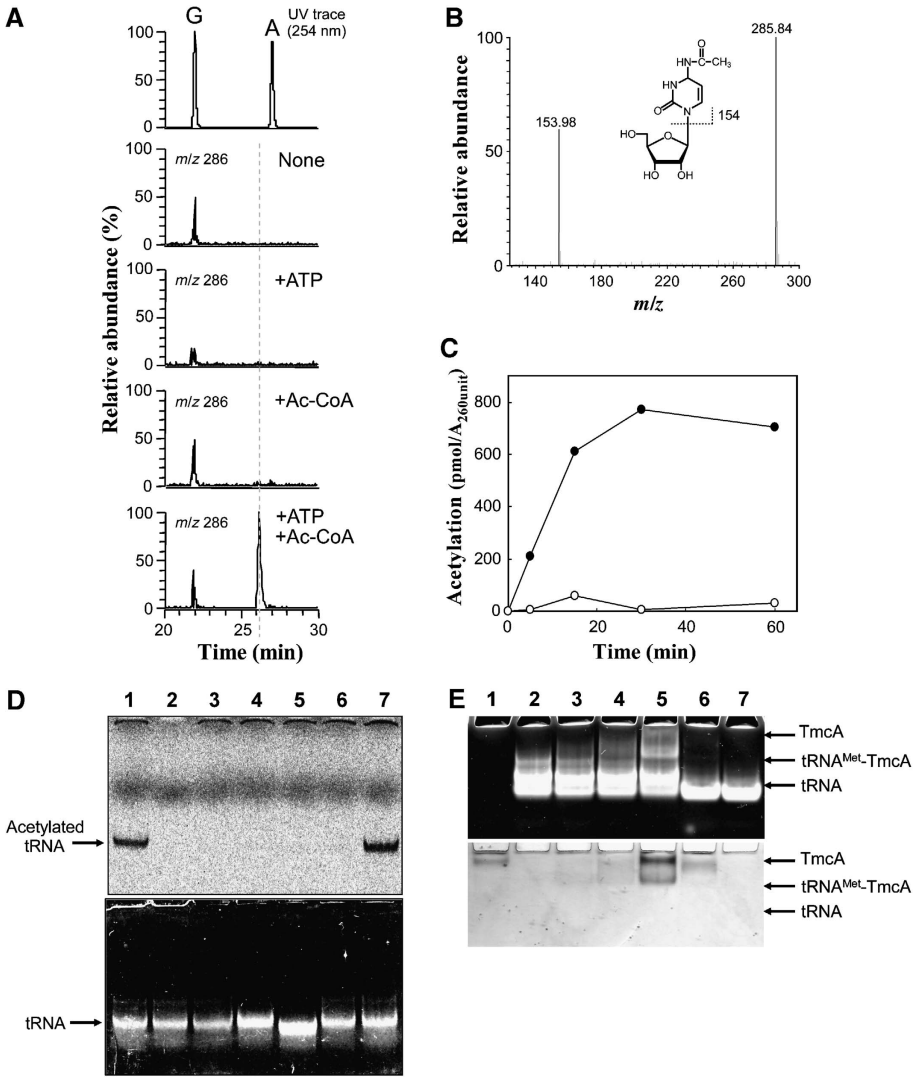

Figure 4 Reconstitution of ac^{4}C formation *in vitro* using recombinant TmcA. (**A**) Mass spectrometric detection of ac^{4}C formation in the unmodified tRNA^{Met} that was transcribed *in vitro*. After the reaction, the tRNA^{Met} was digested into nucleosides and analysed by LC/MS. Mass chromatograms at *m/z* 286 detected ac^{4}C, which was reconstituted *in vitro* in the absence of both ATP and acetyl-CoA (second panel), in the presence of ATP (third panel), in the presence of acetyl-CoA (fourth panel) or in the presence of both ATP and acetyl-CoA (bottom panel). The ac^{4}C appeared only when both substrates were present. (**B**) Mass spectrum of ac^{4}C that was reconstituted *in vitro*. (**C**) *In vitro* reconstitution of ac^{4}C formation in the presence (filled circle) or absence (open circle) of ATP, which was detected by liquid scintillation counting of ^{14}C-acetyl group. (**D**) *In vitro* reconstitution of ac^{4}C formation under various conditions, the radioactivity of which was visualized by an imaging analyser (FLA-7000, Fujifilm). The upper and lower panels show visualized radioactivity and ethidium bromide staining. The reaction was performed in the presence of ATP (lane 1), GTP (lane 7) and ADP (lane 6), in the absence of ATP (lane 3) and TmcA (lane 2). The mutant tRNA^{Met}, EM(C34G), whose C34 was replaced by G34 (lane 4) and tRNA^{Ile2} (lane 5), were employed for ac^{4}C reconstitution instead of tRNA^{Met}. (**E**) Gel-retardation analysis of tRNA^{Met} with recombinant TmcA. The polyacrylamide gel was stained by ethidium bromide (upper panel) and Coomassie brilliant blue (lower panel). Lane 1, TmcA (80 pmol); lane 2, tRNA^{Met}; lane 3, TmcA (40 pmol) and tRNA^{Met}; lane 4, TmcA (80 pmol) and tRNA^{Met}; lane 5, TmcA (160 pmol) and tRNA^{Met}; lane 6, TmcA (80 pmol) and tRNA^{Ile2}; lane 7, tRNA^{Ile2}. Amount of tRNA was 80 pmol in all conditions.

insufficient, element for ac^{4}C formation on tRNA^{Ile2}. Additional introduction of C30-G40 or G44 with the C27-G43 mutation is therefore required for the acetylation of tRNA^{Ile2}. 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^{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 Δ*tmcA* strain was unexpectedly observed. Thus, ac^{4}C is a dispensable modification of tRNA^{Met}, at least in the presence of the AUA codon-specific tRNA^{Ile2}. The only phenotype we observed in this study was a cold-sensitive phenotype for the double-deletion strain (Δ*tmcA*/Δ*dusC*) (Figure 2B). We have no plausible explanation why the lack of ac^{4}C in tRNA^{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).

Table I Kinetic parameters for ATP/GTP hydrolysis

NTP	$tRNA^{Met}$ (2.5 μM)	Acetyl-CoA (200 μM)	K_m (μM)	k_{cat} (10^{-3} s^{-1})	k_{cat}/K_m (10^{-3} s^{-1} μM^{-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

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 eukaryotic tRNA metabolism is involved in the *E. coli* system. It is also possible to speculate that a functional defect of the hypomodified $tRNA^{Met}$ 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^4C 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^4C was not reported at the wobble position in other sequenced $tRNAs^{Met}$ from *Bacillus subtilis*, *Mycoplasma capricolum* or *Thermus thermophilus*, which are not γ-proteobacteria (Sprinzl and Vassilenko, 2005). The limited distribution of TmcA and ac^4C at the wobble position of $tRNA^{Met}$ 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 archea and eukaryotic species (KOG2036 in eukaryotic cog database). It is known that ac^4C occurs at the wobble positions of several tRNAs in archea (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^4C in its 3′-terminal region in some eukaryotes (Thomas *et al*, 1978; McCarroll *et al*, 1983). Further studies should reveal whether eukaryotic homologues of *tmcA* are actually involved in ac^4C formation on tRNA or rRNA.

TmcA contains an ATPase domain in its N-terminal region and an *N*-acetyltransfease domain related to the histone acetyltransferase family in its C-terminal region. *In vitro* reconstitution of ac^4C formation revealed that TmcA specifically acetylates C34 of elongator $tRNA^{Met}$ 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 acetyltransfease. ATP hydrolysis had a higher k_{cat}/K_m value (7.10×10^{-3} s^{-1} μM^{-1}) than that of GTP hydrolysis (0.807×10^{-3} s^{-1} μM^{-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 $tRNA^{Met}$, indicating that TmcA can hydrolyse ATP/GTP independently of its RNA acetylation activity. In the presence of acetyl-CoA or $tRNA^{Met}$, the k_{cat} value of ATP hydrolysis increased, suggesting a functional interplay between ATPase and GNAT domains in TmcA. The mechanisms of ATP hydrolysis-driven ac^4C formation needs to be elucidated, although it can be speculated that some conformational change of TmcA induced by the binding of acetyl-CoA or $tRNA^{Met}$ 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 (s^2U), 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 (SGG × 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 ATP is hydrolysed. As TmcA hydrolyses the β–γ phosphate bond of ATP (or GTP), this indicates that the mechanism of ac^4C formation utilizing ATP hydrolysis is completely different from the biogenesis of L or s^2U.

The Walker-type ATPase domain is widely found in AAA^+ 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 $tRNA^{Met}$, and/or for the efficient turnover of the reaction by promoting product release.

In vitro acetylation of tRNA variants revealed that TmcA discriminates $tRNA^{Met}$ from the structurally similar $tRNA^{Ile2}$ by recognizing C27-G43, C30-G40 and G44 in the anticodon stem. In fact, when these three elements were introduced into $tRNA^{Ile2}$ simultaneously, the mutant $tRNA^{Ile2}$ acquired full acetylation capability (Figure 5C). C27–G43 in $tRNA^{Met}$ is the

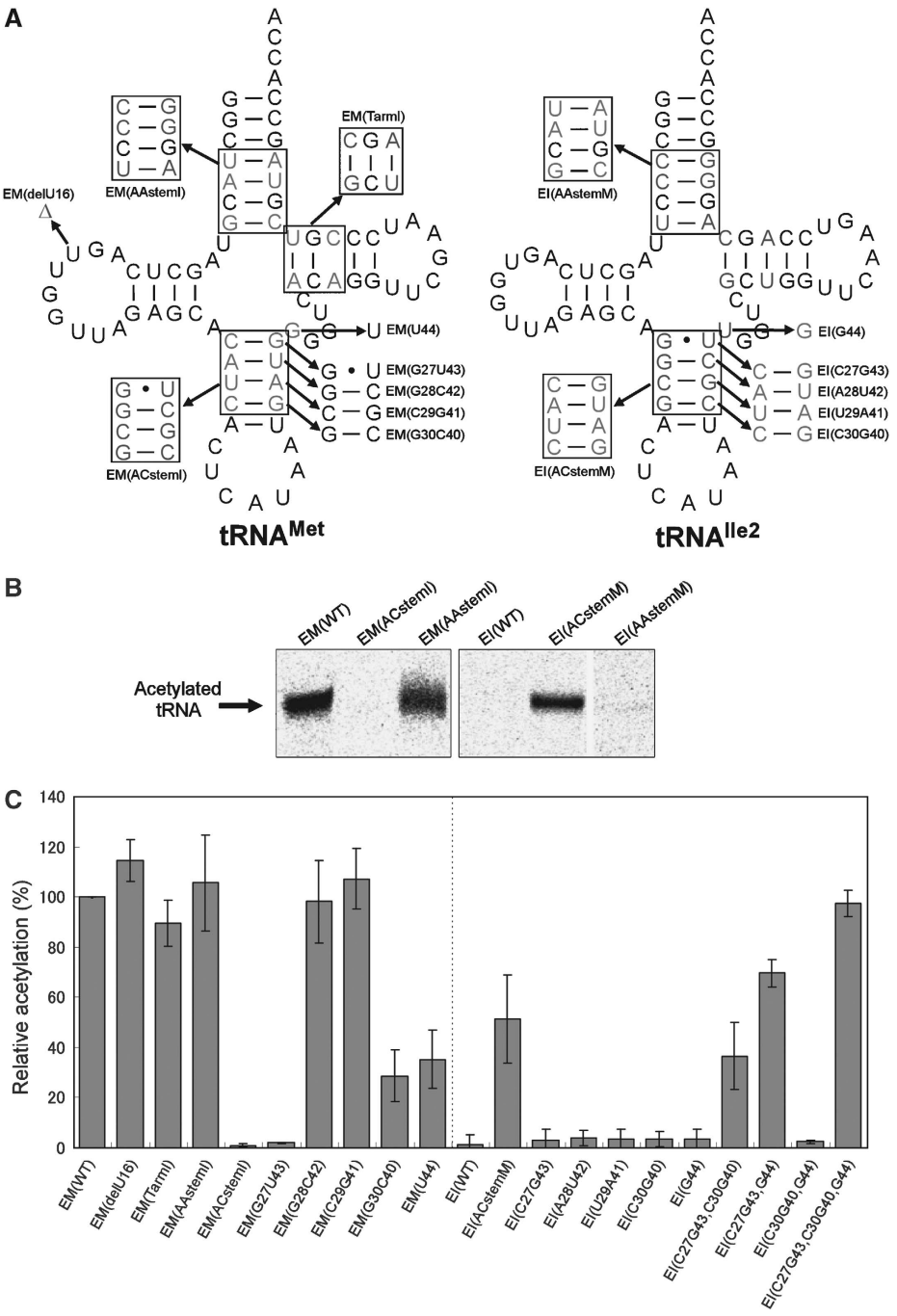

Figure 5 Mutation study of $tRNA^{Met}$ and $tRNA^{Ile2}$ to investigate the positive and negative determinants of ac^4C formation. (**A**) tRNA variants based on *E. coli* elongator $tRNA^{Met}$ (left-hand side) and *E. coli* $tRNA^{Ile2}$ (right-hand side) that were used in this study. The numbering system of the tRNA is based on the tRNA compilation by Sprinzl and Vassilenko (2005). Arrows and boxes indicate the substitutions and deletions made in this study. EM and EI stand for *E. coli* elongator $tRNA^{Met}$ and *E. coli* $tRNA^{Ile2}$, respectively. (**B**) Detection of ac^4C formation in tRNA variants visualized by the imaging analyser. (**C**) Relative acetylation activities of a series of tRNA variants. Radioactivity of ^{14}C-acetylation on each tRNA was quantified by FLA-7000 system (Fujifilm). Radioactivity of ac^4C in EM(WT) was standardized as 100%. A full-colour version of this figure is available at *The Embo Journal* Online.

most critical positive determinant for ac^4C formation, and as a mutant $tRNA^{Met}$ having a G27·U43 mutation (EM(G27U43)) completely failed to be acetylated (Figure 5C), G27·U43 of $tRNA^{Ile2}$ is a negative determinant for ac^4C formation. We previously reported that TilS positively recognizes two consecutive base pairs (C4-G69 and C5-G68) in the acceptor stem of $tRNA^{Ile2}$ for lysidine formation (Ikeuchi *et al*, 2005). In contrast, the corresponding base pairs (U4-A69 and A5-U68) in $tRNA^{Met}$ 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 $tRNA^{Met}$ and $tRNA^{Ile2}$ 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

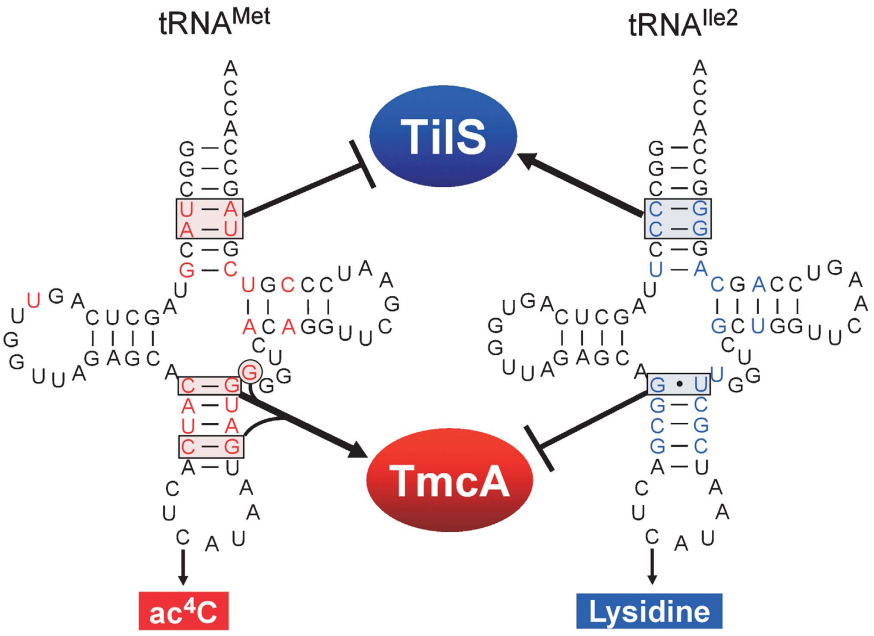

Figure 6 Recognition and discrimination, by TmcA and TilS, of two tRNAs with CAU anticodons. Schematic depiction of tRNA recognition by TmcA in comparison with TilS. In this picture, the anticodon regions of both tRNAs are not highlighted as positive elements for both enzymes, as this region is commonly recognized by both enzymes. TmcA strongly recognizes C27-G43 in the anticodon stem of $tRNA^{Met}$. In addition, C30-G40 and G44 in $tRNA^{Met}$ have an important function for TmcA recognition. G27 · U43 in $tRNA^{Ile2}$ exerts an effect as a negative determinant for TmcA recognition. On the other hand, TilS strongly recognizes two consecutive base pairs, C4-G69 and C5-G68, in the acceptor stem of $tRNA^{Ile2}$ for lysidine formation, whereas U4-A69 and A5-U68 in $tRNA^{Met}$ exert an effect as negative determinants for TilS recognition.

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 $tRNA^{Met}$ and $tRNA^{Ile}$ 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 *E. coli* genomic-deletion strains (OCL/R-series) derived from MG1655sp (MG1655 *rpsL polA*12 *Zih*::Tn10), each of which lacks about 20 kbp (~20 genes), were kindly provided by Dr Junichi Kato (Hashimoto *et al*, 2005). OCL58 (MG1655sp Δ(*ypfH-yfgD*)::kan) specifically lacked ac^4C. The *E. coli* K-12 strain BW25113 (*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$) was used for the 'one-step inactivation of chromosomal genes' procedure (Datsenko and Wanner, 2000; Baba *et al*, 2006). To amplify DNA fragments of the chloramphenicol acetyltransferase gene (Cm^r) with 40-nt extensions at both ends homologous to the flanking sequences of the *ypfI* gene, pairs of oligo DNAs, ΔypfI-F(Cm^r) (5′-gcaatacttttggtaaaagcatttaacttccggggcagggactaaatcagtaagt tggcagc-3′) and ΔypfI-R(Cm^r) (5′-aagtgcgaacagcgcctgcgcggcctcttccc gctgacggaaccagcaatagacataagcggc-3′) were used to construct BW25113 Δ*ypfI*::Cm. The pBT vector (Stratagene) was used as a template of Cm^r.

A series of single-deletion strains having a kanamycine-resistant marker (Km^r) (Δ*ypfI*, Δ*thiI*, Δ*dusA*, Δ*dusB*, Δ*dusC*, Δ*trmH*, Δ*truA*, Δ*trmB*, Δ*trmA* and Δ*truB*) (Baba *et al*, 2006) were obtained from the Genetic Stock Research Center, National Institute of Genetics, Japan. To construct a series of double-deletion strains (for example, Δ*ypfI*/Δ*dusC*), Δ*ypfI*::Cm was transferred to a series of single-deletion strains (Km^r) by P1-transduction. The *E. coli* strains were grown in 5 ml of LB medium in 24-well plates at 37 °C overnight.

Mass spectrometry

The total RNA was extracted from grown cells using an acid-guanidinium thiocyanate-phenol–chloroform reagent (ISOGEN, Nippon Gene, Japan). The extracted RNAs were digested into nucleosides and analysed by LC/MS using ion-trap mass spectrometry as described previously (Ikeuchi *et al*, 2006; Noma *et al*, 2006; Suzuki *et al*, 2007).

Construction and purification of recombinant protein

Oligonucleotides YpfI-NdeI-F (5′-gagatatacatatggctgaactgactgcgcttc aca-3′) and YpfI-XhoI-R (5′-ggtgctcgaggtgaaataattgccattgcgttatg-3′) were used to amplify *ypfI* from the *E. coli* genome by PCR. The PCR products were cloned into the *Nde*I and *Xho*I sites of pET21b (Novagen) to generate pET21-TmcA. *E. coli* BL21(DE3) was used as the host for the expression of recombinant TmcA. The C-terminal 6× 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 dithiothreitol 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.

Preparation of tRNA variants

A series of tRNA variants were transcribed *in vitro* using T7 RNA polymerase as described (Ikeuchi *et al*, 2005). Templates for *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 tRNAs were gel-purified by running them on 10% polyacrylamide gel containing 7 M urea.

In vitro ac^4C formation

ac^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 $MgCl_2$, 10 mM DTT, 1 mM ATP, 120 μM [1-^{14}C] acetyl-CoA (American Radiolabeled Chemicals, 55 mCi mmol^{-1}), 5.0 μg of recombinant TmcA protein and 0.04 A_{260} units (60 pmol) of *in vitro*-transcribed $tRNA^{Met}$. An aliquot (10 μl) of reaction mixture taken at various time points was phenol–chloroform-extracted and ethanol-precipi-

tated to remove free [1-^{14}C] 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 ac^{4}C, 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, ac^{4}C 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 $MgCl_2$, 10 mM DTT, 1 mM ATP, 1 mM acetyl-CoA, 1.4 μg of recombinant TmcA and 0.1 A_{260} 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 $MgCl_2$, 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 Coomasie brilliant blue to visualize the protein.

Kinetic analysis of ATP/GTP hydrolysis

The experiments of ATP/GTP hydrolysis were basically performed as described (Terasaki *et al*, 2004; Ikeuchi *et al*, 2005) in a reaction mixture (50 μl) consisting of 100 mM Tris–HCl (pH 7.8), 10 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.1–250 μM ATP with 0.25 μl of [α-^{32}P]ATP (~3.7 GBq ml^{-1}, GE Healthcare) or 0.1–250 μM GTP with 0.25 μl of [γ-^{32}P]GTP (~3.7 GBq ml^{-1}, GE Healthcare), and TmcA (50 pmol). To determine the kinetic parameters of ATP/GTP hydrolysis in the presence of tRNA^{Met} or acetyl-CoA, 2.5 μM of *in vitro*-transcribed tRNA^{Met} or 200 μM acetyl-CoA was added to the reaction mixture. An aliquot (10 μl) of the reaction was quenched by formic acid at various time periods (7, 15, 23 and 30 s), spotted onto a PEI-cellulose TLC plate (Polygram Cel 300 PEI/UV254, Machery-Nagel) and developed with 0.75 M KH_2PO_4 (pH 3.5). The radioactivity 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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