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Cys-tRNA_i as a Second Translation Initiator for Priming Proteins with Cysteine in Bacteria

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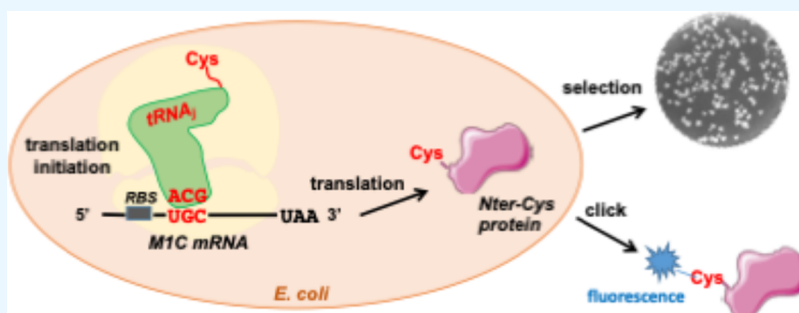
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ABSTRACT: We report the construction of an alternative protein priming system to recode genetic translation in *Escherichia coli* by designing, through trial and error, a chimeric initiator whose sequence identity points partly to elongator tRNA^{Cys} and partly to initiator tRNA_f^{Met}. The elaboration of a selection based on the N-terminal cysteine imperative for the function of glucosamine-6-phosphate synthase, an essential enzyme in bacterial cell wall synthesis, was a crucial step to achieve the engineering of this Cys-tRNA_i. Iterative improvement of successive versions of Cys-tRNA_i was corroborated *in vitro* by using a biochemical luciferase assay and *in vivo* by selecting for translation priming of *E. coli* thymidylate synthase. Condensation assays using specific fluorescent reagent FITC-Gly-cyanobenzothiazole provided biochemical evidence of cysteine coding at the protein priming stage. We showed that translation can be initiated, by N-terminal incorporation of cysteine, at a codon other than UGC by expressing a tRNA_i with the corresponding anticodon. The optimized tRNA_i is now available to recode the priming of an arbitrary subset of proteins in the bacterial proteome with absolute control of their expression and to evolve the use of xenonucleotides and the emergence of a tXNA, *in vivo*.

INTRODUCTION

The initiator tRNA stands as a prominent target for molecular evolution and speciation at the deepest level of biochemical organization.¹ In all three domains of life, Eukarya, Archaea, and Bacteria, initiator tRNA (tRNA_i) is charged by methionyl-tRNA synthetase (MetRS). In bacterial cells, the resulting Met-tRNA_i is further formylated at its amino group to give fMet-tRNA_i. Subsequent pairing between the anticodon of the acylated tRNA_i and the initiator codon, mainly AUG, defines the translational-reading frame and determines the location of genes in mRNAs. The precise modalities of assembly between the mRNA and the ribosome geared to form the first peptide bond vary from one domain to another but constitute the rate-limiting step of gene expression in all known cells.²

We set out to implement a second protein priming system, mediated by an additional initiator tRNA (hereafter designated tRNA_j) and assigned to an amino acid other than methionine, so as to limit the development of a single additional device while keeping the rest of the translational machinery unchanged: elongator tRNAs, aminoacyl-tRNA synthetases,

factors, and ribosomes. Independent attempts to diversify initiator codons and priming amino acids have been successfully carried out by other research groups. On the one hand, the mobilization of elongator aminoacyl-tRNAs was found to marginally trigger initiation of translation using amino acid primers other than methionine.^{3,4} On the other hand, efficient priming was obtained by coexpressing a cognate couple of synthetase and tRNA and incorporating synthetic amino acids chemically related to pyrrolysine at the N-terminus of recoded proteins.^{5–9}

Cysteine (Cys) appeared to be the most suitable protein primer to build a second translation initiation system that incorporates a canonical amino acid. Unlike translation

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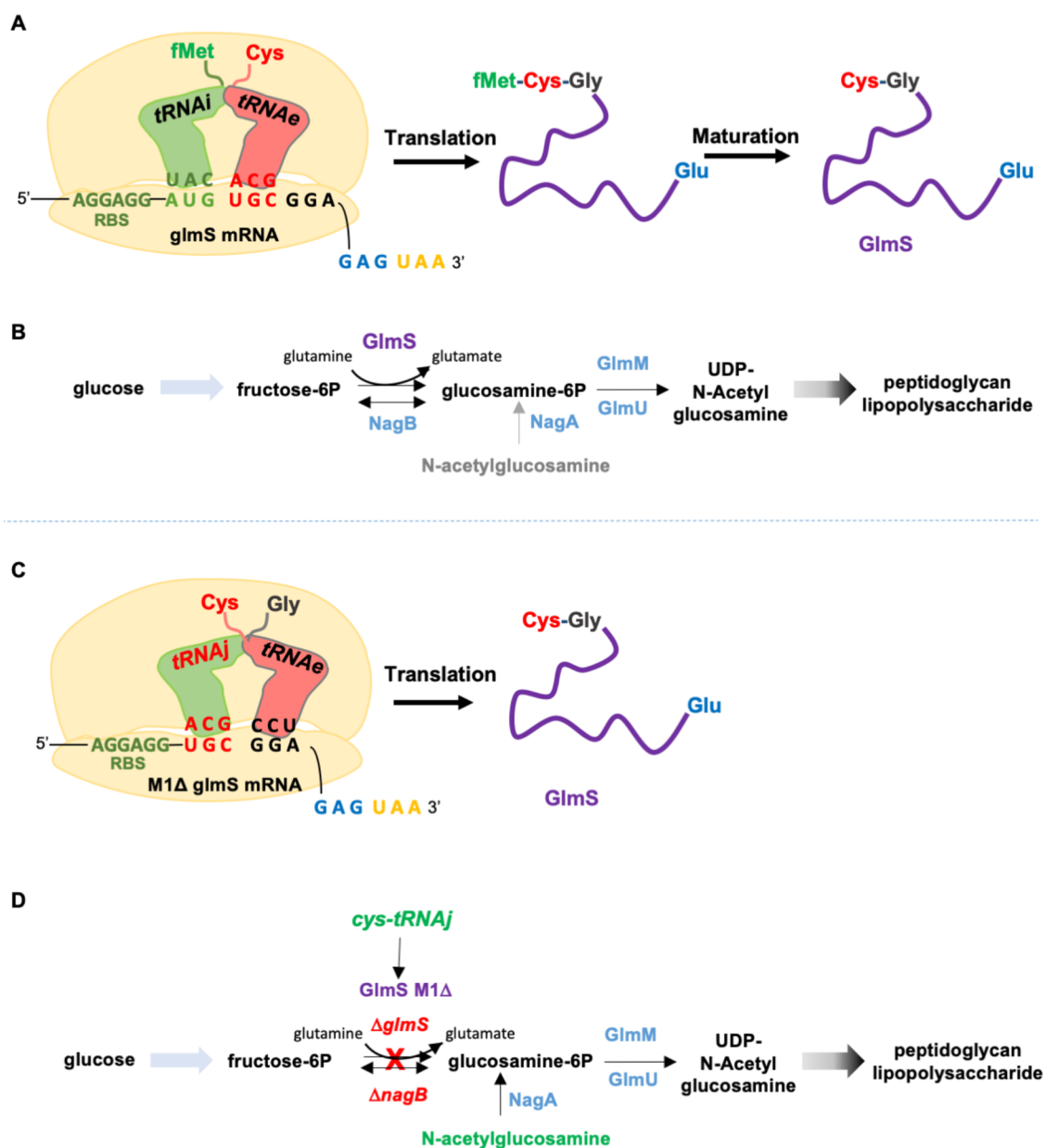


Figure 1. Metabolic selection for translation initiation of L-glutamine—D-fructose-6-phosphate aminotransferase at a cysteine codon by a tRNA_{Cys}. (A) Schematic representation of translation initiation of GlmS mRNA by tRNA_{fMet}. Codons for Cys1, Val2, Glu608, and stop codons of GlmS mRNA are shown. RBS for Ribosome Binding Site is indicated (B) UDP-N-acetyl-D-glucosamine biosynthesis from fructose-6-P involves the three enzymes L-glutamine—D-fructose-6-phosphate aminotransferase, phosphoglucosamine mutase and fused N-acetylglucosamine-1-phosphate uridylyltransferase and glucosamine-1-phosphate acetyltransferase respectively encoded by *glmS*, *glmM* and *glmU*. (C) Schematic representation of translation initiation by a tRNA_{Cys} of the GlmS mRNA encoded by a met1 deleted version of *glmS*. The same GlmS mature protein is produced. (D) In a strain lacking both L-glutamine—D-fructose-6-phosphate aminotransferase (*glmS*) and glucosamine-6-phosphate deaminase (*nagB*), cell growth can be restored if translation is initiated by a tRNA_{Cys} at the met1 deleted allele of *glmS* or by an exogenous supply of N-acetylglucosamine in the medium.

initiation by noncanonical amino acids, a canonical amino acid enables the establishment of metabolic selection *in vivo*. Cysteine has been characterized as a catalytic amino acid at the N-terminus of a few metabolic enzymes in *Escherichia coli*. This strategy differs from previous attempts to diversify translation initiation,^{10,11} by coupling cell growth with the priming of an essential enzyme. Another advantage of cysteine is that it lends itself to functionalization at the N-terminus of proteins due to its unique chemical properties, which opens the way for multiple applications in protein and organism engineering.^{12–14} Cysteine can be easily installed at the N-terminus

of a bacterial protein by specifying a UGU or UGC codon just following the initiator AUG codon. The translation product undergoes the successive action of peptide deformylase and methionine aminopeptidase, truncating fMet-Cys in the nascent protein to Cys in the mature protein.^{15,16} Thus, the same N-terminus of the mature protein can be directly obtained by starting translation with Cys instead of fMet-Cys. Beyond starting a protein with a cysteine, our study aims to control the initiation of translation at a cysteine codon by a Cys-tRNA_{Cys} that we can select *in vivo* for its efficiency and orthogonality. Our first step was to validate the functionality

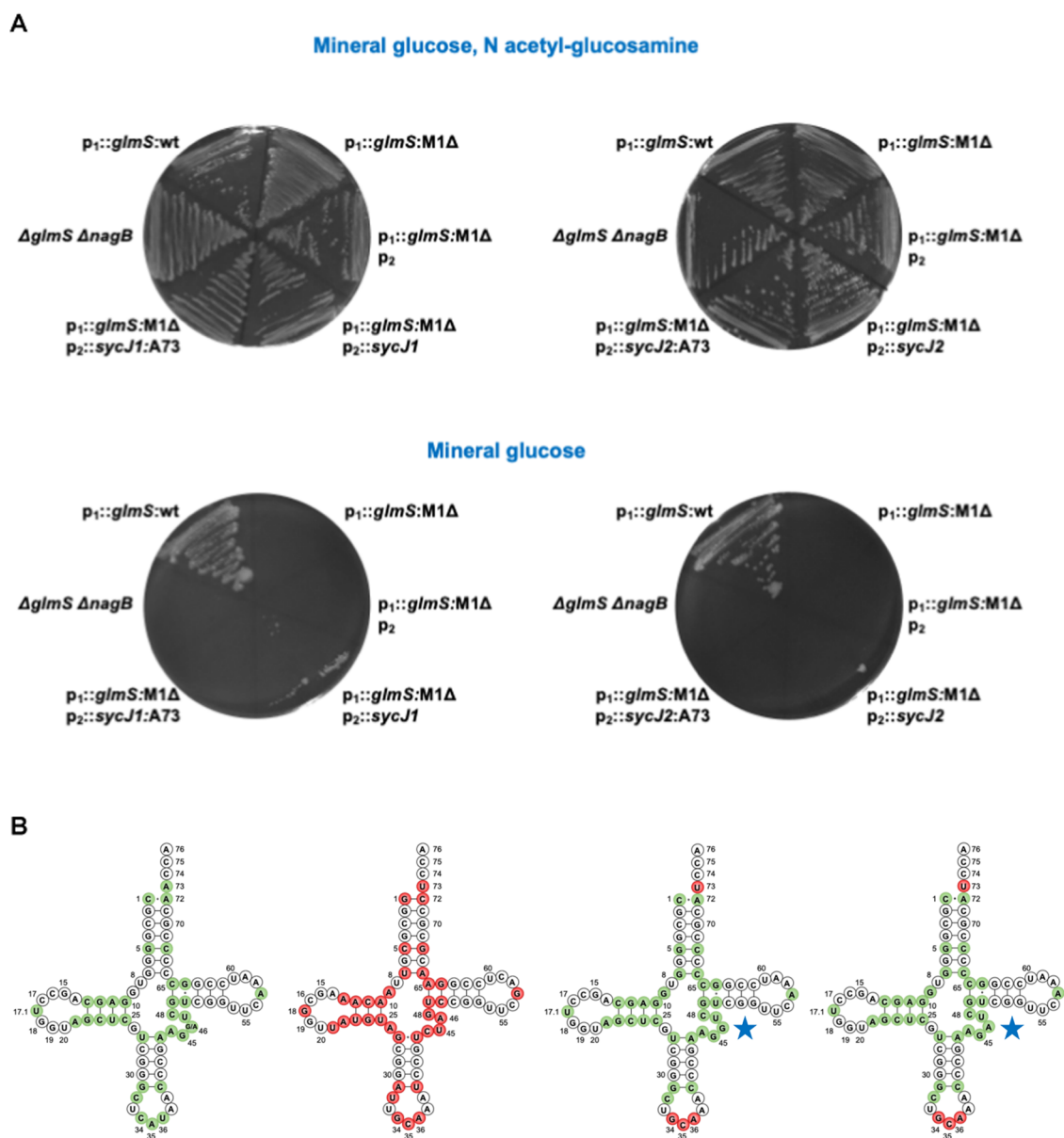


Figure 2. Growth assay validating the GlmS selection to initiate translation at a cysteine start codon by tRNA_i and cloverleaf depiction of tRNAs. (A) Growth of strains on mineral glucose medium plates supplemented with N-acetylglucosamine (top) and growth of corresponding strains on mineral glucose medium plates (bottom) after 5 days of incubation. “ $\Delta glmS$, $\Delta nagB$ ” denotes the GlmS selection strain (EX160). Other strains on the left plates are EX160 transformed with a plasmid harboring *glmS:wt* (XE3723), expressing the *sycJ1* gene (XE3727) or *sycJ1:A73* (XE4039). Right plates: growth of GlmS selection strains expressing the *sycJ2* gene (XE3732) or the *sycJ2:A73* gene (XE4038). (B) Schematic representations of *E. coli* initiator tRNA_i^{Met}; tRNA_i^{Met} (G46) or tRNA_i^{Met} (A46), - *E. coli* elongator tRNA^{Cys} and the two versions of the engineered tRNA_i: tRNA_i¹ (G46, blue star) and tRNA_i² (A46, blue star).

and utility of Cys-tRNA_i for bacterial cell growth. Our long-term goal is to reprogram the chemical structure of tRNA_i into XNA¹⁷ in order to mobilize noncanonical nucleotides in genetic translation well beyond the sequence diversity and post-transcriptional modifications of natural tRNAs.

Pallanck and Schulman showed early on that exchanging the anticodon and discriminator positions, totaling four nucleotides, of the *E. coli* tRNA_i for the corresponding bases of the cysteine elongator tRNA resulted in protein priming with cysteine.^{18,19} Starting from their chimeric construct, we systematically generated 176 variants and selected an initiator Cys-tRNA_i, deviating by up to 13 base changes, capable of priming mRNA translation at a rate up to 63% of the rate obtained for wild-type fMet-tRNA from *E. coli*. By taking

advantage of the catalytic cysteine at the N-terminus of the enzyme glucosamine-6-phosphate synthase, which is essential for bacterial proliferation, we validated the gene for tRNA_i as a dominant and minimalist 77-nucleotide genetic marker that exerts absolute nutritional selection of recoded *E. coli* strains in growth medium devoid of glucosamine. Using an *in vitro* readout of luciferase recoded at its N-terminus, it could be confirmed that a UGC cysteine codon preceded by a ribosome binding site triggers gene expression if and only if cells encode a cognate Cys-tRNA_i. The presence of the N-terminal cysteine of proteins, produced in our alternative translation initiation system, was demonstrated by coupling its thiol function to a fluorescent reactive compound.

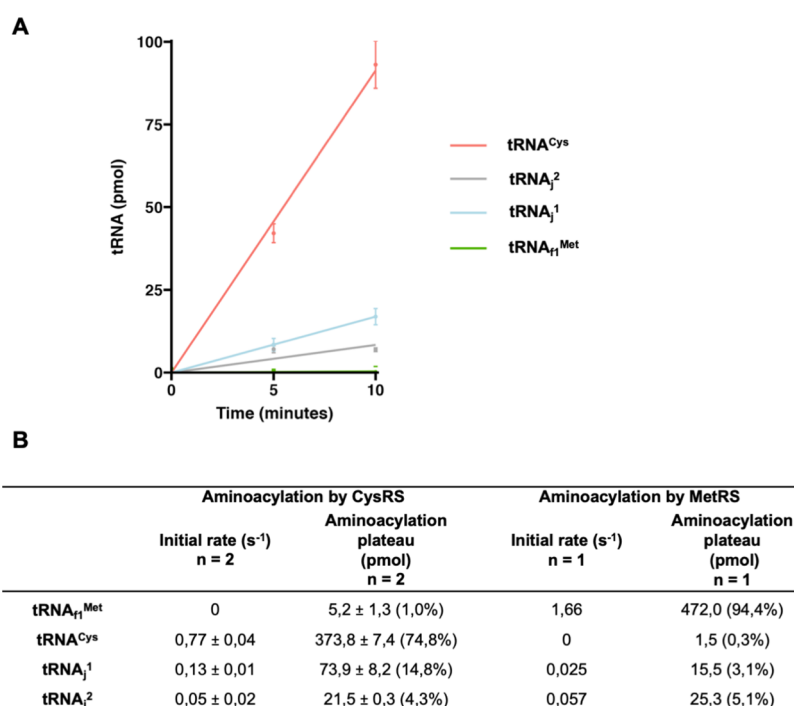


Figure 3. *In vitro* aminoacylation assays of tRNA^{Cys}, tRNA_f^{Met}, and tRNA_j by CysRS and MetRS. The initial rates of aminoacylation were determined using 2 nM enzyme and the plateau with 1 μM enzyme. tRNA concentration in the 100 μL assays was 5 μM. (A) Initial rate of aminoacylation of tRNA^{Cys}, tRNA_f^{Met}, and tRNA_j by the cysteinyl-tRNA synthetase. (B) Table summarizing *in vitro* aminoacylation assays with CysRS and MetRS. The initial rates of aminoacylation reactions and aminoacylation plateaus are indicated. Data are presented as mean ± SD (n = 2). The low methionylation plateaus of tRNA^{Cys} and tRNA_j can be accounted for by contamination of the tRNA preparations with *E. coli* tRNA_f^{Met}. Indeed, the initial rates of methionylation correspond to those expected for tRNA_f^{Met} present at concentrations that can be calculated from the plateau. Thus, the aminoacylation of tRNA_j by MetRS is not detectable. For aminoacylation plateaus, values in parentheses represent the percentage of aminoacylated-tRNA relative to tRNA used in the assay.

RESULTS

GlmS Metabolic Selection Scheme for Cysteine-Primed Translation. The L-glutamine-fructose-6-phosphate aminotransferase protein (also known as glucosamine-6-phosphate synthase) encoded by the essential *glmS* gene is one of the two proteins in *E. coli*, which harbors an N-terminal cysteine crucial for catalysis (Figure 1A). This enzyme catalyzes the conversion of fructose-6-phosphate into glucosamine-6-phosphate, a reaction of the UDP-*N*-acetyl-D-glucosamine biosynthesis pathway (Figure 1B).^{20–22} The latter (UDP-*N*-acetyl-D-glucosamine) is a precursor of hexosamines, which are key compounds of cell wall peptidoglycans, polysaccharides, and enterobacterial common antigens. Glucosamine-6-phosphate deaminase (encoded by *nagB*) is responsible for the reverse activity of GlmS (Figure 1B) and can suppress the defect of a *glmS* mutant when overexpressed.²³ An *E. coli* strain deleted in both *glmS* and *nagB* genes (EX160 strain) requires either a supply of glucosamine or *N*-acetylglucosamine in mineral medium²⁴ or the expression of a functional GlmS protein from a plasmid for growth (Figure 1D). We first showed that *glmS* expressed from a pUC-derived plasmid (pXEN1147 called p₁) could restore the growth of a *glmS* and *nagB* deleted strain in mineral medium lacking glucosamine or *N*-acetylglucosamine (Figure 2A). The cysteine requirement for GlmS activity was confirmed by constructing *glmS* variants, where the Cys-2 codon was either deleted or changed into a GCA (Ala) one. None of these variants could restore EX160 strain growth on a mineral medium (Figure S1).

To initiate translation at the Cys codon of the *glmS* mRNA, the wild-type initiator tRNA_f^{Met} sequence (Figure 2B) was modified by mutating the CAU anticodon into GCA (complementary to the UGC cysteine codon) as well as the discriminator base A73 into U which is required for cysteinyl-tRNA synthetase (CysRS) recognition (Figure 2B).²⁵ The modified initiator tRNA was called tRNA_j. Two versions, tRNA_j¹ and tRNA_j² were designed corresponding to the two different alleles of *E. coli* tRNA_f^{Met} *metZWV* (G46, hereafter named tRNA_f^{Met}) and *metY* (A46, hereafter named tRNA_f^{Met}), respectively (Figure 2B). The genes encoding tRNA_j¹ and tRNA_j² were named *syncJ1* and *syncJ2* and cloned into a pSU-derived plasmid (pEVL649 called p₂). We then tested the ability of these two tRNA constructs to restore growth on mineral medium of EX160 expressing a *glmS* version where the Met1 codon was deleted, thus using the UGC Cys codon as the initiator (Figure 1C). Only *syncJ1* gene expression, not that of *syncJ2*, was able to restore *E. coli* growth (Figure 2A). We confirmed that the initiation of translation by tRNA_j¹ required both the GCA anticodon and U73, as no cell growth was observed upon expression of tRNA_f^{Met} carrying A73 and the GCA anticodon (Figure 2A). The absolute nature of this selection was validated by the absence of growth of the selection strain expressing *glmS* M1Δ (XE3722) after one month at 37 °C.

To investigate the difference in translation initiation efficiency between the two tRNA_j versions (tRNA_j¹ and tRNA_j²), we performed *in vitro* aminoacylation assays using *E. coli* CysRS or methionyl-tRNA synthetase (MetRS) (Figure 3). This analysis corroborated the effects observed *in vivo*, with

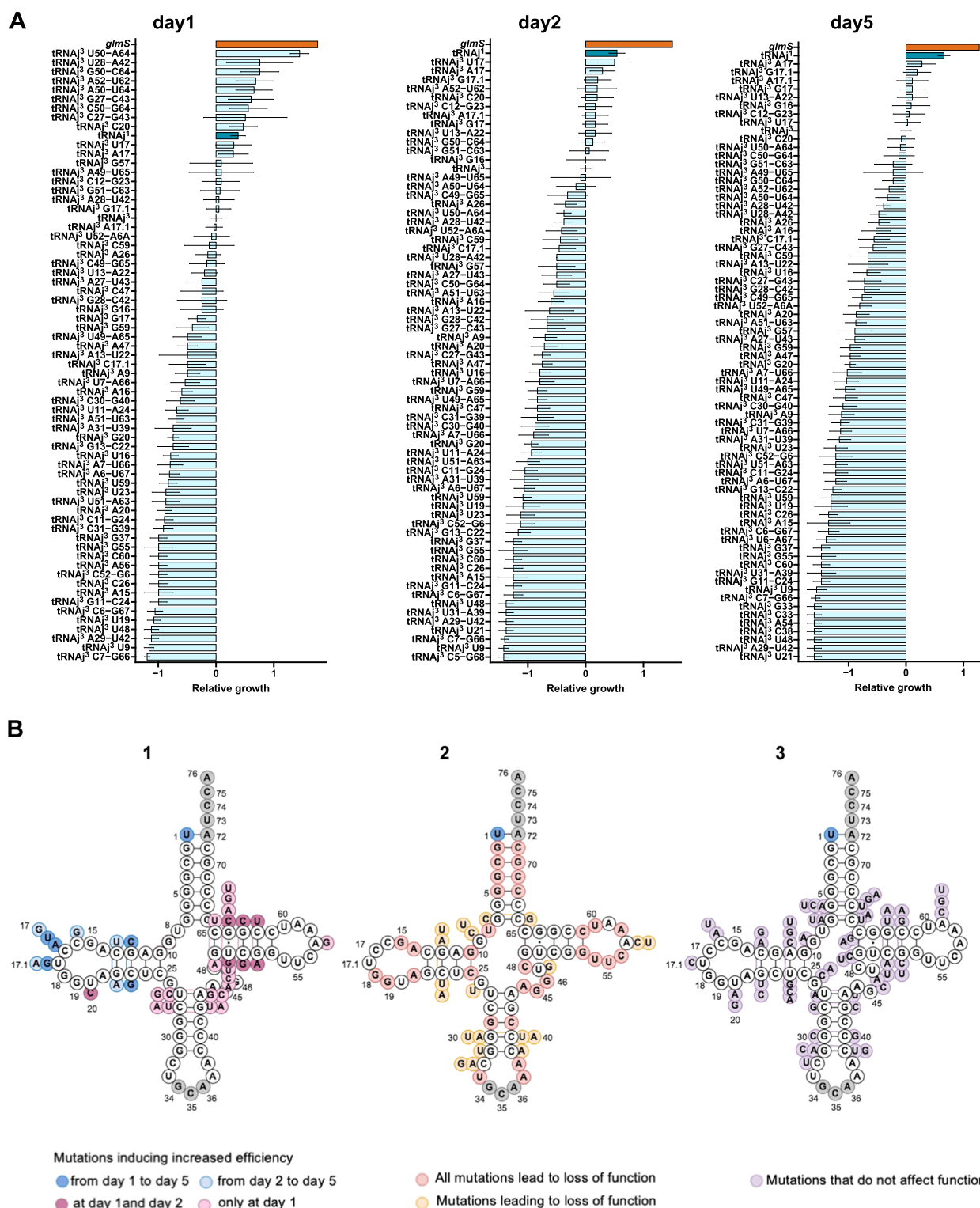


Figure 4. Growth assay of GlmS selection strains expressing a tRNA³ metZ single mutant on mineral medium and schematic representation of mutations and their impact on tRNA³ activity. (A) Positive control, which corresponds to EX160 ($\Delta glsM \Delta nagB$) transformed with a plasmid carrying *glsM wt* gene, is shown in orange. Other selection strains arise from EX160 transformed with two plasmids carrying *glsM M1* Δ and a tRNA³ single mutant, respectively. The original tRNA³ is colored dark blue. For readability, only the names of the mutations of tRNA³ are indicated on the graphs. Bacteria were streaked on mineral medium, incubated at 37 °C for 5 days, and results were collected on day 1, day 2, and day 5. Data are presented as mean \pm SD ($n \geq 4$). (B) Single tRNA³ mutations that enhanced the growth of the GlmS selection strain expressing GlmS M1 Δ compared with the growth expressing tRNA³ at the same incubation time. Noncolored positions are not mutated. Dark blue are mutations that lead to increased efficiency all along the assay (days 1 to 5). Light blue are mutations increasing efficiency at the end of the assay (day 2 to 5), dark pink at the beginning of the assay (day 1 to 2) and light pink at day 1. Single mutations in tRNA³ that abolish tRNA₃ function are highlighted in yellow. tRNA₃ positions leading to a nonfunctional tRNA₃, whatever the mutation, are shown in red. Single mutations in tRNA₃ that decrease tRNA₃ efficiency are highlighted in purple.

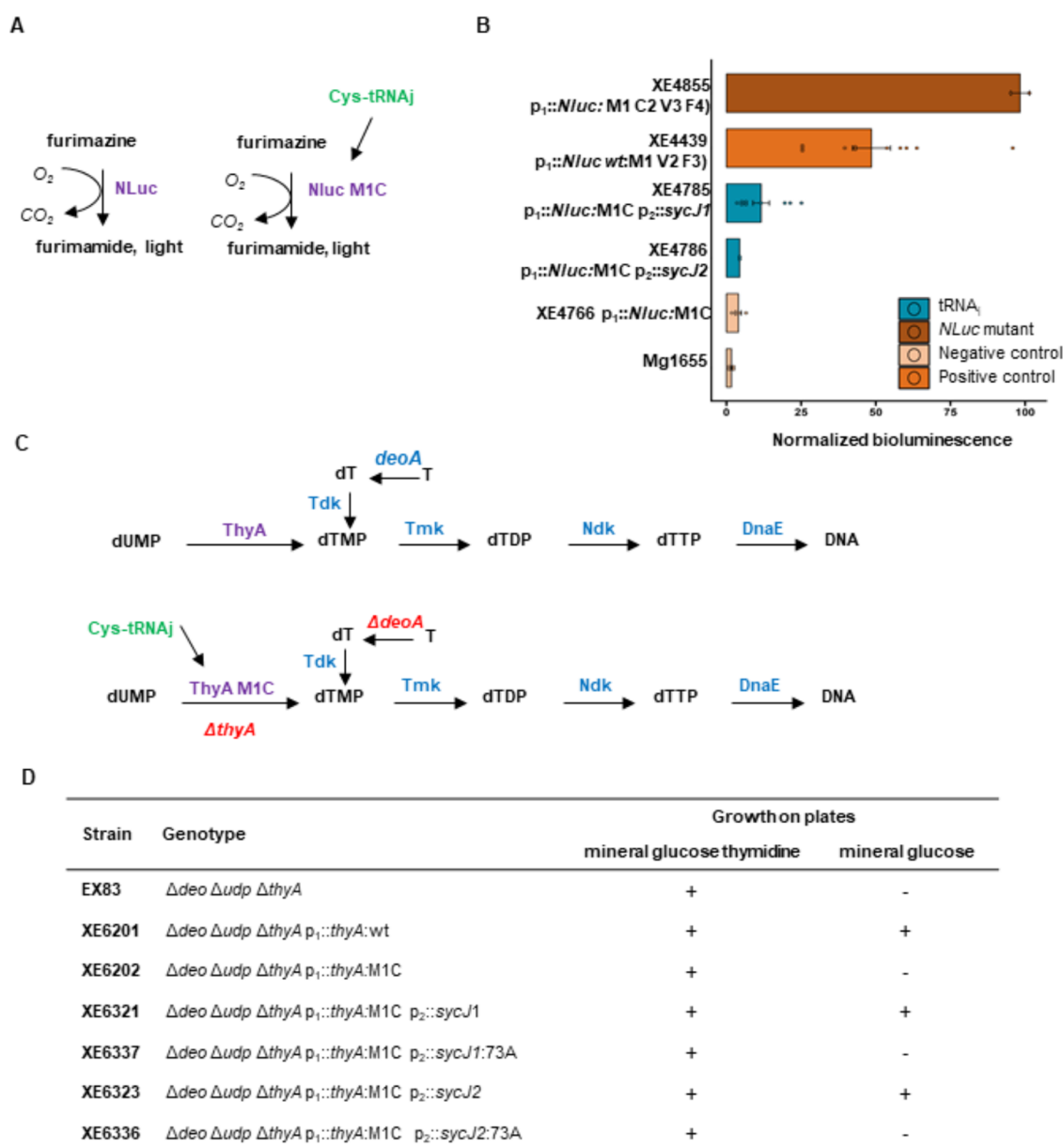


Figure 5. Validation of translation initiation at a cysteine codon by tRNA_j using the NanoLuc assay and thymidylate synthase selection. (A) NanoLuc protein catalyzes the conversion of furimazine to furimamide with light emission in the presence of oxygen. In the presence of an M1C allele of NLuc, protein activity can be restored only if a tRNA_j is supplied and active. (B) Normalized bioluminescence of the different strains tested in the NanoLuc assay. All strains are derived from MG1655 expressing wild-type NanoLuc protein (XE4439), or NanoLuc with an additional Cys at position 2 (XE4855), NanoLuc M1C alone (XE4766) or in combination with tRNA_j¹ (XE4785) or tRNA_j² (XE4786). (C) Biosynthesis of dTTP from dUMP involves the three enzymes thymidylate synthase, thymidylate kinase, and nucleoside diphosphate kinase, respectively, encoded by the *thyA*, *tmk*, and *ndk* genes. The nucleobase thymine (T) and the deoxynucleoside thymidine (dT) are salvaged through the action of the recycling enzymes thymidine phosphorylase and thymidine kinase, respectively encoded by the *deoA* and *tdk* genes. (D) In a strain devoid of both thymidylate synthase and thymidine phosphorylase, cell growth can be restored if translation is initiated by a Cys-tRNA_j at a met deleted allele of *thyA* or by an exogenous supply of dT in the medium.

CysRS aminoacylation rates and aminoacylation plateaus three times higher with tRNA_j¹ than with the tRNA_j². Moreover, *in vitro* tRNA aminoacylation assays with MetRS revealed that the two tRNA_j¹ and tRNA_j² are not aminoacylated by this enzyme, as expected for tRNAs that do not carry the CAU (Met) anticodon (Figure 3).

Selection of tRNA_j Variants Improving Translation Initiation Efficiency and Orthogonality. To improve translation initiation efficiency and orthogonality of the tRNA_j¹, we decided to construct a mutant library of this

tRNA. In a first step, we searched for the best nucleotide pair at position 1–72. Indeed, position C1:A72 is important for tRNA_f^{Met} recognition by methionyl-tRNA_f^{Met} transformylase and for its rejection by EF-Tu.^{26,27} We generated the *sycJ1* variants U1:A72, A1:U72, C1:G72, and G1:C72 and cloned them into the p₂ vector. Only expression of tRNA_j U1:A72 or A1:U72 enabled growth of the selection strain, with U1:A72 being slightly more efficient. This variant was called tRNA_j³ (Figure S2A). In order to select more efficient and orthogonal tRNA_j variants, we generated a systematic library of tRNA_j³

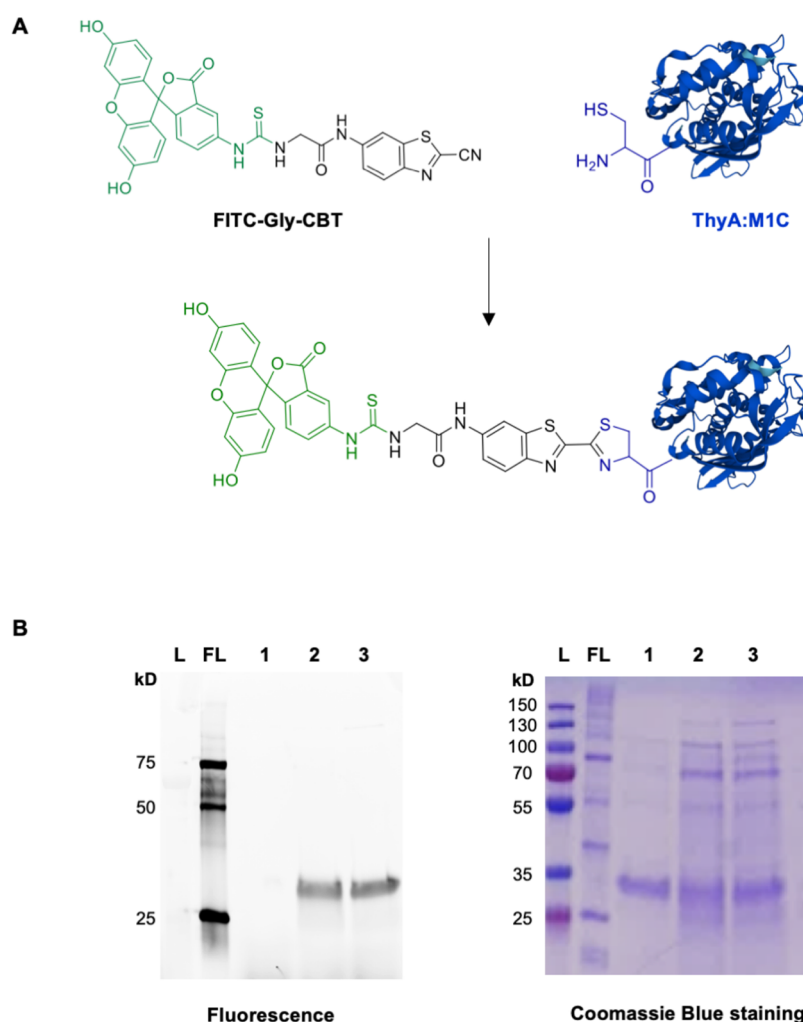


Figure 6. *In vitro* labeling of the N-terminal cysteine of ThyA protein variants with CBT-Gly-FITC. (A) Condensation reaction between the N-terminal Cys of ThyA protein and the CBT-Gly-FITC conducts to a ThyA fluorescent protein. (B) Fluorescence (left) and white light (right) images of a gel loaded with ThyA proteins labeled with CBT-Gly-FITC. ThyA proteins were produced in the E83 selection strain in mineral medium. L: ladder, FL: fluorescent ladder, Lane 1: ThyA wt protein (M1K2), lane 2 ThyA (M1CK2) protein in the presence of the tRNA₄⁴ and ThyA M1C2K3 (ThyA with an additional Cysteine at position 2) as positive control of labeling.

point mutants. The 145 oligonucleotides allowing mutation of *sync3* at each position were synthesized and used for PCR amplification before cloning by restriction enzymes in the p₂ plasmid. To analyze the effect of each point mutation, we ranked the growth efficiencies of strains transformed with these different tRNA₃³ mutants on minimal medium during a five-day-growth test. Cell growths were compared, after one, two, and 5 days of incubation, to the growth of a strain transformed with p₂-tRNA₃³ (Figure 4A). Figure 4B summarizes the impact of the mutations on the tRNA₃³ cloverleaf representation.

Of 145 mutants produced, 56 enabled growth of the selection strain. Twenty-one mutants enabled the strain to grow better than with the tRNA₃³ at some point of the assay (Figure 4B1), and 8 of them appeared to be more efficient than tRNA₃³ at day 5 (Figure 4B1, mutations in light and dark blue). These latter mutations are all located in the D-loop of the tRNA₃³. This systematic mutagenesis revealed 25 positions in tRNA₃³ that result in a complete loss of its functionality regardless of the base change (Figure 4B2, in red) and identified mutations that abolish its functionality (Figure 4B2, in yellow) or do not affect its function (Figure 4B3).

We then constructed and tested various multiple mutants of tRNA₃³ (Figures S2 and S3), favoring combinations of selected mutations in the D-loop that induced a growth advantage over the entire duration of the test (dark blue mutations in Figure 4B) (Figures S2 and S3).

Of the 17 combined mutants tested, 16 were functional within 5 days of testing: the best growth was obtained with expression of the C12, G17.1 double mutant, then called tRNA₄⁴ (Figures S2B and S4), the second best mutant was C12, A17 from day 2 to day 5 (Figures S2C and S4), followed by C12 U13 G16 U17 G17.1 mutant on days 1 and 2 (Figures S2D and S4).

NanoLuc Assay to Quantify Efficiency of Translation Priming by Cys-tRNA₃ at a Cysteine Codon. In order to quantify the efficiency of tRNA₃ mutants in initiating translation, we chose NanoLuc luciferase. This protein is an optimized luciferase, catalyzing the conversion of furimazine to light-producing furimamide (Figure 5A).²⁸ To construct this reporter system, we first mutated the ATG initiation codon into TGC and verified that no functional protein was expressed from the p₁ plasmid (encoding the NLuc M1C protein) in a wild-type strain of *E. coli*. We also checked that a cysteine at

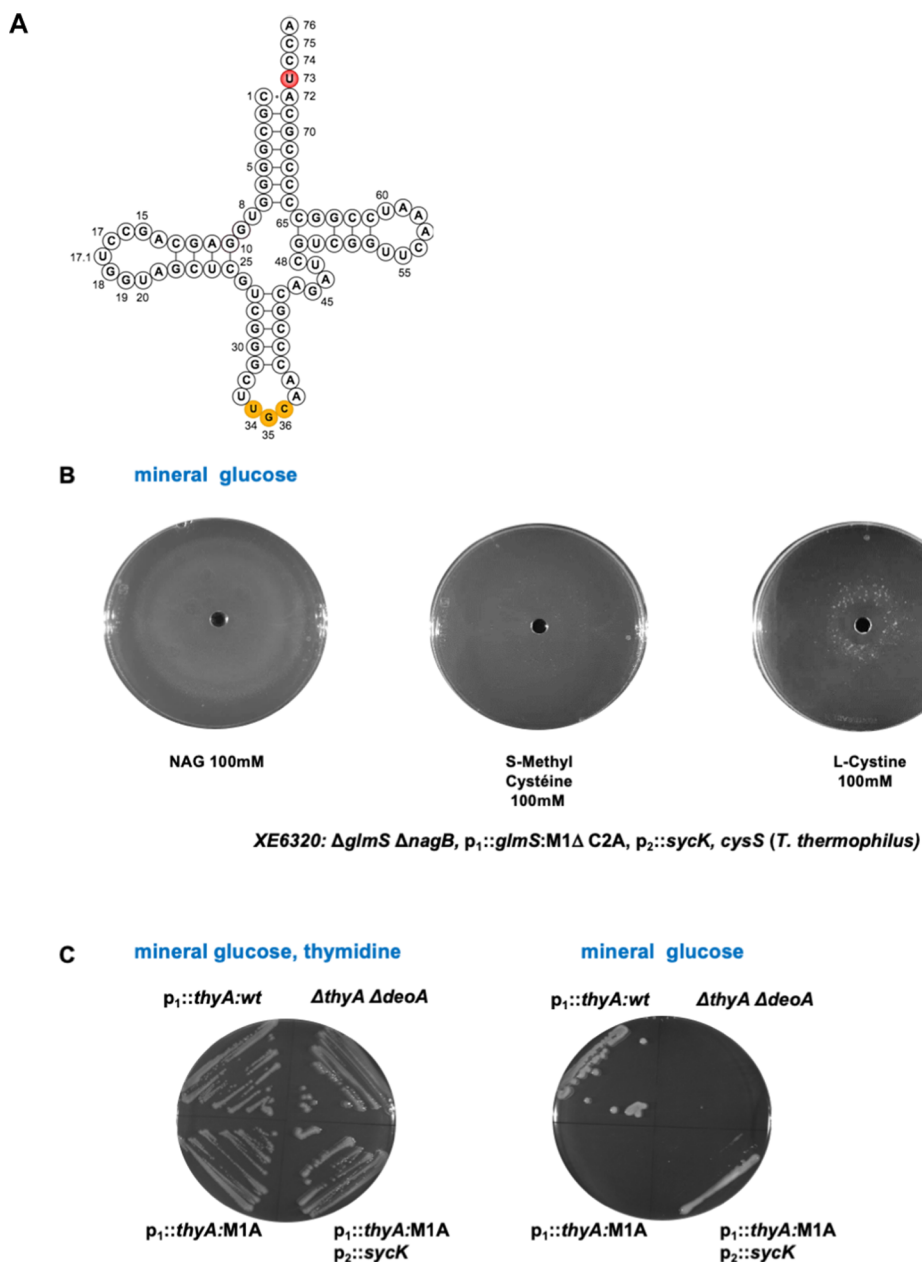


Figure 7. Microbiological assays validate translation initiation at an Ala codon by the $tRNA_{Ala}$. (A) Schematic representation of $tRNA_{Ala}$, which differs from $tRNA_{Ala}^1$ by its UGC anticodon. (B) Growth of the GlnS selection strain named XE6320 ($\Delta glmS \Delta nagB$, $p_1::glmS:M1\Delta C2A$, $p_2::sycK$, $cysS$ *S-T. thermophilus*) in mineral glucose medium with a central well containing N-acetyl-glucosamine, S-methyl-L-cysteine or L-cysteine, each at a concentration of 100 mM, after 5 days of incubation at 37 °C. (C) Growth of the ThyA selection strains on mineral medium with or without thymidine after 2 days of incubation at 37 °C. EX83 ($\Delta thyA \Delta deo$) corresponds to the ThyA selection strain, transformed by a plasmid expressing *thyA* wt (XE6202), expressing *thyA:M1A* (XE6454) or expressing *thyA:M1A* and *sycK* (XE6410).

the N-terminus was not deleterious to the enzyme activity (Figure 5B). Then, we showed that coexpression of *sycJ1* and *NLuc* MIC triggered luciferase synthesis (Figure 5B). In this system, the efficiency of $tRNA_{Ala}^3$ mutants could be evaluated by measuring the emitted luminescence (Figure S5), which is a more accurate assessment than the evaluation of bacterial growth on plates in GlnS selection. In contrast to GlnS selection, $tRNA_{Ala}^3$ was slightly better than $tRNA_{Ala}^1$ but the best double mutant in this quantitative luciferase expression assay corresponds to $tRNA_{Ala}^4$, which is also the most efficient in the GlnS selection (Figure S5B). G16 and C12 single mutants are among the best mutants identified in this assay (Figure S5A). Consistent with these results, *in vitro* aminoacylation of $tRNA_{Ala}^3$

and $tRNA_{Ala}^4$ with CysRS showed that the aminoacylation rates were increased by factors of two and three as compared to those of $tRNA_{Ala}^1$ (Figure S6).

ThyA Metabolic Selection Scheme for Cysteine-Primed Translation. To confirm previous results obtained with the GlnS selection, we considered the use of the Thymidylate Synthase (*thyA* gene) conditional selection screen in *E. coli*.^{29,30} This selection is based on the absolute requirement for this enzyme when thymidine (dT) is not supplied to the cell (Figure 5C). In the selection strain, $tRNA_{Ala}^1$ or $tRNA_{Ala}^2$ are both capable of initiating translation of an MIC variant of *thyA* and restoring cell growth on mineral medium (Figure 5D). Given that $tRNA_{Ala}^2$ was unable to initiate

translation in GlmS assay, this result suggested that the amount of ThyA required for cell growth might be less than that of GlmS. This selection confirmed the potential of tRNA_j to initiate translation of a protein essential for cell growth *in vivo* but constituted a less sensitive assay for testing the efficacy of tRNA_j mutants.

Aquifex aeolicus tRNA_i as a Model for Further Improving Efficiency and Orthogonality of tRNA_j. *Aquifex aeolicus* is a thermophilic species that belongs to one of the earliest-branching phyla of bacteria.³¹ This species has a unique tRNA_f^{Met}, containing almost only G:C pairs in the stems and A, U bases localized in the loops (Figure S7). This tRNA_f^{Met} resembles the tRNA_f^{Met} of *E. coli* (G46) (Figure S7A) and differs from it by five bases (Figure S7B). The specific mutations corresponding to *syj1* (U73, G34, C35, and A36), *syj3* (U73, G34, C35, and A36, U1-A72), and *syj4* (U73, G34, C35, and A36, U1-A72, C12-G23, and G17.1), were introduced into the *A. aeolicus* tRNA_f^{Met} gene. The corresponding genes *syj5*, *syj6* and *syj7* produced tRNA_j⁵, tRNA_j⁶ and tRNA_j⁷, respectively (Figure S7CDE). Their expression in our three systems (GlmS, ThyA, and NanoLuc) enabled efficient initiation of the reporter proteins at a Cys codon (Figure S8). Moreover, *in vitro* aminoacylation assay by *E. coli* CysRS using tRNA_j⁶ showed a very good aminoacylation efficiency, 3.7 fold better than that of tRNA_j¹ (Figure S6B). *A. aeolicus* tRNA_j⁷ induced the strongest growth of GlmS (Figure S8A) and ThyA (Figure S8B) selection strains, and enabled the highest luminescence emission (Figure S8C) among all tRNA_j tested.

ThyA N-terminal Cysteine Coupling to CBT-gly-FITC. The production of proteins starting with a cysteine allows coupling to various compounds of interest using the chemical properties of the free N-terminal thiol function. We tested the condensation of the ThyAM1C protein, produced in the presence of tRNA_j⁴, with the 2-cyanobenzothiazole (CBT) conjugated to a fluorophore (FITC) (Figure 6A), previously shown to specifically label only N-terminal cysteines.³² A sequence of six histidines was added to the C-terminus of the ThyA proteins to facilitate their purification. The proteins produced in the selection strain, in mineral medium, were purified on a NiTED column and incubated for 2 h with the CBT-Gly-FITC at room temperature under the experimental conditions previously described.³² A fluorescent product was observed only with the M1C protein produced with the tRNA_j⁴, at the expected size, on a SDS PAGE polyacrylamide gel (Figure 6B, lane 2). The same gel stained with Coomassie Blue confirmed that the wild-type ThyA protein is not labeled with CBT-gly-FITC (Figure 6B, lane 1). As a positive control, we constructed and produced a ThyA protein with an additional cysteine at position 2 (M1C2K3), which after deformylation and methionine cleavage by *E. coli* methionine aminopeptidase provides a ThyA protein with an N-terminal cysteine.

This experiment demonstrated the presence of a reactive N-terminal cysteine in the ThyA M1C protein produced by tRNA_j expression and the possibility of labeling this N-terminal cysteine residue in a biocompatible, controllable, and efficient reaction.

Coexpression of Orthogonal Cys-tRNA Synthetases to Enhance Translation Initiation by tRNA_j. tRNA_j must compete with endogenous elongator tRNA^{Cys} for aminoacylation by CysRS. Thus, in parallel with systematic mutagenesis of tRNA_j¹, we tested a second strategy to improve

the efficiency of tRNA_j¹ aminoacylation by the expression of an orthogonal CysRS. In addition to improving efficiency, we also sought to improve the orthogonality of our system by focusing on potentially more robust and flexible enzymes. We selected CysRS from bacteria living in extreme environments (*Thermus thermophilus*, *Dictyoglomus thermophilum*, *Dictyoglomus turgidum*), from ancient phylogenetic origin (*Aquifex aeolicus*, *Thermotoga maritima*) or from organisms expressing deviant tRNA^{Cys} (*Thermodesulfovibrio yellowstonii*, *Desulfotignum. phosphitoxidans*, *Desulfacinum hydrothermale*, *Desulfacinum infernum*, and *Desulfomonile tiedjei*). The genes encoding these CysRS were cloned in the plasmid containing *syj1* and were coexpressed with *glmS* in the selection strain. Of the ten CysRS tested, four (*T. thermophilus*, *D. thermophilum*, *T. yellowstonii*, and *D. phosphitoxidans*) improved cell growth compared to XE3727 expressing the tRNA_j¹ alone (Figure S9). Thus, we identified among heterologous CysRS suitable candidates that could be further engineered to select the most efficient CysRS/tRNA_j¹ pair.

Translation Initiation at an Alanine Codon by tRNA_k. To generalize initiation of protein synthesis with a cysteine at a chosen initiation codon, we first attempted to use an alanine codon in our two *in vivo* selection systems. The *syj1* gene was mutated from GCA to TGC at the anticodon and cloned into the p₂ vector to produce the corresponding tRNA_k (Figure 7A). In the GlmS selection, translation initiation by the tRNA_k at a glmS M1ΔC2A (GCA) could only be achieved in the presence of an orthogonal *T. thermophilum* CysRS and by adding L-cystine to increase cellular cysteine concentration³³ (Figure 7B). As expected, the anticodon change led to a decrease in the aminoacylation efficiency of tRNA_k by *E. coli* CysRS.^{34,35} This effect may be counterbalanced by the expression of heterologous CysRS and L-cystine. The essential requirement of cysteine for GlmS activity ascertained that growth was indeed the result of cysteine loading on the tRNA_k. Expression of *syjK* and of a mutated *thyA* allele M1A (GCA) in the EX83 (Δ*thyA* Δ*deoA*) strain effectively restored growth in a minimal medium lacking thymidine (Figure 7C). This result confirms that GlmS selection is more demanding than ThyA selection.

Assay of tRNA_k as a Translation Elongator. We tested whether the construct tRNA_k, equipped with the UGC anticodon, which could initiate translation initiation at an alanine GCA start codon, would also be functional in elongation. The ThyA protein has a catalytic cysteine essential for its enzymatic activity at position 146. A previous *in vivo* study³⁶ showed that substitution of this Cys146 codon by other codons can be suppressed by an elongator tRNA^{Cys} with the corresponding anticodon, thus restoring ThyA activity and cell growth in the absence of thymidine. We used this selection assay to investigate whether tRNA_k could act as an elongator. An inactive version of the *thyA* gene in which Cys146 is mutated to an alanine GCA codon was coexpressed with *syjK* into the EX83 strain. The inability of this strain to grow in the absence of thymidine suggested that Cys-tRNA_k fails to suppress Ala146 and recover enough active ThyA for sustaining cell proliferation (Figure S10).

DISCUSSION

Using the *glmS* gene, which encodes an essential N-terminal cysteine, we set up a tight and absolute metabolic selection in *E. coli* to screen for efficient translation initiation at a Cys codon using a repurposed initiator tRNA. This unique way to

initiate protein translation has been validated using another selectable metabolic pathway of *E. coli* (*thyA*) and straightforwardly quantifiable NanoLuc reporting system. In all three systems, the results were highly consistent, validating the same tRNA_j⁴ variant as the most efficient variant for translation initiation at a cysteine codon. Biochemical proof of the incorporation of cysteine at the N-terminal position was established by a simple bioconjugation reaction, highlighting the biotechnological potential of N-terminal cysteine. *In vitro* aminoacylation assays corroborated the *in vivo* results, revealing enhanced aminoacylation of this mutated tRNA_j⁴ by CysRS, associated with undetectable aminoacylation by MetRS, confirming its orthogonality and the lack of interference with the initiation of canonical translation. For the ThyA and GlmS selections, tRNA_j efficacy and assay sensitivity are closely related to the expression levels of these proteins required for the physiological functioning of an *E. coli* strain (2250 GlmS molecules per cell, 600 ThyA molecules per cell).³⁷ As a result, GlmS selection offers a greater scope for selecting a more efficient translation initiation.

Systematic point mutagenesis of tRNA_j has made it possible to select a more efficient, orthogonal tRNA_j and identify the positions critical to its functionality. We chose to mutate, at each position, the U1:A72 mutant (tRNA_j³) as it did not induce a significant decrease in translation initiation in our selection systems. This mutation, while deviating from the natural tRNA_j, could also contribute to better aminoacylation by the CysRS of tRNA_j³, compared with unpaired tRNA_j¹ C1:A72.³⁸ This hypothesis was confirmed by the results of *in vitro* aminoacylation assays. Analysis of the mutants suggests that formylation of tRNA_j³ probably plays an important role in its initiation efficiency since no mutations are tolerated in the first three G:Cs of the acceptor stem, and any modification of A11-U24 decreases the efficiency of tRNA_j³.^{26,39,40}

As the D-stem-loop is heavily involved in interactions with CysRS,³⁵ most of the mutations that resulted in higher efficiency were consistently located in this part of the tRNA, probably favoring the interaction. It should be noted that essential determinants for interaction with CysRS were unable to tolerate mutations, such as 15G,⁴¹ but also 14A and 21A, by analogy with the two residues 13A and 22A at the start of the tRNA^{Cys} D-loop, known for stacking interactions with 15G.^{35,42} Mutations in the D-loop could enhance (positions 16, 17, 17.1) or block (positions 18,19) interactions with the T-loop, thereby modulating the tRNA overall L-shape and structure.⁴³ These mutations located in the tRNA elbow probably impact interactions with IF3, the ribosomal P site, and RNase P.

As a part of the anticodon stem, none of the tRNA_j³ 29–41 mutants were functional, and only a few were functional for pairs 30–40 and 31–39. This result is consistent with the role of these three consecutive G-C base pairs in the interaction with the ribosomal P site.^{39,44–46} The tRNA_j³ anticodon loop has the advantage of containing three residues identical to elongator tRNA^{Cys} (U33, A37, and A38), which are important for CysRS recognition and have been shown to be mutation-intolerant while preserving the specific tRNA_i structure (in particular position 37).^{34,43,47}

Many of the T-stem mutants did enable slightly better growth than tRNA_j³ in the context of assays. These mutations could reduce interactions with EF-Tu, which are likely enhanced by the U1-A72 mutation of the tRNA_j³. We finally found that the T-loop has proved extremely resistant to

mutagenesis. The results of systematic point mutagenesis of tRNA_j³ are highly consistent with the nature of the different positions known to interact with the various initiation players but also with the determining positions known for recognition by CysRS.

Because of its ancient phylogenetic origin (and few bases that differentiate it from *E. coli*), we assumed that *A. aeolicus* tRNA would be a good orthogonal candidate, very robust, and amenable to mutations. Interestingly, the five bases of *A. aeolicus* tRNA_f^{Met} that differ from that of *E. coli* tRNA_{f1}^{Met} (C27-G43, C50, and G51-C63) correspond to mutations found to be slightly favorable to tRNA_j³ activity during systematic point mutagenesis. *A. aeolicus* tRNA_j⁶ can therefore be considered a triple mutant of tRNA_j³. Finally, tRNA_j⁷ validated our goal of obtaining the most efficient and orthogonal tRNA_j possible.

Precise control of translation initiation is of major interest in the production of genetically engineered microorganisms (GEMs) to produce molecules of interest and to dissociate the synthesis of these molecules from those of other cellular proteins. We therefore considered the use of orthogonal components to improve the efficiency of tRNA_j initiation and to limit interference with cellular physiological functions. Expression of certain heterologous CysRS resulted in improved growth of the selection strain in the presence of the tRNA_j¹ clearly suggesting that aminoacylation is a limiting factor that could be improved in this system. Our *in vivo* approach opens the way to CysRS engineering, which can be considered with the best-selected tRNA_j mutants, for the selection of a specialized CysRS/tRNA_j pair with enhanced functionality.^{48,49}

Translation initiation at an Ala codon revealed that the concentration of free cysteine in the cytoplasm could also constrain the efficiency of translation initiation, which can be alleviated by the addition of L-cystine but not by S-methyl-L-cysteine. The use of tRNA_k in the elongation assay demonstrated the specificity of tRNA_j to initiate translation, minimizing interference with the cell's protein biosynthesis in agreement with the absence of cellular toxicity observed by tRNA_j expression.

In conclusion, we have developed a minimalist and robust system to prime protein synthesis on the ribosome with cysteine. This system adds without interfering with the canonical processes of formyl-methionine initiation and cysteine elongation. It enables the absolute control of gene expression and carries the potential to partition the proteome into a subpopulation primed by fMet and a subpopulation primed by Cys. The inescapable character of cysteine assignment at the N-terminus of GlmS paves the way to the customization of additional initiation factors cognate to tRNA_j, as well as to the in-depth engineering of tRNA_j and the *in vivo* recruitment of tXNA_j, i.e., a xenonucleotide-containing initiator, through the use of nucleoside triphosphate transporter.^{50,51} All of these perspectives should favor evolutionary speciation at the translation level as well as the containment of such reprogrammed species.

MATERIALS AND METHODS

Materials. Strains used in this study are derivatives of the wild-type *E. coli* K12 strain MG1655. The cloning steps were carried out in the strain β 2033 (pro thi rpsL hsdS Δ lacZ F'(Δ lacZ M15 lacIq traD36 proA⁺ proB⁺) strain. For the construction of strains and plasmids, the *E. coli* bacteria were cultivated at 37 °C in Luria–Bertani (LB) broth supplemented

with carbenicillin (100 mg/L, Fisher), chloramphenicol (25 mg/L, Sigma), N-acetylglucosamine (1 mM, Sigma), and thymidine (300 μ M, Sigma), L-cystine (100 mM, Sigma), S-methyl-cysteine (100 mM, Sigma), and L-Cysteine hydrochloride (Sigma) when required. FITC-Gly-CBT was purchased at Iris Biotech.

Plasmids Constructions. Oligonucleotides are listed in Table EV1 and plasmids in Table EV2. The *glmS* and *thyA* genes were PCR amplified from MG1655 genomic DNA, and the *NLuc* gene was amplified from pSB1C3_Bba_K1680009 (iGEM). The different mutated genes *glmS* M1 Δ , *glmS* C2 Δ , *thyA* M1C, and *NLuc* M1C were cloned into the high-copy plasmid pXEN1147 (derived from pUC18 called p₁) under the control of a strong and constitutive *cpdP* (*Vibrio fischeri*) promoter and using PacI and NotI restriction sites.

For screening and selection of functional tRNA_j, tRNAs were cloned into the p15A plasmid pEVL649 called p₂⁵² under the control of the *lac* promoter and using SacI and XbaI restriction sites. The 6bp preceding (TTCAGG) and following (TTTCC) *metY* genes were included to ensure correct maturation. *sysJ* and *sysK* genes were cloned using two different techniques: (i) Restriction-ligation following a PCR amplification of oligonucleotides. Whenever applicable, PCR products were digested twice with DpnI (NEB, 3 h 37 °C incubation) before digestion with restriction enzymes. (ii) Annealing and phosphorylation of ordered DNA oligonucleotide: 300 pmol of each oligo were mixed in equimolar amounts in the annealing buffer (Tris-HCl 100 mM pH 7.5–8.0, EDTA 10 mM, NaCl 10 mM in 50 μ L), incubated at 95 °C for 2 min and slowly cooled down (0.1 °C/s) to room temperature. Annealed oligos were phosphorylated using T4 polynucleotide kinase (10 U, NEB) along with its buffer and ATP (1 mM) and then ligated with a compatible digested vector. CysRS were PCR amplified from genomic DNA and restriction-ligated into p₂-derived plasmids containing a *sysJ* or *sysK* gene between PacI and NotI. For *in vitro* aminoacylation assays, *sysJ1*, *sysJ2*, *sysJ3*, *sysJ4*, *sysJ6*, and *cysT* genes were cloned into pBSTNAV3S⁵³ using SacII and PstI restriction sites. pXEN2023 was constructed by cloning *cysS* gene into pET15b-lpa vector with an N-terminal 6x histidine tag and under the control of the T7 promoter, using X3215 and M13 fwd PCR primers and NdeI and NotI restriction sites. A linker encoding 5 alanines, one serine and 6 histidines were added to the *thyA* wt, the M1C *thyA*, and the M1C2K3 *thyA* genes by PCR amplifications using primer pairs X3684/X3948, X3685/X3948, and X3996/X3948, respectively, and subsequent cloning in p₁ via PacI and NotI restriction sites.

GlmS, ThyA, and Nanoluc Assays. Strains are listed in Table EV3. GlmS complementation assays were conducted on mineral medium agar plates with and without N-acetylglucosamine supplementation. The relative growth of each strain containing a tRNA_j³ mutant was established by comparison with the strain expressing tRNA_j³. This evaluation was carried out as follows: the reference strain expressing tRNA_j³ received an arbitrary score of 2, tRNA_j³ mutants allowing higher growth than the tRNA_j³ received a score of 3, tRNA_j³ mutants allowing lower growth received a score of 1, and tRNA_j³ mutants not allowing growth received a score of 0. Relative growth was calculated on 3 independent growth comparison experiments (four for tRNA_j activity assays). The average score of each tRNA_j³ mutant was calculated on day 1, day 2, and day 5 and normalized with the average tRNA_j³ score of the same day. ThyA complementation assays were conducted similarly, using

thymidine instead of N-acetylglucosamine, at least in duplicates. For luciferase assays, the measured luminescence was normalized with the OD₆₀₀.

Titration of CysRS Active Sites. The specific activity of L-[³⁵S]-cysteine was determined by CysRS active site titration, using a procedure adapted from.⁵⁴ Reactions were performed in a 20 mM Tris-HCl pH 7.5 buffer containing 20 mM MgCl₂, 50 μ M ZnCl₂ and in the presence of 2 mM ATP, 25 μ M L-[³⁵S]-Cys isotopic dilution prepared from commercial L-[³⁵S]-PerkinElmer), 5 μ M tRNA and 1.5 or 3 μ M CysRS (diluted in a 10 mM Tris-HCl pH 7.5, 2 mM DTT, 0.2 mg/mL BSA buffer). Reactions were incubated for 10 min at 25 °C in a final volume of 100 μ L and spotted immediately on BA85 nitrocellulose membranes (Schleicher & Schuell). Filters were rinsed with 20 mM Tris-HCl, 10 mM MgCl₂, pH 7.5 cold buffer, and radioactivity due to enzyme-bound cysteyl-adenylate was determined by scintillation counting. The results were used to calculate the specific activity of the L-[³⁵S]-Cys preparation.

Aminoacylation Reactions. Cysteine aminoacylation reactions were performed in a 20 mM Tris-HCl buffer pH 7.5 containing 7 mM MgCl₂, 15 mM KCl, 2 mM DTT, 50 μ M ZnCl₂, pH 7.5; adapted from ref 25 in the presence of 2 mM ATP, 25 μ M L-Cys (L-[³⁵S]-Cys, PerkinElmer, 28 dpm/pmol as determined by active site titration), 5 μ M tRNA and CysRS (1 μ M for plateau assays and 2 nM for kinetics, in a 10 mM Tris-HCl pH 7.5, 2 mM DTT, 0.2 mg/mL BSA buffer). Reactions were incubated for 0–10 min at 25 °C (100 μ L volume), and then tRNAs were precipitated by adding (i) 3 mL of cold trichloroacetic acid (TCA) containing 0.5% w/v DL-Met, 100 μ M L-Cys and 10 mM DTT and (ii) 15 μ L of carrier RNA (4 mg/mL). The precipitate was recovered by filtration on Whatman GF-C disks. Filters were rinsed with cold TCA containing 0.5% (w/v) DL-Met, 100 μ M L-Cys, and 10 mM DTT. Radioactivity was determined by scintillation counting. Methionine aminoacylation reactions were performed as described⁵⁵ in the standard buffer and in the presence of 2 mM ATP, 24 μ M L-[¹⁴C]-methionine (Hartman Analytic, 2.04 GBq/mmol), 5 μ M tRNA, and MetRS (1 μ M for plateau assays or 2 nM for initial rate measurements). Reactions were incubated, and tRNA precipitated and recovered by filtration as above, using TCA containing 0.5% w/v DL-methionine. Radioactivity retained on the filters was measured by scintillation counting. The background was determined from reactions in which no enzyme was added and subtracted from the counting data. Aminoacylation rates were calculated by linear regression from the results at different reaction times.

Coupling to FITC-Gly-CBT. As previously described,³² 10 μ M of purified protein were incubated in PBS Buffer pH 7.4 in the presence of 2 mM TCEP (tris(2-carboxyethyl)phosphine) and 50 μ M of FITC-Gly-CBT in a final volume of 20 μ L for 2 h at 25 °C. Reactions were quenched by adding L-Cysteine at 50 μ M before loading samples on 12% SDS polyacrylamide gel.

Data Analysis. Data were retrieved from Excel files using readxl (version 1.4.3) and filtered using dplyr (version 1.1.3) and tidyr (1.3.0). Data were visualized with R (version 4.2.1) using ggplot2 (version 3.4.4), ggpubr (version 0.6.0), and ggprism (version 1.0.4,⁵⁶).

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.4c08326>.

PXEN1147 plasmid sequence, growth assays, schematic representations of tRNAs sequences, luciferase assays, *in vitro* aminoacylation assays, list of oligonucleotides, plasmids, and strains. (PDF)

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Notes

The authors declare no competing financial interest.

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