Dual pathways of tRNA hydroxylation ensure efficient translation by expanding decoding capability

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In bacterial tRNAs, 5-carboxymethoxyuridine (cmo⁵U) and its derivatives at the first position of the anticodon facilitate non-Watson-Crick base pairing with guanosine and pyrimidines at the third positions of codons, thereby expanding decoding capabilities. However, their biogenesis and physiological roles remained to be investigated. Using reverse genetics and comparative genomics, we identify two factors responsible for 5-hydroxyuridine (ho⁵U) formation, which is the first step of the cmo⁵U synthesis: TrhP (formerly known as YegQ), a peptidase U32 family protein, is involved in prephenate-dependent ho⁵U formation; and TrhO (formerly known as YceA), a rhodanese family protein, catalyzes oxygen-dependent ho⁵U formation and bypasses cmo⁵U biogenesis in a subset of tRNAs under aerobic conditions. E. coli strains lacking both trhP and trhO exhibit a temperature-sensitive phenotype, and decode codons ending in G (GCG and UCG) less efficiently than the wild-type strain. These findings confirm that tRNA hydroxylation ensures efficient decoding during protein synthesis.

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RNA modifications confer chemical diversity on simple RNA molecules, expanding their functional repertoires. To date, more than 140 species of RNA modifications have been identified in RNA molecules from all domains of life.

In protein synthesis, tRNA serves as an adapter molecule to connect codons on mRNA with the corresponding amino acids. After they are transcribed, tRNAs undergo chemical modifications mediated by site-specific tRNA-modifying enzymes. These modifications play critical roles in stabilizing tRNA tertiary structure and fine-tuning the decoding process. A wide variety of modifications are present at the first (wobble) position of the anticodon in tRNA (position 34). The wobble modification modulates codon recognition, thereby promoting accurate decoding during protein synthesis.

In the original wobble rule, guanosine at the wobble position (G34) base-pairs with U and C at the third letter of the codon, likewise, uridine at the wobble position (U34) base-pairs with A and G at the third letter of the codon. In Mycoplasma species and mitochondria, however, U34 recognizes any of the four bases in a family box through a mechanism called four-way wobbling. To decode two codon sets ending in purine (NNR), the wobbling

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Fig. 1 Decoding capacity of tRNAs is expanded by (m)cmo5U. a Secondary structure of E. coli tRNAAla1 with post-transcriptional modifications: 4-thiouridine (s4U), dihydrouridine (D), 2'-O-methyluridine (Um), 5-methoxycarbonylmethoxyuridine (mcmo5U), 7-methylguanosine (m7G), 5-methyluridine (m5U), and pseudouridine (Ψ). A pair of gray triangles indicate the RNase T1 cleavage positions for analysis of RNA fragments containing the wobble position. b Anticodon–codon pairing in six codon boxes decoded by tRNAs bearing cmo5U and mcmo5U. Each of the three tRNA genes in parenthesis was individually deleted. In the family boxes (four codons specify a single amino acid), many codons are redundantly decoded by two or three isoacceptors (tRNAs charging with the same amino acid). For example, CUU codon is decoded by tRNALeu2 with GAG anticodon as well as tRNALeu3 with cmo5UAG anticodon.

Results

E. coli yegQ is involved in tRNA hydroxylation. We recently reported that RlhA is responsible for ho5C formation at position 2501 in E. coli 23S tRNA30. RlhA belongs to a family of proteins, which contain the peptidase U32 motif. This finding prompted us to speculate that other paralogs of peptidase U32-containing proteins are involved in ho5U34 formation in tRNAs. The E. coli genome contains four paralogs of peptidase U32-containing proteins, rlhA, yegQ, yhbU, and yhbV (Supplementary Fig. 1, Supplementary Data 1). We extracted total RNA from each of the respective knockout strains, digested with RNase T1, and subjected the digests to capillary LC-nano-ESI-mass spectrometry (RNA-MS) to detect tRNA fragments containing cmo5U (shotgun analysis). RNA fragments were detected as multiply charged negative ions (Supplementary Table 1). We clearly detected an anticodon-containing RNA fragment of tRNAVal1 from total RNA of wild-type (WT) E. coli cells (Fig. 2a). As reported previously13, cmo5U34 was present as a major wobble cation in this tRNA, whereas little unmodified fragment (U34) was detected. In a knockout strain lacking yegQ (ΔyegQ), which encodes a peptidase U32-containing protein, cmo5U34 frequency decreased markedly, to about 30% of the WT level, and a corresponding fragment containing unmodified U34 appeared, indicating that yegQ is partially responsible for the initial step of cmo5U34 formation. We reasoned that other paralogs of peptidase U32-containing proteins might be involved in this process. Accordingly, we constructed a quadruple mutant strain, ΔyegQ/ΔyhbU/ΔyhbV/ΔrlhA, lacking all paralogs of peptidase U32-containing proteins, and analyzed the RNA fragment of tRNAVal1. However, the cmo5U34-containing fragment persisted in this strain (Fig. 2a), clearly indicating that another hydroxylation

Both enzymes cause a temperature-sensitive phenotype and decreases the efficiency with which codons ending in G (GCG and UCG) were decoded, indicating that ho5U34 formation ensures efficient decoding during translation.
pathway plays a redundant role in the formation of ho\textsuperscript{5}U34 in tRNAs.

To analyze the modification status of each tRNA species in the \(\Delta yegQ\) strain, we used the reciprocal circulating chromatography (RCC) method\textsuperscript{42} to isolate six tRNA species (tRNA\textsubscript{Ala1}, tRNA\textsubscript{Leu3}, tRNA\textsubscript{Pro3}, tRNA\textsubscript{Ser1}, tRNA\textsubscript{Thr4}, and tRNA\textsubscript{Val1}) bearing a cmo\textsuperscript{5}U34 or mcmo\textsuperscript{5}U34 modification from both WT and \(\Delta yegQ\) strains. Each tRNA was digested by RNase T1 and subjected to RNA-MS to analyze the anticodon-containing fragments (Fig. 2b, Supplementary Fig. 2, Supplementary Table 1). Hypomodified fragments were further sequenced by collision-induced dissociation (CID) analysis (Supplementary Fig. 3). In tRNA\textsubscript{Ala1}, mcmo\textsuperscript{5}U34 was present as a major wobble modification in the WT, but its levels were reduced to about 50% (concomitant with appearance of the unmodified fragment) in \(\Delta yegQ\) (Fig. 2b). Similarly, mcmo\textsuperscript{5}U34 levels were reduced in
tRNA\textsubscript{Ser} and tRNA\textsubscript{Thr} and cmo5U34 from ΔyegQ (Fig. 2c, Supplementary Fig. 2). The level of cmo5U34, a major modification in WT tRNA\textsubscript{Val}, was also reduced about 50% in ΔyegQ (Fig. 2c, Supplementary Fig. 2). By contrast, the levels of cmo5U34 dropped sharply, to less than 3% of WT levels, in the ΔyegQ strain (Fig. 2c, Supplementary Fig. 2). These results indicate that yegQ is involved differently in ho5U34 formation in each tRNA. In particular, tRNA\textsubscript{Leu3} and tRNA\textsubscript{Pro3} are major targets of YegQ. Accordingly, we renamed yegQ as trhP. 

**Biogenesis of mo5U34 in Bacillus subtilis.** Instead of cmo5U34 or cmo5U34, B. subtilis uses mo5U34 as an xo5U-type wobble modification. Upon depletion of intracellular AdoMet, mo5U is replaced by ho5U\textsuperscript{3+}, indicating that ho5U is the precursor and AdoMet is the methyl donor for mo5U formation. We identified two orthologs of trhP in B. subtilis, yrrN and yrrO (Supplementary Fig. 1, Supplementary Data 1), both of which are encoded tandemly in the same operon (Fig. 2d). YrrO has a peptidase U32 motif and a characteristic C-terminal motif also found in E. coli ThrP, whereas YrrN only has a peptidase U32 motif.

We investigated whether these two paralogs are actually involved in mo5U34 formation by shotgun analysis of total RNAs extracted from B. subtilis ΔyrrN and ΔyrrO strains. Similar to our observation in the E. coli ΔtrhP strain (Fig. 2a), mo5U34 frequency in tRNA\textsuperscript{Val} decreased, and an RNA fragment containing unmodified U34 appeared in both ΔyrrN and ΔyrrO strains (Fig. 2e), indicating that both yrrN and yrrO were necessary for hydroxylation of U34 to form ho5U34 in B. subtilis. Accordingly, we renamed yrrO and yrrN as trhP1 and trhP2, respectively. To determine whether these two paralogs act redundantly to form ho5U34, we constructed the double-deletion strain ΔtrhP1/ΔtrhP2 and analyzed the modification status of tRNA. The same level of mo5U34 was detected in this strain as in the single-knockout strains (Fig. 2e), suggesting that both paralogs are involved in synthesizing about 50% of mo5U34 in the cell, and another pathway is required for the remainder, as also observed for formation of cmo5U34 in E. coli.

We then focused on yrrM, which is encoded within the same operon as trhP1 and trhP2 (Fig. 2d). B. subtilis YrrM has high sequence similarity to the AdoMet-dependent catechol O-methyltransferase that catalyzes methylation of the hydroxyl group on the aromatic ring, suggesting that this protein is a methyltransferase responsible for mo5U34 formation. As expected, mo5U34 in tRNA\textsuperscript{Val} disappeared and was converted to ho5U34 in a ΔyrrM strain (Fig. 2e). We then generated recombinant YrrM protein and performed in vitro reconstitution of mo5U34 in E. coli tRNA\textsubscript{Thr} containing ho5U34, which had been isolated from a ΔcmoB strain. mo5U34 was successfully synthesized in the presence of both YrrM and AdoMet (Supplementary Fig. 4). The product was confirmed by CID analysis (Supplementary Fig. 4). Taken together, these findings indicate that YrrM is an AdoMet-dependent methyltransferase that converts ho5U34 to mo5U34 in B. subtilis. During the preparation of this manuscript, B. subtilis yrrM was demonstrated to be a ho5U34-methyltransferase and renamed trmR\textsuperscript{44}.

**Alternative pathway for tRNA hydroxylation.** E. coli trhP and B. subtilis trhP1/trhP2 are partially involved in the initial step of cmo5U34 and mo5U34 formation, respectively, indicating the existence of another redundant pathway for ho5U34 formation in both species. To search for the gene(s) responsible for this pathway, we used a comparative genomic approach. In bacteria, we found several organisms with cmo5U and cmo5U homologs, but no trhP homolog (Fig. 3a). Similarly, some bacteria had a trmR homolog, but no trhP homologs (Fig. 3a). Given that ho5U34 is a common precursor for cmo5U34 synthesis mediated by cmoA and cmoB, and mo5U34 synthesis mediated by trmR, the bacterial species lacking trhP homologs should have another gene responsible for ho5U34 formation independent of the trhP pathway. We identified seven bacterial species with cmoA and cmoB homologs, but no trhP homolog (Fig. 3a). Among 4746 E. coli ORFs, we selected 141 genes (Fig. 3b) commonly present in all seven species, as well as in B. subtilis. We then narrowed down the list of candidates to seven genes with unknown functions (Fig. 3b). Among them, a yceA homolog was identified as a strong candidate because its genomic locus is close to that of cmoA in cyanobacteria, and yceA is encoded as a fusion protein with trmR in three bacterial species, Phytophthora sojae, Phytophthora ramorum, and Phaeodactylum tricornutum (Fig. 3b). To determine whether yceA is responsible for the second pathway of ho5U34 formation, we isolated tRNA\textsuperscript{Val} from E. coli knockout strains of trhP and yceA, and analyzed the status of wobble modifications. The residual mo5U34 observed in the ΔtrhP strain completely disappeared in the double-deletion strain ΔtrhP/ΔyceA (Fig. 3c), indicating that yceA is responsible for the second pathway of ho5U34. Hereafter, we refer to yceA as trhO (tRNA hydroxylation O). However, the amount of unmodified U34-containing fragment increased slightly in the single-deletion strain ΔtrhO (Fig. 3c), indicating that the trhP-mediated pathway plays the predominant role in ho5U34 formation especially in the absence of trhO.

B. subtilis ybfQ is an ortholog of E. coli trhO. To determine whether ybfQ is involved in the second pathway for ho5U34 formation in B. subtilis, we constructed a triple-knockout strain, ΔtrhP1/ΔtrhP2/ΔybfQ, and analyzed the status of tRNA wobble
Phylogenetic distribution of trhP and trhO. To investigate the phylogenetic distribution of trhP, trhPI, trhP2, and trhO, we generated a phylogenetic tree of organisms possessing or lacking each homolog (Supplementary Fig. 6, Supplementary Data 2). trhO orthologs predominated in bacteria and eukaryotes (40%; 232 of 584), whereas trhP orthologs (yegQ type in Supplementary Fig. 1) and trhP1/trhP2 orthologs (PepU32#5/PepU32#3 in Supplementary Fig. 1) were only present in bacteria, and were less widely distributed [14% (84 of 584) and 5% (32 of 584), respectively] (Supplementary Fig. 6, Supplementary Data 2). trhP orthologs were mainly detected in γ- and β-proteobacteria, in addition to some desulfovibrio bacteria in δ-proteobacteria. trhP1/trhP2 orthologs always co-occurred, and were present in phyllum Firmicutes and some members of Tenericutes and Cyanobacteria. Given that TrhP1 is a related family with TrhP (Supplementary Fig. 1), TrhP1 might have branched out from TrhP, and evolved to require a paralogous protein TrhP2 that might be generated by gene duplication. Supporting this speculation, trhP1 and trhP2 are tandemly encoded in the same operon in B. subtilis (Fig. 2e). Also, we can explain the reason why trhP orthologs and trhP1/trhP2 orthologs show a mutually exclusive distribution in bacteria (Supplementary Fig. 6). Intriguingly, over half of organisms bearing trhP or trhP1/trhP2 orthologs also harbor trhO orthologs [49 of 84 (58%) organisms bearing trhP, P = 0.0003 (Fisher’s exact test); 22 of 32 (69%) organisms bearing trhP1/trhP2, P = 0.0007 (Fisher’s exact test)] (Supplementary Fig. 7). This significant overlap suggests that harboring both pathways for tRNA hydroxylation might help organisms to adapt to two different environments, i.e., aerobic and anaerobic conditions.

Phenotypes of E. coli strains lacking tRNA hydroxylation. We then measured the growth rate of a series of E. coli knockout strains involved in (m)cmo5U34 modifications. No growth reduction was observed in the ΔcmoM strain, as reported previously13 (Fig. 4a), indicating that the terminal methylation of mcm5U34 has little impact on cell growth. A slight increase of doubling time was observed in the ΔcmoB strain, in which ho5U34 accumulated, indicating that the carboxymethyl group of (m)cmo5U34 contributes to efficient growth of E. coli cells. Notably, the ΔtrhP/ΔtrhO strain grew more slowly than the WT and ΔcmoB strains, providing a clear evidence for the functional importance of 5-hydroxyl group of (m)cmo5U34 in cells. To characterize phenotypic features of the ΔtrhP/ΔtrhO strain, we further knocked out tRNA genes responsible for G-ending codons [serU (tRNA^Ser2) for the UCG codon, thrW (tRNA^Thr2) for the AGC codon, and proK (tRNA^Pro1) for the CCG codon], because these codons are redundantly deciphered by the respective tRNA and the isodecoder with the (m)cmo5U34 modification in each codon box. Growth reduction of the ΔtrhP/ΔtrhO strain relative to the ΔcmoB strain was observed upon knockout of serU, thrW, and proK (Fig. 4a), indicating that the 5-hydroxyl group of (m)cmo5U34 plays a critical role in deciphering G-ending codons, especially in the absence of the respective isodecoder.

Next, we examined the temperature sensitivity of a series of knockout strains involved in (m)cmo5U34 modifications. The ΔtrhP1/trhP2 strain, which was present at 50% of the WT level, completely disappeared in the triple-deletion strain (Supplementary Fig. 5). However, as observed in the E. coli ΔtrhO strain, little reduction in the m5U34 level was observed in the single-deletion strain ΔybfQ (Supplementary Fig. 5).
on LB plates at 30 °C, but slowly at 37 °C and not at all at 42 °C (Fig. 4b). The growth defect of this strain at 42 °C was restored by introduction of plasmid-encoded trhP (Supplementary Fig. 8), indicating that the temperature-sensitive phenotype of this strain can be attributed to hypomodification of tRNA^Ser^1. Curiously, the ΔtrhP/ΔserU strain did not exhibit temperature sensitivity, although the frequency of mcmo^5^U34 actually decreased in tRNA^Ser^1 (Fig. 2c), indicating that trhO-mediated hydroxylation compensates for the growth defect when the trhP-mediated hydroxylation pathway is impaired. This result highlights the importance of redundant hydroxylation pathways for formation of xo^5^U. Similarly, we observed a severe growth reduction in the ΔtrhP/ΔtrhO/ΔthrW strain cultivated on M9 minimum plates, even at 40 °C (Fig. 4c). At 42 °C, this strain was unable to grow,
and ΔcmoB strain exhibited a growth defect. These results clearly demonstrated that the absence of the 5-hydroxyl group of (m) cmo5U34 causes a temperature-sensitive phenotype, especially in the absence of the isodecoder responsible for G-ending codons.

Decoding properties without tRNA hydroxylation. To investigate the functional role of (m)cmo5U34 modification in terms of decoding efficiency, we conducted dual-luciferase reporter assays based on the RF2 recoding system18,45. The reporter constructs consisted of Renilla luciferase (Rluc) fused with firefly luciferase (Fluc) in a +1 frame via a slippery linker derived from the +1 frameshift signal of the RF2 recoding site, so that Fluc expression requires a +1 frameshift at the linker sequence. The UGA codon at the recoding site was substituted with GUC and UCG as test codons to examine their ability to be decoded by tRNAAla1 and tRNAser1, respectively (Fig. 4d). We also prepared a control reporter in which the recoding site was replaced with GG (zero frame). These reporters were introduced to a series of reporter constructs, because in the absence of isodecoder responsible for G-ending codons.

\[ \Delta \text{trhO} \Delta \text{serU} (\text{U34}) \] had significantly higher +1 frameshift activity than ΔcmoB/ΔserU (ho5U34) (Fig. 4e), indicating that the 5-hydroxyl group of ho5U34 on tRNAser1 can decode the UUC codon, especially in the absence of isodecoder tRNAser2.

Characterization of trhP-mediated tRNA hydroxylation. According to our recent study49, the shikimate pathway is associated with rhlA-mediated ho5C2501 formation in 23S tRNA. A series of genetic studies revealed that prephenate is an essential metabolite for the first step of this modification. Given that TrhP belongs to a family of peptidase U32-containing proteins, we asked whether prephenate is also required for trhP-mediated ho5U34 formation. Consistent with this possibility, previous studies reported that the initial step of cmo5U34 formation is associated with chorismate biogenesis in E. coli, B. subtilis, and Salmonella typhimurium46,49. Chorismate is an end product of the shikimate pathway and a common precursor for aromatic amino acids and vitamins in bacteria and plants47. Shotgun analyses of E. coli total RNA revealed that cmo5U34 formation was significantly impaired in an ΔaroC strain, in which no chorismate was detected (Fig. 5a). To dissect these two enzymatic activities, we constructed a series of genetic studies revealing that prephenate or its downstream metabolites are required for trhP-mediated ho5U34 formation.

\[ \Delta \text{trhP} \] strain (U34) was detected in an ΔaroC strain (Supplementary Fig. 10). In E. coli, chorismate is converted into five metabolites: isochorismate (catalyzed by the products of entC and menF), 4-hydroxybenzoate (ubIC), 4-amino-4-deoxycytidine (padB), and prephenate (pheA and tyrA) (Fig. 5a). Among these pathways, the ubIC-mediated pathway was excluded because 4-hydroxybenzoate does not restore cmo5U34 formation in Salmonella ΔaroD strain46. As observed in the ΔaroC strain, cmo5U34 formation was only impaired in the pheAΔ/ΔtyrA strain, but not in the ΔentC/ΔmenF, ΔpadB, and ΔtrpE strains (Supplementary Fig. 9), indicating that prephenate or its downstream metabolites are required for ho5U34 formation.

Prephenate is converted to downstream metabolites via three pathways (Fig. 5a). cmo5U34 formation was unchanged in ΔtyrA and ΔtrpB strains (Supplementary Fig. 9). pheA encodes a fusion of chorismate mutase (CM) and prephenate dehydratase (PDT), which synthesizes prephenate and phenylpyruvate, respectively (Fig. 5a). To dissect these two enzymatic activities, we constructed a pheA variant possessing only the CM activity [phea(CM)] by introducing an active-site mutation in the PDT domain46. To determine whether prephenate is responsible for ho5U34 formation, we constructed the quadruple-knockout strain ΔpheAΔtyrAΔcmoAΔtrhO, and then introduced plasmid-encoded phea(CM), resulting in accumulation of prephenate. ho5U34 levels were restored relative to those in a mock...
transformant (Fig. 5b). Furthermore, when prephenate was directly added to a culture medium of the quadruple-knockout strain, ho^5^U34 clearly appeared (Fig. 5c). These results demonstrated that prephenate is required for ho^5^U34 formation.

To characterize the peptidase U32 domain of trhP, we mutated each of six conserved residues in this domain (Fig. 5d, Supplementary Fig. 11) and examined their activities in vivo by complementation of the ΔtrhP/ΔtrhO strain. Shotgun analyses revealed that cmo^5^U34 was fully restored by WT trhP, but not by any of the mutants examined in this study (Fig. 5e), indicating that the peptidase U32 domain plays a key role in trhP-mediated tRNA hydroxylation.

### Characterization of trhO-mediated tRNA hydroxylation

Given that Fe(II)- and 2-OG-dependent oxygenases use O_2_ as a
Fig. 5 Characterization of trhP-mediated tRNA hydroxylation. a A shikimate pathway and related metabolism. Chemical structures of metabolites and the responsible genes (italicized) at each step are shown. Two or three genes at each step indicate a redundant pathway [e.g., prephenate is redundantly synthesized from chorismate mediated by pheA(CM) and tyrA]. Black or gray arrows represent pathways indispensable or dispensable for trhP-mediated ho3U34 formation, respectively (Supplementary Fig. 9). White arrows represent pathways not examined in this study. b Genetic complementation of ho3U34 formation. Mass-spectrometric shotgun analysis of total tRNAs obtained from the E. coli ΔpheA/ΔtyrA/ΔcmoA/ΔtrhO strain transformed with a control plasmid (left panels) or pMW-phaCM (right panels). XICs show multiply charged negative ions of anticodon-containing fragments of tRNAVal with U34 (upper panels) and ho5U34 (lower panels). Sequence, m/z value, and charge state of each fragment are shown on the right. c Metabolic complementation of ho5U34 formation. Mass-spectrometric shotgun analysis of total tRNAs obtained from the E. coli ΔtreP/Δα cmoA/ΔtrhO strain cultured in the absence (left panels) or presence (right panels) of 1 mM prephenate. XICs show multiply charged negative ions of anticodon-containing fragments of tRNAVal with U34 (upper panels) and ho5U34 (lower panels). Sequence, m/z value, and charge state of each fragment are shown on the right. d Domain organization of E. coli TrhP, which contains Peptidase_U32 (PF01136) and Peptidase_U32_C (PF06325) domains. Six residues in the Peptidase_U32 domain that are essential for TrhP-mediated hydroxylation are indicated. e Mutation study of trhP. Mass-spectrometric shotgun analysis of total RNA in the E. coli ΔtrhP strain transformed with plasmid-encoded trhP WT or mutants, as indicated. XICs show multiply charged negative ions of the anticodon-containing fragments of tRNAVal with U34 (black lines) and cmo5U34 (red lines) in total tRNAs. Sequence, m/z value, and charge state of each fragment are shown on the right.

Discussion

In this study, we identified two independent pathways, mediated by trhP and trhO, involved in tRNA hydroxylation in the early steps of (m)cmo5U34 formation. We confirmed that (m)cmo5U34 was completely converted to unmodified U34 in a ΔtrhP/ΔtrhO strain. This finding enabled us to analyze the physiological roles of (m)cmo5U34. This strain grew more slowly than a ΔcmoB strain, which has ho5U34, confirming the physiological importance of the O5 oxygen atom of (m)cmo5U34. In addition, the ΔtrhP/ΔtrhO strain exhibited severe growth defects and temperature-sensitive phenotypes when each of the tRNA genes (serU, thrW, and proK) responsible for G-ending codons was simultaneously deleted. These genetic interactions strongly indicate that (m)cmo5U34 plays a functional role in efficiently deciphering G-ending codons. The luciferase reporter assay revealed that decoding of UCG was significantly impaired in the ΔtrhP/ΔtrhO strain relative to the ΔcmoB strain in the absence of tRNAser2, suggesting that the ho5UGA anticodon decodes the UCG codon more efficiently than the UGA anticodon. These observations demonstrate the direct involvement of the O5 oxygen of the xo5U34 modification in codon recognition in vivo.

According to the crystal structure of the ribosome 30S subunit in complex with the anticodon-stem loop (ASL) of tRNA, cmoU-G pairing forms Watson–Crick geometry (Supplementary Fig. 17), which is more stable than U-G wobble geometry due to the stacking interaction with the neighboring base pair (i.e., the second letters of codon and anticodon)19. The O5 oxygen of cmo5U34 makes a hydrogen bond with 2’OH of U33 to pre-structure the ASL, presumably reducing entropic cost to base pair with any codons. In addition, the O5 oxygen may induce keto-enol tautomer conversion of the uracil base to stabilize cmo5U-G pairing in Watson–Crick geometry. Moreover, because (m)cmo5U34 decodes G-ending codons more efficiently than...
ho5U3413,18, the carboxymethyl group and terminal methylation of (m)cmo5U34 contribute further to efficient codon recognition. Structural analysis has shown that the carboxymethyl group of cmo5U34 forms a hydrogen bond with the O4 carbonyl oxygen of U in the first letter of the codon, implying that the cmo5U side chain is directly involved in codon recognition19. Because ho5U34 has a phenolic hydrogen, and the pKa value of O5 is 7.7851, ho5U34 is ionized to some extent under neutral pH conditions. Thus, the carboxymethylation of cmo5U34 and methylation of mo5U34 might confer efficient codon recognition by suppressing the ionization of ho5U34.

Our findings reveal that the TrhP-dependent pathway requires prephenate, whereas the TrhO-dependent pathway requires molecular oxygen. Thus, these two pathways are biochemically independent with respect to their requirement for metabolites. The existence of redundant and robust pathways for ho5U formation emphasizes that the xo5U34 modification is essential for bacteria to survive in a harsh environment. This is the unique instance of the RNA modification synthesized by two independent pathways in the same organism. According to phylogenetic distribution analysis in all domains of life (Supplementary Fig. 6), some organisms possess both trhP and trhO genes, whereas other...
organisms possess just one of them. Considering that anaerobes preceded aerobes in the early evolution on Earth, the \textit{trhO} pathway might have been established in anaerobic bacteria before the \textit{trhO} pathway arose, assuming that xo\textsuperscript{5}U34 was present in such ancestral organisms. Essentially, the \textit{trhO} pathway was acquired by aerobic bacteria after the O\textsubscript{2} concentration increased on Earth. The \textit{trhP} pathway is required for anaerobic bacteria, whereas the \textit{trhP} and \textit{trhO} dual pathways are useful for organisms that live in both anaerobic and aerobic environments.

\textit{TrhP} is a peptidase U32-containing protein. Phylogenetic analysis has shown that peptidase U32-containing proteins can be classified into 12 subfamilies (Supplementary Fig. 1)\textsuperscript{30}. We showed previously that three of these families include the RhA proteins (RhA1, RhA2a, and RhA2b) responsible for ho\textsuperscript{5}C formation in 23S rRNA\textsuperscript{30}. It is plausible that other subfamilies are also involved in hydroxylation of RNA or other biomolecules. \textit{Clostridia} species harbor a member of the PepU32\#1 family and \textit{trmR}, but no homologs of \textit{trhO} or \textit{trhP}, indicating that mo\textsuperscript{5}U34 is present and that PepU32\#1 family proteins are functional homologs of \textit{trhP} in these species. \textit{Helicobacter pylori}, a representative of the e-proteobacteria, possesses a PepU32\#2 family protein (HP0169), moA, and cmoB, but no homologs of \textit{trhO} or \textit{trhP}, indicating that cmo\textsuperscript{5}U34 is present and suggesting that HP0169 is responsible for prephenate-dependent ho\textsuperscript{5}U34 formation in this species. Intriguingly, HP0169 is required for gastric colonization by \textit{H. pylori}\textsuperscript{52}. Similarly, in \textit{Salmonella enterica}, a \textit{trhP} ortholog is associated with chicken macrophage infection\textsuperscript{53}. Together, these findings suggest that xo\textsuperscript{5}U34 contributes to bacterial infection and pathogenesis.

We found that prephenate is required for \textit{trhP}-dependent ho\textsuperscript{5}U34 formation. Given that prephenate is also a substrate for cmo\textsuperscript{5}U34 formation mediated by CmoA and CmoB\textsuperscript{24}, it is a critical metabolite involved in the entire pathway of cmo\textsuperscript{5}U34 biogenesis. Prephenate is generated from chorismate, which in turn is a common precursor of multiple metabolites, including aromatic amino acids, quinones, folate, and siderophores\textsuperscript{47}. Thus, cmo\textsuperscript{5}U34 modification might be tightly associated with the shikimate pathway and biogenesis of aromatic amino acids. The frequency of cmo\textsuperscript{5}U34 might be regulated by the cellular concentration of prephenate under some environmental stress conditions.

\textit{TrhP} is a paralog of RhA in the same family of peptidase U32-containing proteins. RhA is responsible for prephenate-dependent ho\textsuperscript{5}C2501 formation in \textit{E. coli} 23S rRNA\textsuperscript{30}, strongly implicating the involvement of the peptidase U32 motif in the C5-hydroxylation of pyrimidine base. Here, we showed that three conserved residues (E162, C170, and C177) in the motif of \textit{TrhP} are essential for ho\textsuperscript{5}U34 formation. Additionally, the corresponding residues (E161, C169, and C176) in RhA were also required for ho\textsuperscript{5}C2501 formation\textsuperscript{30}, demonstrating that the peptidase U32 motif is directly involved in the hydroxylation of RNA. To date, we have no evidence that \textit{TrhP} and RhA directly catalyze the hydroxylation of RNA molecules. Given that RhA is directly bound to the 50S subunit and its precursor in the cell, RhA might be the hydroxylase responsible for ho\textsuperscript{5}C2501 formation. By analogy, \textit{TrhP} might be a hydroxylase for tRNA. Regarding the role of prephenate in ho\textsuperscript{5}U34 formation, several possibilities should be considered. Prephenate might serve as an oxygen donor for ho\textsuperscript{5}U34 formation, or alternatively as a coenzyme for the reaction. Moreover, we cannot exclude the possibility that unknown metabolites derived from prephenate are involved in ho\textsuperscript{5}U34 formation. Further studies are necessary to elucidate the molecular mechanism underlying ho\textsuperscript{5}U34 formation mediated by \textit{TrhP} and prephenate.

\textit{trhO} homologs are present in many aerobes and facultative anaerobes, but not in obligate anaerobes such as \textit{Bacteroides}, \textit{Clostridium}, and \textit{Bifidobacterium}. \textit{trhO} homologs are distributed in a wide range of bacteria, including \alpha-, \beta-, and \gamma-proteobacteria, Bacilli, actinobacteria, the FCB group, cyanobacteria, and a subset of phylum Tenericutes (Supplementary Fig. 6). Intriguingly, \textit{trhO} homologs are also widely distributed in vertebrates and other eukaryotes. This finding suggests the presence of an xo\textsuperscript{5}U-type modification in eukaryotes.

We also showed that \textit{TrhO} directly catalyzes oxygen-dependent ho\textsuperscript{5}U34 formation. \textit{TrhO} is related to rhodanese, which is involved in persulfide formation during detoxification of cyanide; however, the functions of most rhodanese family proteins remain unclear. In the context of RNA modifications, Tum1p is a rhodanese protein that mediates a persulfide sulfur for 2-thiouridine synthesis in eukaryotes\textsuperscript{38}. Bacterial YbbB (also known as MnmH) is another rhodanese family protein responsible for biogenesis of 2-selenouridine (se2U)\textsuperscript{54} and geranyl-2-thiouridine (ges2U)\textsuperscript{55}. Mutation study of \textit{TrhO} revealed that the active-site loop of the rhodanese domain is responsible for ho\textsuperscript{5}U34 formation, suggesting that the rhodanese domain plays a critical role in hydroxylation of uracil base. Future studies should seek to clarify the mechanism by which rhodanese catalyzes this reaction.

We now have a complete picture of xo\textsuperscript{5}U34 formation in bacteria (Fig. 7). In the first step, U34 is redundantly hydroxylated by \textit{TrhP} and \textit{TrhO} to form ho\textsuperscript{5}U34 in tRNAs responsible for decoding NYN codons. \textit{TrhP} requires prephenate as a metabolite for ho\textsuperscript{5}U34 formation, whereas \textit{TrhO} uses a molecular oxygen for this purpose under aerobic conditions. In \textit{E. coli}, \textit{TrhP}
is involved in ho5U34 formation of all six tRNA species, but has a preference for tRNAAla, tRNAPhe, and tRNAPro, whereas ThrO mainly hydroxylates the other four species. CmoA employs prephenate and AdoMet to generate SCM-SAH, a metabolite used for carboxymethylation of ho5U34 to yield cmo5U34. CmoB uses SCM-SAH to carboxymethylate ho5U34 to yield cmo5U34. Four tRNAs (for Ala1, Ser1, Pro3, and Thr4) are further methylated by CmoM to yield mcmo5U34. In Gram-positive bacteria, including B. subtilis, ho5U34 is methylated by TrmR to yield mo5U34 instead.

**Methods**

**Strains and media.** A series of single-knockout strains of *E. coli* and their parent strain were obtained from the National BioResource Project (NBRP), National Institute of Genetics (NIG), Japan (Keio collection)48. Other knockout strains were generated by homologous recombination using pCP20 transformation50. The KanR marker was removed by pCPl transformation51. Genotyping of each construct was performed by colony PCR. A series of knockout strains were isolated from genomic DNAs of strains which lack prephenate dehydratase activity due to T278A mutation48, and a series of point mutants of pMW-trhP and pMW-trhO were constructed by QikChange site-directed mutagenesis (Agilent Technology). For expression vectors of TrhO (YceA) and TrmR (YrrM), plasmids of *E. coli* and *B. subtilis* were constructed by Sanger sequencing. All primers used in this study are listed in Supplementary Data 3.

**RNA extraction and tRNA isolation.** Total RNA from each *E. coli* strain was extracted by phenol under acidic conditions49. The cells suspended in 1× TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA) was mixed with an equal volume of water-saturated phenol, followed by freeze and thaw twice and vigorous mixing for 1 hour at room temperature. The aqueous phase was separated by centrifugation, transferred to a new tube, washed with chloroform, and then precipitated using 2-propanol at −20°C, and resuspended in TE buffer. Total RNA was recovered by 2-propanol precipitation. Individual tRNAs were isolated with ethanol from the supernatant. For RNA-MS shotgun analyses of *E. coli* strains, total RNA was extracted by phenol under acidic conditions49. The cells suspended in 1× MOPS buffer (10 mM MOPS, 0.6 mM Glu/Glu/Val, 0.3 mM Arg/Asp/Leu/Lys/Phe/Pro/Thr, 0.2 mM His/Met/Tyr, and 0.1 mM Cys/Trp), 2 M guanidinium thiocyanate (Glu/Glu/Val), 2 M guanidinium thiocyanate (Arg/Asp/Leu/Lys/Phe/Pro/Thr), 0.2 M guanidinium thiocyanate (His/Met/Tyr), and 0.1 M guanidinium thiocyanate (Cys/Trp), 500 uM thiamine hydrochloride, 0.02 M calcium pantothenate, 0.02 M 4-amino benzoic acid, 0.02 M 4-hydroxybenzoic acid, 0.02 mM 2,3-dihydroxybenzoic acid), 0.4% glucose, and appropriate antibiotics, in the presence or the absence of 1 mM prephenate (Sigma-Aldrich, St. Louis, MO, USA), was used to determine the metabolite required for the trhP pathway.

**Plasmid construction.** For the genetic rescue study, CDSs of trhP, trhO, and pheA with their 200 bp upstream sequences (including native promoter regions) were PCR-amplified from the *E. coli* genome and cloned into pMW118 (Nippon Gene) to yield pMW-trhP, pMW-trhO, and pMW-pheA, respectively. The pMW-pheA(CM), which lacks prephenate dehydratase activity due to the T278A mutation48, and a series of point mutants of pMW-trhP and pMW-trhO were constructed by QikChange site-directed mutagenesis (Agilent Technology). For expression vectors of TrhO (YceA) and TrmR (YrrM), CDSs of *E. coli* and *B. subtilis* were PCR-amplified from genomic DNAs of *E. coli* genome and *B. subtilis* genome, respectively, and cloned into pET21b (Novagen) to yield pET-trhP and pET-trmR. All constructs were confirmed by Sanger sequencing. All primers used in this study are listed in Supplementary Data 3.

**RNA extraction and tRNA isolation.** Total RNA from each *B. subtilis* strain was extracted by phenol under acidic conditions49. The cells suspended in 1× TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA) was mixed with an equal volume of water-saturated phenol, followed by freeze and thaw twice and vigorous mixing for 1 hour at room temperature. The aqueous phase was separated by centrifugation, transferred to a new tube, washed with chloroform, and then precipitated using 2-propanol at −20°C, and resuspended in TE buffer. Total RNA was recovered by 2-propanol precipitation. Individual tRNAs were isolated with ethanol from the supernatant. For RNA-MS shotgun analyses of *B. subtilis* strains, total RNA was extracted by phenol under acidic conditions49. The cells suspended in 1× MOPS buffer (10 mM MOPS, 0.6 mM Glu/Glu/Val, 0.3 mM Arg/Asp/Leu/Lys/Phe/Pro/Thr, 0.2 M His/Met/Tyr, and 0.1 M Cys/Trp), 2 M guanidinium thiocyanate (Glu/Glu/Val), 2 M guanidinium thiocyanate (Arg/Asp/Leu/Lys/Phe/Pro/Thr), 0.2 M guanidinium thiocyanate (His/Met/Tyr), and 0.1 M guanidinium thiocyanate (Cys/Trp), 500 uM thiamine hydrochloride, 0.02 M calcium pantothenate, 0.02 M 4-amino benzoic acid, 0.02 M 4-hydroxybenzoic acid, 0.02 mM 2,3-dihydroxybenzoic acid), 0.4% glucose, and appropriate antibiotics, in the presence or the absence of 1 mM prephenate (Sigma-Aldrich, St. Louis, MO, USA), was used to determine the metabolite required for the trhP pathway.
follows\textsuperscript{13,35}. For RNA fragment analyses of individual tRNAs and shotgun analyses, 1.25 pmol of tRNA or 50 ng of the small RNA fraction was digested with 50 units RNase T1 (Thermo Scientific) in 20 mM NH\textsubscript{4}OAc (pH 5.3), followed by digestion of an equal volume of 0.1 M triethylamine hydrochloride (TEAA; pH 7.0). The RNA T\textsubscript{1} digests were subjected to the trap column for desalting and chromatographed by H\textsuperscript{2}O, C\textsubscript{18} and 

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[97.5\% 18O\textsubscript{2}, 1\% 17O\textsubscript{2}, 1.5\% 16O\textsubscript{2}], 80\% N\textsubscript{2}\textsuperscript{[44x211]} was obtained commercially (Tatsuoka, Japan). The

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the separation. XICs were plotted according to the theoretical m/z of each fragment (Supplementary Table 1). LC/MS nucleoside analysis was performed using Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) equipped with a Dionex UltiMate 3000 LC System (Thermo Fisher Scientific) essentially as described\textsuperscript{13,39}. Twenty micrograms of the small RNA fraction were digested at 37 °C for 3 h in 60 ml of solution containing 20 mM trimethylamine-HCl (TMA-HCl) (pH 7.0), 0.05 U of nuclease P1, and 0.1 U of RAP. The digests mixed with acetonitrile (f.c. 90%) were chromatographed by ZIC-cHILIC column (3 mm inner diameter, 2.1 × 20 mm, Merck Millipore) using a solvent system consisted of 5 mM TMA-HCl (pH 7.0), 20 mM Mg(OAc)\textsubscript{2}, 100 mM KCl, 10 mM MgCl\textsubscript{2} and 7 mM 2-mercaptoethanol in cold room. The gel was first stained with SYBR Safe (Thermo Fisher Scientific) to detect RNA and then with Coomassie brilliant blue (Nalcaci Tesque) to detect protein.

Electrophoresis mobility shift assay. EMSA was performed essentially as described\textsuperscript{34}. Recombinant TrhO (0, 10, 20, 40, or 80 pmol) and in vitro transcribed tRNA\textsubscript{Ala} or tRNA\textsubscript{Leu} (20 pmol each) were incubated at 37 °C for 1 h in 10 ml of reaction mixture [50 mM HEPES-KOH (pH 7.5), 5 mM Mg(OAc)\textsubscript{2}, 100 mM KCl, 1 mM spermine, 1 mM DTT]. The mixtures were electrophoresed in 6% native polyacrylamide gel with running buffer [50 mM HEPES-KOH (pH 7.5), 5 mM Mg(OAc)\textsubscript{2}, and 1 mM DTT] in cold room. The gel was stained with SYBR Safe (Thermo Fisher Scientific) to detect tRNA and then with Coomassie brilliant blue (Nalcaci Tesque) to detect protein.

Comparative genomics. The comparative genomics approach used to identify the trhO (yea1) gene was performed with the IMG database\textsuperscript{25}. The gene occurrence profile was used to select seven organisms in which cmo\textsubscript{BA} and yrrM homologs were present and trhP (yea2) or trhP/trhR homologs were absent: Ehrlichia canis strain Jake, Erwinia pyrifoliae DSM12163, Alcanivorax borkumensis SK2, Candidatus Phytomyxa asteris onion yellow OY-M, Pontibacter actinarium sp. BAB170, Dacyloccoccus salina PCC 8305, and Psychrobacter arcicrus 273-4. Using the phylogenic profiler, E. coli genes that are conserved in these seven organisms and \textit{B. subtilis} were identified. According to the UniProt gene annotation, seven uncharacterized genes were picked as candidates.

Phylogenetic analysis. The phylogenetic tree of peptidase U32 (Supplementary Fig. 1) was generated as described\textsuperscript{36}. Species names matched to proteins were retrieved from UniProt. The occurrence profiles of trhO, trhP, trhP1, trhP2, cmoA, cmoB, trhR, trhM, and cmo homolog (Supplementary Fig. 6) were retrieved from GTOP\textsuperscript{62} or the Interpro database\textsuperscript{63}. To generate the phylogenetic tree, 584 organisms that are registered in the databases we used, i.e., species listed in GTOP, phytoL, and Pfam, were selected.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

A reporting summary for this Article is available as a Supplementary Information file. The source data for graphs and gels are provided as a Source Data file. All data is available from the corresponding author upon reasonable request.

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Preparation of recombinant protein. \textit{E. coli} BL21(DE3) transformed with pET-trhO was cultured at 37 °C until OD\textsubscript{600} reached ~0.7, supplemented with 0.4% lactose, and further cultured at 16 °C for 24 h. The \textit{E. coli} Rosetta(DE3) transformed with pET-trhO was cultured at 37 °C until OD\textsubscript{600} reached ~0.7, supplemented with 0.4% lactose, and further cultured at 16 °C for 24 h. The \textit{E. coli} Rosetta(DE3) transformed with pET-trhO was cultured at 37 °C until OD\textsubscript{600} reached ~0.7, supplemented with 0.4% lactose, and further cultured at 16 °C for 24 h. The \textit{E. coli} Rosetta(DE3) transformed with pET-trhO was cultured at 37 °C until OD\textsubscript{600} reached ~0.7, supplemented with 0.4% lactose, and further cultured at 16 °C for 24 h. The \textit{E. coli} Rosetta(DE3) transformed with pET-trhO was cultured at 37 °C until OD\textsubscript{600} reached ~0.7, supplemented with 0.4% lactose, and further cultured at 16 °C for 24 h. The \textit{E. coli} Rosetta(DE3) transformed with pET-trhO was cultured at 37 °C until OD\textsubscript{600} reached ~0.7, supplemented with 0.4% lactose, and further cultured at 16 °C for 24 h.

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**Author contributions**

Y.S. and S.K. performed a series of experiments. All authors discussed the results. Y.S. and T.S. wrote this paper. Y.S., S.K., and T.S. designed the research structure. T.S. supervised all the work.

**Additional information**

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