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Research Paper

A general approach for the generation of orthogonal tRNAs

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Abstract

Background: The addition of new amino acids to the genetic code of *Escherichia coli* requires an orthogonal suppressor tRNA that is uniquely acylated with a desired unnatural amino acid by an orthogonal aminoacyl-tRNA synthetase. A $tRNA^{Tyr}_{CUA}$–tyrosyl-tRNA synthetase pair imported from *Methanococcus jannaschii* can be used to generate such a pair. In vivo selections have been developed for selecting mutant suppressor tRNAs with enhanced orthogonality, which can be used to site-specifically incorporate unnatural amino acids into proteins in *E. coli*.

Results: A library of amber suppressor tRNAs derived from *M. jannaschii* $tRNA^{Tyr}$ was generated. $tRNA^{Tyr}_{CUA}$s that are substrates for endogenous *E. coli* aminoacyl-tRNA synthetases were deleted from the pool by a negative selection based on suppression of amber nonsense mutations in the barnase gene. The remaining $tRNA^{Tyr}_{CUA}$s were then selected for their ability to suppress amber nonsense codons in the β-lactamase gene in the presence of the cognate *M. jannaschii* tyrosyl-tRNA synthetase (TyrRS). Four mutant suppressor tRNAs were selected that are poorer substrates for *E. coli* synthetases than *M. jannaschii* $tRNA^{Tyr}_{CUA}$, but still can be charged efficiently by *M. jannaschii* TyrRS.

Conclusions: The mutant suppressor $tRNA^{Tyr}_{CUA}$ together with the *M. jannaschii* TyrRS is an excellent orthogonal tRNA–synthetase pair for the in vivo incorporation of unnatural amino acids into proteins. This general approach may be expanded to generate additional orthogonal tRNA–synthetase pairs as well as probe the interactions between tRNAs and their cognate synthetases.

Keywords: Aminoacyl-tRNA synthetase; In vivo selection; In vivo unnatural amino acid mutagenesis; Orthogonal suppressor tRNA

1. Introduction

In vitro methods have been developed to site-specifically introduce unnatural amino acids into proteins by suppression of amber mutations with chemically acylated tRNAs [1,2]. The ability to directly incorporate unnatural amino acids site-specifically into proteins in living cells would greatly expand our ability to manipulate protein structure and function as well as probe cellular processes. Our strategy to expand the genetic code of *Escherichia coli* involves the generation of a suppressor tRNA that is not acylated by any of the *E. coli* synthetases (orthogonal suppressor tRNA), and a synthetase that does not acylate any *E. coli* tRNAs, but efficiently charges the orthogonal suppressor tRNA. The specificity of this synthetase is then altered so that it charges the orthogonal suppressor tRNA with a desired unnatural amino acid, and none of the common 20 amino acids [3,4].

Several strategies have been developed to generate orthogonal suppressor tRNA–synthetase pairs in *E. coli*. One involves destroying an existing *E. coli* tRNA's affinity toward its cognate synthetase (but not its ability to function in translation) by mutating nucleotides at the tRNA–synthetase interface. A mutant synthetase is then evolved that uniquely recognizes the orthogonal tRNA. Such an orthogonal tRNA has been generated from *E. coli* $tRNA^{Gln}_2$, but no mutant *E. coli* GlnRS has been evolved that charges the derived orthogonal tRNA more efficiently than wild-type *E. coli* $tRNA^{Gln}_2$ [4,5]. A second strategy involves importing a tRNA–synthetase pair from another organism into *E. coli* if cross-species aminoacylation is inefficient, and the suppressor tRNA derived from the tRNA is not charged by *E. coli* synthetases. Two orthog-

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onal suppressor tRNA–synthetase pairs have been developed based on the *Saccharomyces cerevisiae* $tRNA_2^{Gln}$–GlnRS and $tRNA^{Asp}$–AspRS pairs [6,7]. Another approach involves the use of a heterologous synthetase as the orthogonal synthetase but a mutant initiator tRNA of the same organism or a related organism as the orthogonal tRNA. Two pairs have been generated, a mutant human initiator tRNA–*E. coli* GlnRS pair for use in *S. cerevisiae* and a mutant *E. coli* initiator $tRNA_2^{fMet}$–mutant yeast TyrRS pair for use in *E. coli* [8].

The development of additional orthogonal tRNA–synthetase pairs may allow the simultaneous incorporation of multiple unnatural amino acids into proteins. Moreover, different aminoacyl-tRNA synthetases may be better starting points for generating active sites with particular specificities, e.g. large hydrophobic vs. small hydrophilic amino acids. We recently reported the generation of an orthogonal $tRNA_{CUA}^{Tyr}$–TyrRS pair by expressing the *Methanococcus jannaschii* $tRNA_{CUA}^{Tyr}$–TyrRS pair in *E. coli* [9]. In comparison to the Gln and Asp orthogonal pair, the TyrRS charges its cognate amber suppressor tRNA with much higher efficiency. However, the $tRNA_{CUA}^{Tyr}$ derived from *M. jannaschii* is also a better substrate for the endogenous *E. coli* synthetases than the *S. cerevisiae* $tRNA_{CUA}^{Gln}$ and $tRNA_{CUA}^{Asp}$. In order to improve the utility of this $tRNA_{CUA}^{Tyr}$–TyrRS pair, and to develop additional such pairs, we have developed a general strategy for selecting mutant tRNAs with enhanced orthogonality. Mutant suppressor tRNAs selected from libraries derived from *M. jannaschii* $tRNA_{CUA}^{Tyr}$ have been generated and characterized.

2. Results and discussion

2.1. Suppressor tRNA library design and construction

Because of the complex nature of tRNA–synthetase interactions that are required to achieve a high degree of fidelity in protein translation, the rational design of orthogonal tRNA–synthetase pairs is difficult. Consequently, we have taken an approach that exploits the poor cross recognition of some interspecies tRNA–synthetase pairs, coupled with subsequent in vivo evolution of tRNAs with enhanced orthogonality. The $tRNA^{Tyr}$ of *M. jannaschii*, an archaebacterium, has different identity elements from those of *E. coli* $tRNA^{Tyr}$. In particular, the *E. coli* $tRNA^{Tyr}$ has a G1C72 pair in the acceptor stem while the *M. jannaschii* $tRNA^{Tyr}$ has a C1G72 pair. We have shown that an amber suppressor tRNA derived from *M. jannaschii* $tRNA^{Tyr}$ is not efficiently aminoacylated by the *E. coli* synthetases, but functions efficiently in protein translation in *E. coli* [9]. In addition, the *M. jannaschii* TyrRS, which has only a minimalist anticodon-loop-binding domain, does not aminoacylate *E. coli* tRNAs [10], but still efficiently aminoacylates its own suppressor $tRNA_{CUA}^{Tyr}$ [9].

To test the orthogonality of this suppressor tRNA in *E. coli*, an amber codon was introduced at a permissive site (Ala184) in the β-lactamase gene [6]. Those tRNAs that can be charged by *E. coli* synthetases will suppress the amber codon and allow cells to live in the presence of ampicillin. The *M. jannaschii* $tRNA_{CUA}^{Tyr}$ suppresses the amber codon in the β-lactamase gene with an IC_{50} value of 56 μg/ml ampicillin [9]. In contrast, the orthogonal $tRNA_{CUA}^{Gln}$ derived from *S. cerevisiae* $tRNA_2^{Gln}$ has an IC_{50} of 21 μg/ml ampicillin when tested in the same assay [6]. The IC_{50} for *E. coli* in the absence of any suppressor tRNA is 10 μg/ml ampicillin. This result shows that the *M. jannaschii* $tRNA_{CUA}^{Tyr}$ is a better substrate for *E. coli* synthetases than the $tRNA_{CUA}^{Gln}$. Consequently, if the *M. jannaschii* $tRNA_{CUA}^{Tyr}$ is used in vivo to deliver unnatural amino acids into proteins in *E. coli*, it may also be mischarged with natural amino acids by *E. coli* synthetases, leading to heterogeneous amino acid incorporation.

The improvement of the orthogonality of the *M. jannaschii* $tRNA_{CUA}^{Tyr}$ requires the introduction of 'negative recognition determinants' to prevent recognition by endogenous *E. coli* synthetases. On the other hand, these mutations should not strongly interfere with the tRNA's interaction with its cognate *M. jannaschii* TyrRS or the ribosome. Since *M. jannaschii* TyrRS lacks most of the anticodon-binding domain [10], mutations introduced at the anticodon loop of the tRNA are expected to have a minimal effect on TyrRS recognition. An anticodon-loop library with four randomized nucleotides was constructed to test this notion (Fig. 1). Given the various combinations and locations of identity elements for various *E. coli* tRNAs, mutations at additional positions may increase the likelihood of finding a mutant tRNA with the desired properties. Thus, a second library containing mutations at nonconserved positions in all of the tRNA loops (all-loop library) was also constructed (Fig. 1). Conserved nucleotides were not randomized so as to maintain the tertiary interactions that stabilize the 'L'-shaped structure of the tRNA [11,12]. Stem nucleotides were also not mutated since substitution of one such nucleotide requires a compensatory mutation. The 11 nucleotides (C16, C17, U17a, U20, C32, G37, A38, U45, U47, A59, and U60) were therefore randomized (Fig. 1). The theoretical size of this library is 4.19×10^6, and a library with a size of 1.93×10^8 colony forming units was constructed to ensure complete coverage of the mutant library.

2.2. A general selection for orthogonal suppressor tRNAs in E. coli

To select for a member of the *M. jannaschii* tRNA library with enhanced orthogonality, we used a combination of negative and positive selections in the absence and presence of the cognate synthetase (Fig. 2). In the negative selection, amber nonsense codon(s) are introduced in a toxic gene at a nonessential position. When a member of

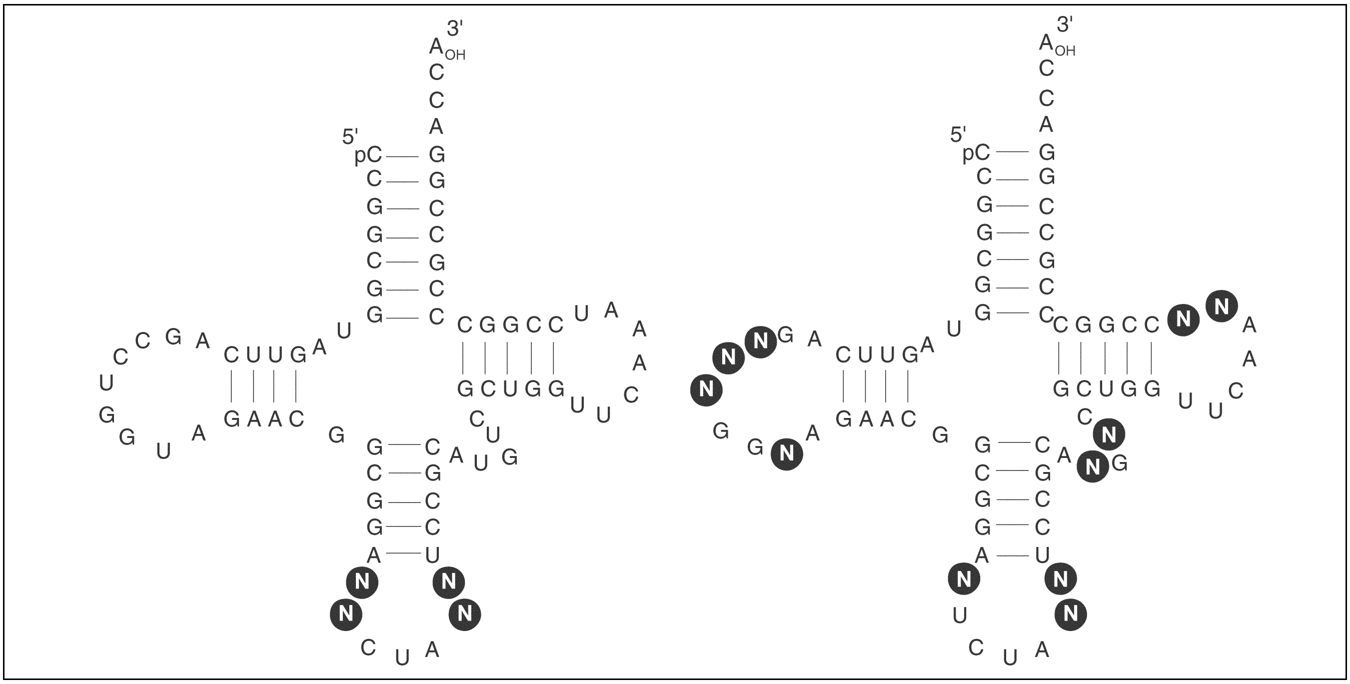

Fig. 1. Anticodon-loop tRNA library (left) and all-loop tRNA library (right) derived from *M. jannaschii* $tRNA^{Tyr}_{CUA}$. Randomly mutated nucleotides (N) are shaded in black.

the suppressor tRNA library is aminoacylated by endogenous *E. coli* synthetases (i.e. it is not orthogonal to the *E. coli* synthetases), the amber codon is suppressed and the toxic gene product produced leads to cell death. Only cells harboring orthogonal tRNAs or nonfunctional tRNAs can survive. All survivors are then subjected to a positive selection in which an amber codon is placed in a drug resistance gene at a nonessential position. tRNAs are then selected for their ability to be aminoacylated by the coexpressed cognate synthetase and to insert an amino acid in response to this amber codon. Cells harboring nonfunctional tRNAs, or tRNAs that cannot be recognized by

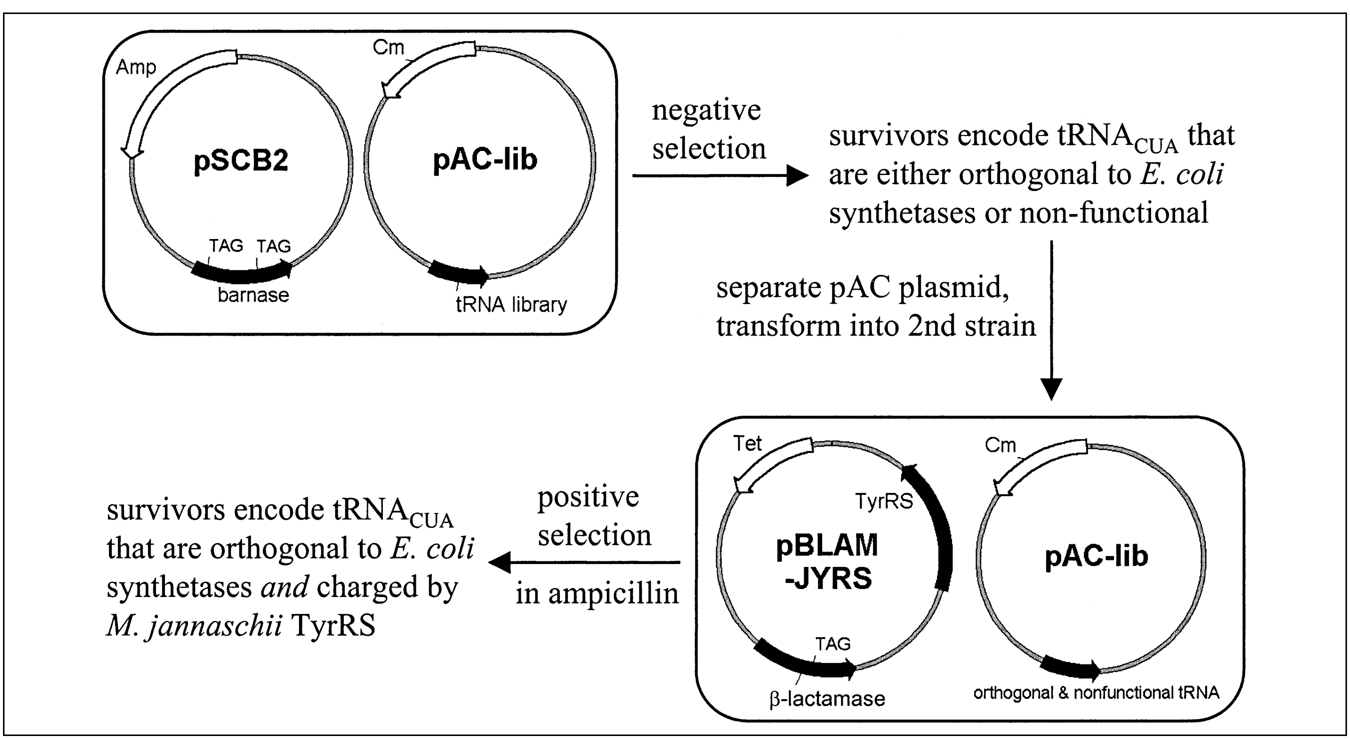

Fig. 2. A general selection for suppressor tRNAs that are poor substrates for the *E. coli* synthetases and charged efficiently by a cognate synthetase of interest.

the synthetase of interest will be sensitive to antibiotic. Therefore, only tRNAs that (1) are not substrates for endogenous *E. coli* synthetases; (2) can be aminoacylated by the synthetase of interest; (3) are functional in translation will survive both selections.

A negative selection was chosen that takes advantage of the toxicity of barnase when produced in *E. coli* in the absence of its natural inhibitor barstar [13]. Amber codons were introduced at nonessential positions in the barnase gene based on analysis of the three-dimensional structure of barnase [6]. Because of barnase's extreme autotoxicity, a low copy number pSC101 origin was placed in the plasmid expressing barnase. In addition, different numbers of amber codons were tested to modulate the stringency of the selection. Plasmid pSCB2 was used to express a barnase mutant with two amber stop codons at Gln2 and Asp44; plasmid pSCB3 contained an additional amber stop codon at Gly65.

To optimize the selection conditions, two suppressor tRNAs were used that are known to be poorly recognized by the *E. coli* synthetases. A mutant suppressor tRNA^{Tyr} derived from *S. cerevisiae* (sc-tRNA$^{Tyr}_{CUA}$, expressed in pAC-YYG1) suppresses the amber codon (Ala184TAG) in the β-lactamase gene affording an IC_{50} value of 12 μg/ml ampicillin for *E. coli* cells; and the suppressor tRNA^{Tyr} derived from *M. jannaschii* (mj-tRNA$^{Tyr}_{CUA}$, expressed in pAC-JY) affords an IC_{50} of 56 μg/ml ampicillin for host cells [9]. For comparison, the suppressor tRNA$^{Gln}_{CUA}$ derived from *S. cerevisiae* tRNA$^{Gln}_{2}$ has an IC_{50} of 21 μg/ml ampicillin when tested in the same assay, and has been demonstrated to be orthogonal to *E. coli* synthetases in vitro and in vivo [6]. Therefore, a negative selection that eliminates cells expressing mj-tRNA$^{Tyr}_{CUA}$, but allows the growth of cells expressing sc-tRNA$^{Tyr}_{CUA}$ should delete nonorthogonal suppressor tRNAs. Cells were grown in liquid minimal media containing 1% glycerol and 0.3 mM leucine (GMML) with appropriate antibiotics to maintain plasmid pSCB2 and the pAC plasmid. Arabinose was added to one set of cells (set 1) to induce the expression of the barnase, while in set 2 no arabinose was added. The fraction of cells surviving the selection was determined by the ratio of cell densities in set 1 relative to set 2 (Fig. 3): cells harboring the control plasmid pAC-Cm (without suppressor tRNA) and plasmid pAC-YYG1 survived, while cells harboring plasmid pAC-JY largely died. When plasmid pSCB3 was used, cells harboring plasmid pAC-JY started to grow in 24 h (data not shown). Therefore, the negative selection was carried out using pSCB2, which encodes the barnase gene containing two amber codons under the above conditions for the library selection.

The positive selection is based on suppression of an amber stop codon introduced at position Ala184 in the TEM-1 β-lactamase gene. Plasmid pBLAM-JYRS encodes

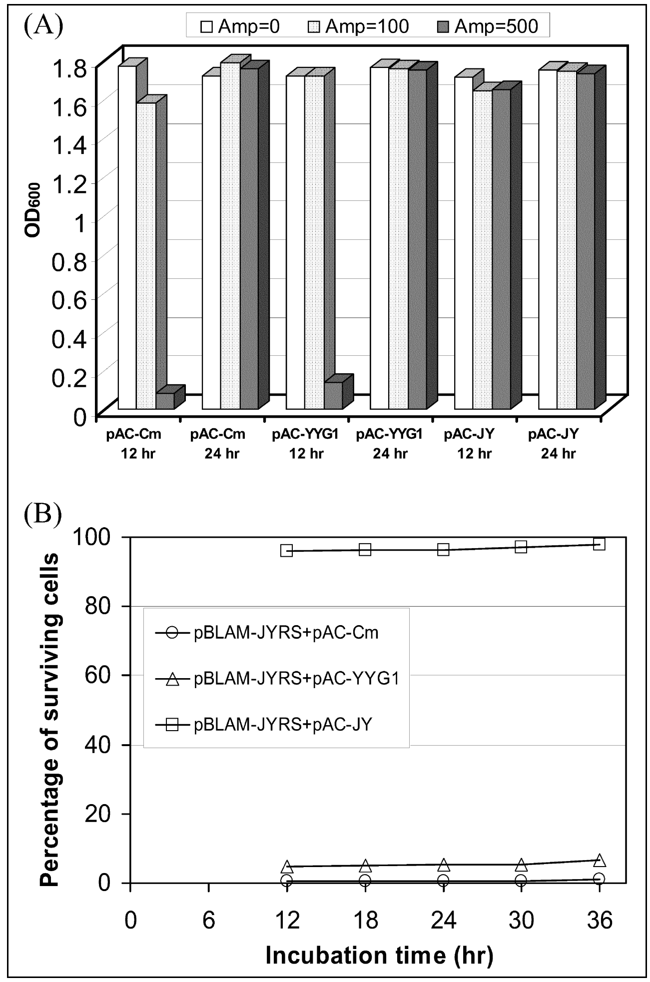

Fig. 4. Positive selection based on the suppression of an amber codon in the β-lactamase gene. pAC plasmid encoding the suppressor tRNA was cotransformed with pBLAM-JYRS in *E. coli* DH10B cells: (A) selection was carried out in liquid 2×YT media with various concentrations of ampicillin; (B) selection was carried out on 2×YT agar plates with 500 μg/ml ampicillin.

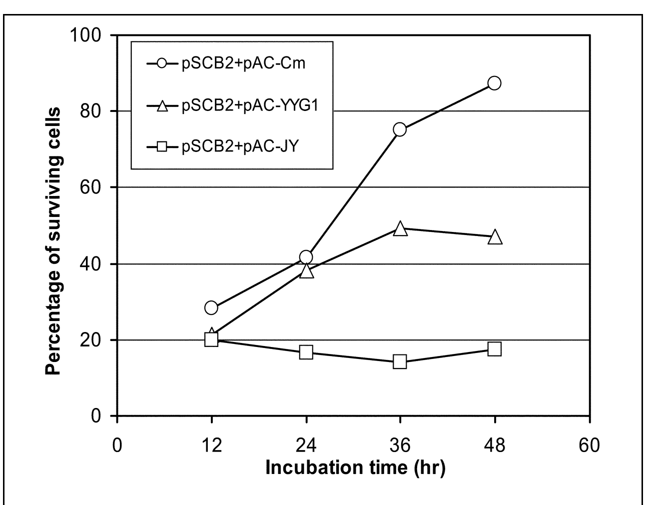

Fig. 3. Negative selection based on the suppression of two amber codons in the barnase gene. Selections were carried out in GMML liquid medium, and 20 mM of arabinose was used to induce barnase expression.

the gene for the *M. jannaschii* tyrosyl-tRNA synthetase and a β-lactamase with an amber mutation at Ala184. pAC plasmids isolated from cells surviving the negative selection were cotransformed with pBLAM-JYRS into *E. coli* DH10B cells. Cells harboring nonfunctional tRNAs or tRNAs that are poor substrates for the *M. jannaschii* synthetase die; those with tRNAs that can be charged by the synthetase survive. To test the feasibility of the positive selection, two model suppressor tRNAs were tested in the presence of *M. jannaschii* TyrRS. The sc-$tRNA^{Tyr}_{CUA}$ has a G1:C72 base pair and is not charged efficiently by *M. jannaschii* TyrRS. When they were coexpressed in cells with the Ala184amber β-lactamase mutant, cells survived to an IC_{50} of 18 μg/ml ampicillin. In contrast, cells containing the *M. jannaschii* $tRNA^{Tyr}_{CUA}$ and the cognate TyrRS survive to an IC_{50} of 1220 μg/ml ampicillin [9]. The model positive selection was first tried in liquid 2×YT medium. The growth of cells harboring pBLAM-JYRS and different pAC plasmids in liquid 2×YT medium with various concentrations of ampicillin are shown in Fig. 4A. Cells transformed with the mj-$tRNA^{Tyr}_{CUA}$ grew at a faster rate and at higher concentrations of ampicillin. However, if cells were grown longer than 24 h, cells transformed with either pAC-Cm or pAC-YYG1 also grew to saturation, making the selection problematic. Therefore, the positive selection was carried out on plates with initial cell densities between 10^3 and 10^5 per plate (Fig. 4B). The survival ratio (number of colonies on plates with ampicillin relative to plates without ampicillin) did not change significantly with different initial cell densities, and was stable over the growth time. The positive selection on ampicillin plates resulted in preferential growth of cells with mj-$tRNA^{Tyr}_{CUA}$ expressed. Therefore, for the library selection the positive selection was carried out on plates instead of in liquid medium.

2.3. Selection results

The negative and positive selections were carried out tandemly as described above on both the anticodon-loop and all-loop libraries. The selected suppressor tRNAs were isolated and retransformed into *E. coli* DH10B harboring pBLAM to test the tRNA's orthogonality to *E. coli* synthetases. The tRNAs were then retransformed into *E. coli* harboring pBLAM-JYRS to test how efficiently the tRNA can be charged by *M. jannaschii* TyrRS. Sequencing of the clones resulting from one round of negative and positive selection of anticodon-loop library revealed that three independent tRNAs were isolated (Fig. 5). When cotransformed with pBLAM, all had lower IC_{50} values than the parent *M. jannaschii* $tRNA^{Tyr}_{CUA}$, indicating they are poorer substrates for *E. coli* synthetases (Table 1). Mutant AA2 also had very high affinity for *M. jannaschii* TyrRS. Unfortunately, although this mutant tRNA could be stably maintained in *E. coli*, it slowed the growth rate of cells for unknown reasons. This effect likely led to the emergence of mutants AA3 and AA4, which both had a mutation outside of the randomization region. Cells harboring AA3 or AA4 grew normally. Nevertheless, AA3 and AA4 were relatively poor substrates for the *M. jannaschii* TyrRS.

Four independent tRNAs were selected from two rounds of negative and positive selections using the all-loop library (Fig. 5). All were poorer substrates for the *E. coli* synthetase than the parent *M. jannaschii* $tRNA^{Tyr}_{CUA}$, yet were still efficiently charged by the *M. jannaschii*

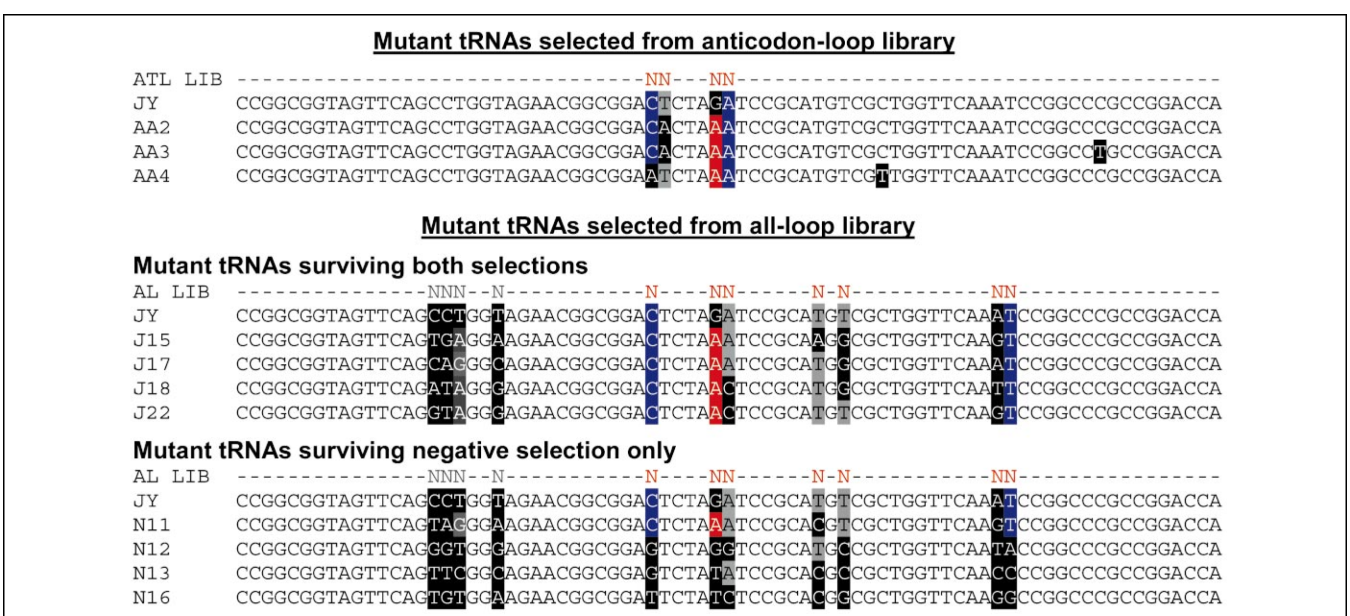

Fig. 5. DNA sequences of mutant suppressor tRNAs selected from anticodon-loop library and all-loop library. JY stands for the wild-type *M. jannaschii* $tRNA^{Tyr}_{CUA}$. Wild-type nucleotides are shaded in blue; mutated nucleotides in red; and semi-conserved nucleotides in gray.

TyrRS as shown by the in vivo β-lactamase assay (Table 1). The IC_{50} value for cells expressing the best mutant, J17, was 12 μg/ml ampicillin, which is even lower than that of cells with the orthogonal $tRNA^{Gln}_{CUA}$ derived from *S. cerevisiae* expressed (21 μg/ml ampicillin). When J17 was coexpressed with the *M. jannaschii* TyrRS, cells survived to an IC_{50} value of 436 μg/ml ampicillin, providing a selection window (ratio of IC_{50} value with TyrRS to IC_{50} value without TyrRS) of 35-fold. In addition, the expression of all these mutant tRNAs did not affect the growth of *E. coli* cells.

To confirm the properties of the selected suppressor tRNAs, they were tested in another in vivo assay based on the suppression of an amber codon in the chloramphenicol acetyltransferase (CAT) gene. In contrast to β-lactamase which is secreted into the periplasm, CAT localizes in the cytoplasm. Moreover, ampicillin is bacteriocidal while chloramphenicol is bacteriostatic. As shown in Table 2, the selected suppressor tRNAs also were orthogonal in the CAT assay, indicating their suitability for CAT selections.

For comparison, we randomly picked four colonies that passed the negative selection only, and tested the tRNAs using the in vivo complementation assay. All of them had very low IC_{50} values when transformed with pBLAM, indicating the negative selection worked well (Table 1). The IC_{50} values were also low when cotransformed with pBLAM-JYRS, revealing that the positive selection functions to delete tRNAs that cannot be charged by the *M. jannaschii* TyrRS.

Analysis of the DNA sequences of the selected tRNAs yielded a characteristic pattern of nucleotide substitutions (Fig. 5). tRNAs that passed both negative and positive selections all had C32 and T60 unchanged, while G37 was mutated to A, and T17a was mutated to either A or G. Some semi-conserved changes included mutation of A38 to either C or A; mutation of T45 to either T or A; mutation of T47 to either G or T. Other mutations had no obvious common pattern. We also sequenced 20 tRNAs that passed the negative selection only, four of which are shown in Fig. 5, and found they all lacked at least one of the common mutations listed above.

Table 1
In vivo β-lactamase assay of selected suppressor tRNAs

Suppressor tRNA	IC_{50} (μg/ml of ampicillin)	
	Coexpressed with pBLAM	Coexpressed with pBLAM-JYRS
mj-$tRNA^{Tyr}_{CUA}$	56	1220
No $tRNA^{Tyr}_{CUA}$	10	10
Mutant tRNAs selected from anticodon-loop library		
AA2	22	1420
AA3	10	110
AA4	12	135
Mutant tRNAs selected from all-loop library		
Mutant tRNAs surviving both selections		
J15	30	845
J17	12	436
J18	20	632
J22	14	459
Mutant tRNAs surviving negative selection only		
N11	11	16
N12	9	18
N13	10	12
N16	9	9

Plasmid pBLAM was used to express the β-lactamase gene with an amber codon at Ala184; plasmid pBLAM-JYRS expressed the amber mutant and the TyrRS of *M. jannaschii*. Suppressor tRNAs were encoded on pAC plasmid and cotransformed with pBLAM or pBLAM-JYRS in the assay.

Table 2
In vivo chloramphenicol acetyltransferase assay of selected suppressor tRNAs

Suppressor tRNA	IC_{50} (μg/ml of chloramphenicol)	
	pYC only	pYC+pBK-JYRS
mj-$tRNA^{Tyr}_{CUA}$	27	308
No $tRNA^{Tyr}_{CUA}$	3	3
J15	11	297
J17	4	240
J18	6	284
J22	5	271

pYC plasmids encoded the chloramphenicol acetyltransferase gene with an amber codon at Asp112 and different suppressor tRNAs listed in the left column of the table. pBK-JYRS was used to express the TyrRS of *M. jannaschii*.

The preferred nucleotides in the selected mutant suppressor tRNAs may play the following roles: (i) they may function as negative determinants for recognition by the *E. coli* synthetases; (ii) they may be identity elements for aminoacylation by *M. jannaschii* TyrRS; or (iii) they may also optimize the tRNA's interaction with *E. coli*'s translational machinery so as to increase the suppression efficiency of the tRNA. It is noteworthy that the G37A mutation was found in tRNAs selected from both the anticodon-loop and all-loop library. This mutation is consistent with previous studies, both theoretical [14] and experimental [15,16], showing that adenine at position 37 enhances amber suppression efficiency. Fechter et al. recently reported that the complete identity set for *M. jannaschii* $tRNA^{Tyr}$ is six nucleotides (C1G72, A73, and anticodon G34U35A36) [17]. The presence of C32 and T60 in all selected mutant suppressors therefore is not required for recognition by *M. jannaschii* TyrRS. All *E. coli* tRNAs have T at position 60 except four tRNAs which have C [18]. Based on the crystal structure of yeast $tRNA^{Phe}$ [19], nucleotide 60 does not interact with other nucleotides. Thus, T60 may maintain the shape of the TΨC loop for productive interaction with the *E. coli* translational machinery. The change of the TΨC loop structure may affect translational fidelity, as the insertion of a nucleotide between T60 and the conserved C61 enables a glycine tRNA to shift reading frame [20]. The role of C32 is not obvious – position 32 in *E. coli* tRNAs includes T, C, and A, and two *E. coli* $tRNA^{Tyr}$s do have C32. As for position 17a, only $tRNA^{Thr}$ has an A at this position.

3. Significance

All of the selected suppressor tRNAs are poorer substrates for *E. coli* synthetases relative to the *M. jannaschii* $tRNA^{Tyr}_{CUA}$, resulting in less mischarging when introduced into *E. coli*. These tRNAs can also be stably maintained in *E. coli* without adverse effects on the growth of host cells. Moreover, they can still be charged efficiently by *M. jannaschii* TyrRS. All these properties make the mutant suppressor tRNA together with the *M. jannaschii* TyrRS a robust orthogonal tRNA–synthetase pair for the selective incorporation of unnatural amino acids into proteins in vivo. Indeed, we have used the J17 mutant suppressor tRNA and an engineered mutant TyrRS to deliver *O*-methyl-L-tyrosine in response to a TAG codon with a fidelity rivaling that of the common 20 amino acids [3]. The in vivo selection strategy described here may be useful for the generation of more orthogonal tRNA–synthetase pairs, and for studies of interactions between tRNAs and synthetases.

4. Materials and methods

4.1. Strains and plasmids

E. coli strain DH10B was obtained from Gibco/BRL. Suppressor tRNA expression plasmids were derived from pAC123 [4]. Plasmids for negative selections were derived from pBATS [21], pYsupA38B2 and pYsupA38B3 [6] as described below.

4.2. Negative selection

A PCR fragment containing the β-lactamase gene and the pSC101 origin was generated from pBATS using the following oligonucleotides: LW115, 5′-ATGCATGCTGCATTAATGAATCGGCCAACG-3′; LW116, 5′-TCCCCGCGGAGGTGGCACTTTTCGGGG-3′. DNA encoding barnase containing two (residues Gln2 and Asp44) or three (residues Gln2, Asp44 and Gly65) amber codons were obtained from pYsupA38B2 and pYsupA38B3, respectively, by digestion with *Sac*II and *Sph*I. Ligation of the above fragments afforded plasmids pSCB2 and pSCB3. The expression of barnase was under arabinose induction. Genes encoding different suppressor tRNAs for in vivo expression were constructed from two overlapping synthetic oligonucleotides (Operon, CA, USA) by Klenow extension and inserted between the *Eco*RI and *Pst*I sites of pAC123 to generate pAC-YYG1 and pAC-JY, respectively, placing transcription under control of the *lpp* promoter and the *rrnC* terminator. pAC-Cm is the control plasmid without any tRNA. To optimize the negative selection conditions, competent DH10B cells harboring pSCB2 or pSCB3 were transformed by electroporation with pAC-Cm, pAC-YYG1, and pAC-JY, separately. Single colonies were picked and grown in 2×YT with chloramphenicol (Cm, 34 μg/ml) and ampicillin (Amp, 100 μg/ml). Cell cultures grown overnight were washed twice with minimal media containing 1% glycerol and 0.3 mM leucine (GMML), and resuspended in GMML with Cm and Amp to an OD_{600} of 0.1. After recovering at 30°C for 10 min, into one culture (set 1) was added 20 mM of arabinose to induce the expression of barnase; no arabinose was added to the second culture (set 2). At different time points, a small amount of cell culture was diluted and plated on 2×YT agar with Cm and Amp to measure cell density. For negative selections of the suppressor tRNA libraries, the pAC plasmids containing the library were transformed into DH10B cells harboring pSCB2. Cells were quenched by addition of SOC medium and recovered at 30°C for 1 h, then were washed with phosphate buffer and GMML, and cultured in 1 l GMML. After recovering at 30°C for 30 min, Cm, Amp, and 20 mM arabinose were added. After 36 h, cells were pelleted and pAC plasmids were isolated and purified by agarose gel electrophoresis.

4.3. Positive selection

Plasmid pBLAM-JYRS was constructed by inserting the *M. jannaschii* TyrRS gene from pBSA50 [10] between *Nde*I and *Pst*I sites of pBLAM-YQRS [6] using oligonucleotides LW104, 5′-GGAATTCCATTAGGACGAATTTGAAATG-3′; and LW105, 5′-AAACTGCAGTTATAATCTCTTTCTAATTGGCTC-3′. To optimize the positive selection conditions, competent DH10B cells harboring pBLAM-JYRS were transformed with pAC-Cm, pAC-YYG1, and pAC-JY, separately. Single colonies were picked and grown in 2×YT with Cm and tetracycline (Tet, 40 μg/ml). In liquid selections, overnight cell cultures were diluted into 2×YT with Cm and Tet at a starting OD_{600} of 0.1. Various concentrations of Amp were added, and cell growth was monitored by OD_{600}. In plate selections, approximately 10^3 to 10^5 cells were plated on two sets of 2×YT agar plates containing Cm and Tet, one set of which contained 500 μg/ml Amp. For selections involving the mutant tRNA library, pAC plasmids isolated from the cells from the negative selection were transformed into competent DH10B cells harboring pBLAM-JYRS. Cells were recovered at 37°C for 45 min, and approximately 10^5 cells were plated onto each 2×YT agar plate containing Cm, Tet and 500 μg/ml of Amp. After 24 h, colonies were picked and re-grown in 6 ml 2×YT containing Cm, Tet and 200 μg/ml of Amp. DNA was isolated and pAC plasmid was purified by agarose gel electrophoresis.

4.4. Construction of the suppressor tRNA library

The sequences of the two overlapping oligonucleotides used to construct the anticodon-loop library are (the tRNA sequence underlined): LW125, 5′-GGAATTC<u>CCGGCGGTAGTTCAGCCTGGTAGAACGGCGGANNCTANNTCCGCATGTCG</u>-3′; LW126, 5′-AAAACTGCAG<u>TGGTCCGGCGGGCCGGATTTGAACCAGCGACATGCGGANNTAGNNTCCGCCGTTCTAC</u>-3′ (where N is equimolar of A, C, T or G). The sequences of oligonucleotides for the all-loop library are: LW145, 5′-GGAATTC<u>CCGGCGGTAGTTCAGNNNGGNAGAACGGCGGANTCTANNTCCGCANGNCGCTGGTTC</u>-3′ and LW146, 5′-AAAACTGCAG<u>TGGTCCGGCGGGCCGGNNTTGAACCAGCGNCNTGCGGANNTAGANTCCGCCGTTC</u>-3′. These genes were inserted into pAC123 similarly as described above to afford the tRNA libraries.

4.5. In vivo complementation assays

The in vivo complementation assay which is based on suppression of an amber codon in the β-lactamase gene was carried out as described [6,9]. In the chloramphenicol acetyltransferase (CAT) assay, an amber codon was substituted for Asp112 in the CAT gene of pACYC184 to afford pACMD112TAG [7]. The genes encoding the suppressor tRNAs under the control of the *lpp* promoter and *rrnC* terminator were excised from pAC plasmids with *Nco*I and *Ava*I, and inserted into the pre-digested pACMD112TAG to afford plasmids pYC-JY, pYC-J15, pYC-J17, pYC-J18, and pYC-J22, respectively. Plasmid pBK-JYRS, a derivative of pBR322, was used to express the *M. jannaschii* TyrRS under the control of the *E. coli* GlnRS promoter and terminator. The survival of *E. coli* DH10B cells transformed with pYC plasmid alone or cotransformed with pYC and pBK-JYRS was titrated against a wide range of chloramphenicol concentrations added to the growth media, and IC_{50} values were interpolated from the curves.

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