A New Orthogonal Suppressor tRNA/Aminoacyl-tRNA Synthetase Pair for Evolving an Organism with an Expanded Genetic Code

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Dedicated to Professor *Albert Eschenmoser* on the occasion of his 75th birthday

Several steps have been completed toward the development of a method for the site-specific incorporation of unnatural amino acids into proteins *in vivo*. Our approach consists of the generation of amber suppressor tRNA/aminoacyl-tRNA synthetase pairs that are orthogonal to all *Escherichia coli* endogenous tRNA/synthetase pairs, followed by directed evolution of the orthogonal aminoacyl-tRNA synthetases to alter their amino-acid specificities. A new orthogonal suppressor tRNA/aminoacyl-tRNA synthetase pair in *E. coli* has been derived from the *Saccharomyces cerevisiae* $tRNA^{Asp}$ and aspartyl-tRNA synthetase, and the *in vitro* and *in vivo* characteristics of this pair were determined. Two different antibiotic resistance selections were compared using this novel pair in an effort to develop a tunable positive selection for a mutant synthetase capable of charging its cognate suppressor tRNA with an unnatural amino acid.

1. Introduction. – In an effort to expand the scope of protein mutagenesis, we previously developed an *in vitro* method for the site-specific incorporation of unnatural amino acids into proteins [1]. This strategy is based on the suppression of amber stop codons by *in vitro* transcribed tRNAs that are chemically aminoacylated with unnatural amino acids. The ability to perform unnatural amino-acid mutagenesis directly in living cells will further enhance the utility of this technique, and provide powerful tools for studying proteins in their native environment [2]. This will require aminoacyl-tRNA synthetases (aaRSs) that uniquely aminoacylate amber suppressor tRNAs *in vivo*. One can obtain functional tRNA/synthetase pairs that are orthogonal (*i.e.*, not catalytically competent) with all *E. coli* endogenous tRNA/synthetase pairs by either evolving an endogenous pair [3], or by importing a suitable pair from another organism [4][5]. To date, one orthogonal pair for use in *E. coli* was generated from the $tRNA^{Gln}$/GlnRS pair from *S. cerevisiae* [4], and one from the $tRNA^{Tyr}$/TyrRS pair from *Methanococcous jannaschii* [5]. Additional pairs may provide better starting points for evolving active sites with particular amino-acid specificities (*e.g.*, size, hydrophobicity, *etc.*), and may enable simultaneous incorporation of multiple unnatural amino acids.

One challenge of finding suitable candidate pairs lies in the paucity of data about cross-species tRNA specificities of synthetases; another lies in the fact that the tRNA anticodon sequence is often critical for recognition by the synthetase (*i.e.*, mutating the anticodon to CUA for amber suppression often results in loss of recognition by the cognate synthetase). In the latter case, it is sometimes possible to introduce compensatory mutations in the acceptor stem-binding domain of the synthetase that rescue recognition of the suppressor tRNA. For example, the *E. coli* AspRS E93K

mutant recognizes $tRNA^{Asp}_{CUA}$ about an order of magnitude better than does native AspRS, albeit poorly [6]. Furthermore, the E93K mutant no longer recognizes the native $tRNA^{Asp}_{GUC}$ [6], making the *E. coli* $tRNA^{Asp}_{CUA}$/AspRS(E93K) pair a candidate for an orthogonal pair in *E. coli*. The only obstacles are the relatively low activity of the E93K mutant and the ability of the endogenous native AspRS to aminoacylate the suppressor $tRNA^{Asp}_{CUA}$ to some degree [6]. It may be possible to increase the expression levels of the AspRS(E93K) and the suppressor tRNA to compensate for the lowered activity, but the ability of the endogenous AspRS to charge the suppressor tRNA would be a significant source of background in the ensuing selections to alter the amino-acid specificity of the orthogonal synthetase.

To avoid this problem, one may be able to take advantage of the known orthogonality of the yeast $tRNA^{Asp}$ in *E. coli* [7][8], if one can alter the anticodon-binding-domain specificity of the yeast AspRS in a similar fashion to that of *E. coli* AspRS. We reasoned that AspRS orthogonal system could be generated by importing the *S. cerevisiae* $tRNA^{Asp}_{CUA}$/AspRS(E188K) pair, because *a*) the anticodon $tRNA^{Asp}$ identity elements are the same for *E. coli* and yeast AspRS [9], *b*) E188 γ-carboxylate forms H-bonds to the same N(1) and N(2) of anticodon base G34 in the yeast AspRS crystal structure [10] as E93 in the *E. coli* structure [11], and *c*) mutagenesis of E188 to alanine showed marked increase in K_D and decrease in specificity for $tRNA^{Asp}$ [12][13], suggesting that E188 has the same function in yeast AspRS as E93 in the *E. coli* enzyme. We describe here the *in vitro* and *in vivo* properties of this new orthogonal pair, and discuss its suitability for evolving the amino-acid binding site of the yeast enzyme.

2. Results and Discussion. – 2.1. *Yeast* $tRNA^{Asp}_{CUA}$ *is an Orthogonal tRNA in* E. coli. One of the requirements of the system for delivery of an unnatural amino acid into proteins site-specifically is a suppressor tRNA that cannot be aminoacylated by any endogenous *E. coli* synthetase. To test whether the yeast $tRNA^{Asp}_{CUA}$ fulfills this requirement *in vivo*, we used a β-lactamase suppression assay. In this assay, the tRNA is under control of the *lpp* promoter and *rrnC* terminator on a pACYC184-derived plasmid ($pACYD_{CUA}$). Suppression of the A184TAG mutation in β-lactamase on a pBR322-derived vector (pBLAM) is measured as the ampicillin concentration at which half of the cells survive, yielding IC_{50} values in units of μg/ml ampicillin [4]. If the newly introduced suppressor tRNA is aminoacylated by any endogenous *E. coli* synthetases, the cells will grow in the presence of ampicillin. The *E. coli* strain DH10B co-transformed with pBLAM and the yeast $tRNA^{Asp}_{CUA}$ can survive only at very low concentrations of ampicillin (18 μg/ml). For comparison, cells bearing pBLAM alone grow to 10 μg/ml, and when co-expressed with *S. cerevisiae* $tRNA^{Gln}_{CUA}$ (A38) (which is an orthogonal tRNA in *E. coli* [4]) under the same conditions, the cells can grow to 21 μg/ml ampicillin. The expression level of the suppressor tRNA was determined by Northern blotting and comparison with the endogenous $tRNA^{Lys}_{UUU}$. When the yeast $tRNA^{Asp}_{CUA}$ expression cassette is on the pTAK-DRS^{E188K}-YD_{CUA} vector, the expression levels of the two tRNAs are equivalent, whereas, on the $pACYD_{CUA}$ plasmid, the suppressor tRNA expression level is 10-fold lower (results not shown). The acceptance of the suppressor tRNA by the ribosome was also determined (see below). These results demonstrate that yeast $tRNA^{Asp}_{CUA}$ is orthogonal in *E. coli* and is suitable for the delivery of an unnatural amino acid into proteins *in vivo*.

2.2. *Yeast AspRS(E188K) Mutant Does Not Aminoacylate* E. coli *tRNAs.* The second requirement for an orthogonal system is that the orthogonal synthetase cannot aminoacylate any of the endogenous *E. coli* tRNAs. Charging of the *E. coli* tRNAs other than $tRNA^{Asp}$ by the yeast AspRS would result in misacylation and cell death. Therefore, the only endogenous tRNA of concern is the *E. coli* $tRNA^{Asp}$. Purified yeast AspRS(E188K) enzyme was assayed *in vitro* under physiological conditions for acylation of whole *E. coli* tRNA with L-[2,3-$^3H_2$]aspartic acid. The E188K mutant shows a marked decrease in activity toward endogeneous *E. coli* tRNA and a slight increase in activity toward the suppressor $tRNA^{Asp}_{CUA}$ (see *Table 1*). The ability of the yeast AspRS(E188K) enzyme to acylate *E. coli* $tRNA^{Asp}$ *in vivo* was assessed by complementing the AspRS temperature-sensitive *E. coli* strain CS89 [14] with the yeast AspRS(E188K) encoded on a pBR322-derived plasmid (pTAK-DRS^{E188K}) under control of *tac* promoter. No colonies were observed after 24 h at non-permissive temperature (43°), compared to the positive controls expressing either wild-type yeast or wild-type *E. coli* AspRS. Together, these results confirmed that the E188K mutation in the yeast AspRS is not only responsible for the marked decrease in specificity for the yeast $tRNA^{Asp}_{GUC}$ [12], but also eliminates the activity toward the *E. coli* $tRNA^{Asp}_{GUC}$.

Table 1. *Activity of Orthogonal Synthetases toward the Corresponding* E. coli *Endogenous tRNA Substrates and the Orthogonal Suppressor tRNAs* (activities are in pmol product per mg enzyme per min)

	Endogenous *E. coli* tRNA	Cognate suppressor tRNA
E. coli GlnRS	560,000 [a])	590 [a])
S. cerevisiae GlnRS	9 [b])	180 [b])
S. cerevisiae AspRS	>16,000	470
S. cerevisiae AspRS(E188K)	240	520

[a]) From [3]. [b]) From [4].

2.3. *Yeast AspRS(E188K) Mutant Aminoacylates Yeast* $tRNA^{Asp}_{CUA}$ In Vitro *and in* E. coli. The remaining requirement for orthogonality is that the synthetase must efficiently acylate its cognate suppressor tRNA. Purified yeast AspRS was assayed *in vitro* for its ability to aminoacylate the yeast suppressor $tRNA^{Asp}_{CUA}$. Its activity under physiological conditions is comparable with the activity of other orthogonal synthetases toward their cognate suppressor tRNAs (*Table 1*) [4]. The *in vivo* evidence for efficient charging of the suppressor tRNA by the orthogonal synthetase and for the acceptance of the suppressor tRNA by the ribosome was obtained with a β-lactamase assay analogous to the one based on pBLAM described earlier. To separate the reporter from the synthetase gene for more convenient vector manipulation, the β-lactamase gene was moved to a lower-copy vector containing a p15A origin. This change also conveniently lowered the reporter copy number, thus reducing the background signal of the assay. Also, this β-lactamase contains an amber stop codon in the coding region of a short poliovirus C3 peptide fused to the N-terminus of β-lactamase (*C3*TAG), as opposed to a location within the reporter (see *Sect. 3.4*). Since the sequence of the N-terminal fusion peptide has no effect on the function of β-lactamase, any amino acid can be inserted at this position, making this fusion a suitable selection marker for a general positive selection. Also, the suppression efficiency at this site is better than at the

internal A184TAG site used previously (*Table 2*). Expression of AspRS(E188K) under a *tac* promoter on the pBR322-derived plasmid bearing the suppressor tRNA (pTAK-DRS^{E188K}-YD$_{CUA}$) cotransformed into *E. coli* with the newly constructed pACBLA^{C3TAG} showed an ampicillin IC_{50} well above that of the control strain expressing the inactive form of AspRS (60 μg/ml compared with 5.4 μg/ml) (*Table 3*). The 11-fold increase in the IC_{50} value results from acylation of the yeast tRNA$^{Asp}_{CUA}$ by the yeast AspRS(E188K), and shows that the ribosome can process the aminoacylated suppressor tRNA$^{Asp}_{CUA}$.

Table 2. *Suppression of β-Lactamase Amber Codons with the Aspartyl Orthogonal Pair.* The ampicillin IC_{50} values for cells cotransformed with one of the β-lactamase amber constructs in pACBLA-YD$_{CUA}$ vector, and a pTAK vector expressing either an active AspRS(E188K) ($IC_{50(act)}$) or inactive AspRS(E188K) mutant containing 5 alanine mutations in the active site ($IC_{50(inact)}$) (see *Sect. 3.2*). Based on the comparison of IC_{50} ratios, the β-lactamase system is most sensitive when a single amber codon resides in the N-terminal fusion peptide.

	$IC_{50(act)}$	$IC_{50(inact)}$	$IC_{50(act)}/IC_{50(inact)}$
C3^{N6TAG}-BLA	135	7.5	18
BLA^{A184TAG}	57	7.1	8
C3^{N6TAG}-BLA^{A184TAG}	4.2	<1	4.2

Table 3. *Comparison of the Orthogonal Pairs* In Vivo *Using the β-Lactamase Selection*

aaRS/tRNA$^{a.a.}_{CUA}$ pair	$IC_{50(act)}$	$IC_{50(inact)}$	$IC_{50(act)}/IC_{50(inact)}$
S. cerevisiae Gln	140	11	13
M. jannaschii Tyr [a])	514	60	8.6
S. cerevisiae Asp	60	5.4	11

[a]) *Lei Wang*, TSRI, unpublished results. Each tRNA was expressed from a construct identical to pACYD$_{CUA}$. The synthetases were expressed from a vector identical to pTAK-DRS, except for the TyrRS gene, which was under control of a weaker *E.coli* GlnRS promoter, because overexpression of TyrRS due to the *tac* promoter was toxic to the cells.

2.4. *Behavior of Orthogonal Pair in a β-Lactamase Selection.* To alter the amino-acid specificities of the orthogonal synthetases, libraries of random or targeted mutants are generated, followed by selection of mutant enzymes with novel amino-acid specificities. One approach involves a general positive selection based on antibiotic resistance. This type of selection relies on suppression of an amber codon in a given antibiotic resistance gene by a suppressor tRNA aminoacylated by an orthogonal synthetase. In principle, higher synthetase activity results in survival of the cells at higher antibiotic concentrations, therefore allowing the experimenter to adjust the selection stringency as desired. A positive selection based on β-lactamase amber suppression was previously reported [4]. To apply this selection to the evolution of the orthogonal aspartyl pair, the properties of the β-lactamase selection have been examined under various conditions, and compared to a selection based on chloramphenicol resistance.

The ratio of the IC_{50} values in the presence and absence of active orthogonal synthetase in *Table 3* is one of the key parameters for comparing the performance of orthogonal pairs in a given antibiotic selection. In principle, a higher ratio means that weaker library members can pass the selection. The ratios can be increased, for

example, by altering the suppressor tRNA to decrease its recognition by an endogenous synthetase, or by mutating the synthetase to increase its activity toward the suppressor tRNA. Alternatively, it may be possible to modify the stringency of the selection by performing the selection in a release-factor-1-deficient strain (see *Sect. 2.6*), or by changing the expression levels of individual selection components. The effects of modulating the expression of individual components are summarized in *Fig. 1.* The lowest selection ratio (5.0) is observed when the suppressor tRNA is coded on a low-copy vector (pACBLA^{C3TAG}-YD$_{CUA}$), and synthetase expression is driven by a relatively weak *E. coli* GlnRS promoter. Changing the orthogonal synthetase promoter to a stronger *tac* promoter (construct pTAK-DRS^{E188K}) had a significant effect on both the IC_{50} and the selection ratio. A similar effect was observed after additional increase in the copy number of the suppressor tRNA gene, which resulted from moving the tRNA expression cassette from the low-copy pACBLA^{C3TAG}-YD$_{CUA}$ plasmid to a higher-copy pTAK-DRS^{E188K} to afford pTAK-DRS^{E188K}-YD$_{CUA}$. This selection system offers the highest sensitivity of all three combinations examined.

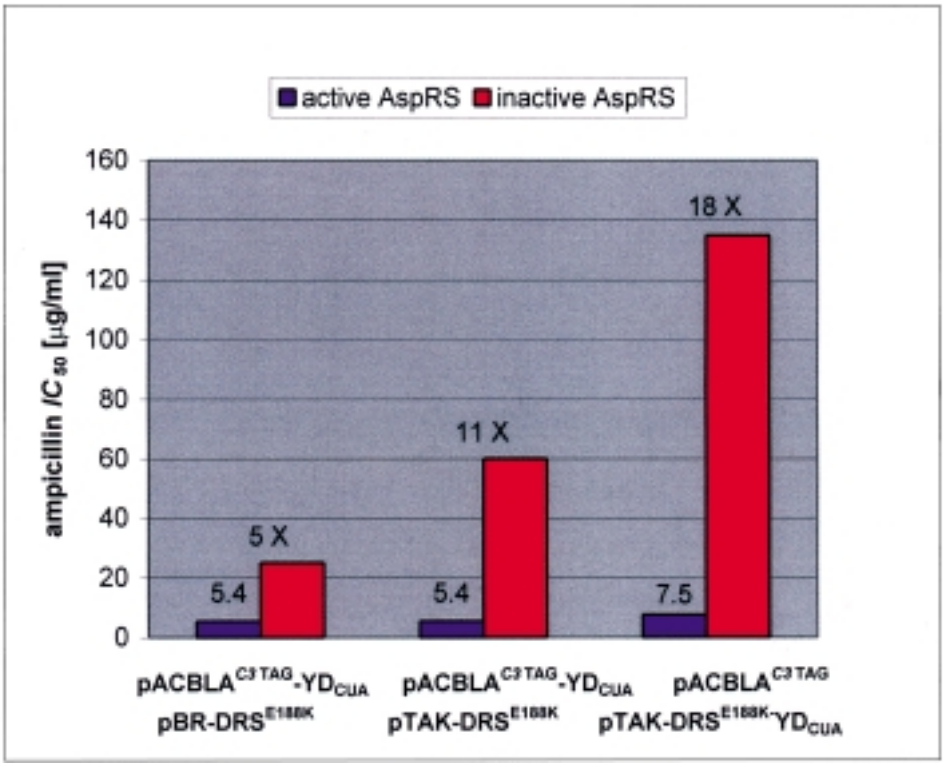

Fig. 1. *Effect of increasing the AspRS and tRNA expression levels on the selection range.* The synthetase expression level was increased by changing the *E. coli* GlnRS promoter in pBR-DRS^{E188K} for the *tac* promoter in pTAK-DRS^{E188K}. The tRNA expression level was increased by moving the tRNA from the low-copy pAC vector (p15A origin), to a high-copy pTAK vector (ColE1 origin).

2.5. *Behavior of Orthogonal Pairs in a Chloramphenicol Acetyl Transferase (CAT) Selection.* The β-lactamase selection has several inherent limitations: *i*) ampicillin IC_{50} values are more cell-density dependent than IC_{50} values for other antibiotics, due to the periplasmic localization of the enzyme, which facilitates 'cross feeding' even in a sparsely populated culture. This places a limit on the starting cell density in a selection; and *ii*) sufficiently high ampicillin concentrations kill the majority of nonresistant cells, making it even more difficult to modulate the selection stringency without losing the weak hits. A selection based on suppression of amber-stop codon in CAT minimizes these concerns, since chloramphenicol is bacteriostatic and the cytoplasmic localization of CAT slows 'cross feeding'. Indeed, experiments with pACM(S27TAG)-based selection demonstrate that the CAT selection is amenable to higher cell densities in liquid media and on plates, and that more active synthetases are enriched over weaker synthetases (*T. J. M.*, unpublished results).

To test the CAT selection, we attempted to append the same N-terminal *C3*TAG sequence to CAT, as in the case of β-lactamase. However, this construct was inactive, even when the amber codon was replaced by an aspartate codon, probably due to the interference of the fused peptide with active CAT trimer formation. Instead, a candidate permissive site in CAT was identified from sequence alignments of homologous CAT proteins [15] and crystal-structure analysis [16], and an amber mutation was introduced into a chloramphenicol gene on pACYC184 vector to generate pACM(D112TAG). To confirm general amino-acid permissivity of this site, 12 different amino-acids were inserted at this position using the *Interchange System* (*Promega*), a set of strains carrying 12 different suppressor tRNAs (*Fig. 2*). The chloramphenicol IC_{50} values obtained in these strains were compared to a suppression profile of another CAT amber mutation in the same plasmid, pACM(T10TAG), provided by the manufacturer. Few significant differences between the three sites were observed, suggesting that all three sites are permissive to most amino acids. However, when the two CAT mutants were compared in presence of the aspartyl orthogonal pair (which constitutes the first Asp suppressor strain), D112TAG was suppressed more efficiently than T10TAG. This fortuitous difference in suppression profile could provide a very specific negative selection against aspartate incorporation by synthetase mutants capable of recognizing their natural amino-acid substrate.

The IC_{50} ratio for the CAT selection is smaller than the IC_{50} ratio of the β-lactamase selection (see $RF1^{wt}$ strain in *Fig. 3*), which indicates that the CAT selection may be more stringent than the β-lactamase selection, at least in the given synthetase activity range. Also, the suppression of D112TAG in CAT by the aspartyl orthogonal pair only results in growth up to 16 μg/ml chloramphenicol, compared to suppression up to *ca.* 300 μg/ml by some of the known suppressor tRNAs (*Fig. 2*). Therefore, we sought to improve the suppression efficiency of the aspartyl orthogonal pair by minimizing the release-factor-1-mediated termination at the amber stop codon.

2.6. *Effect of Release Factor 1 (RF1) on Selections.* Release factors mediate termination of translation at stop codons and effectively compete with suppressor tRNAs. Temperature-sensitive mutations in *E. coli* RF1 are known to increase read-through levels by several suppressor tRNAs *in vivo* [17]. Suppression of amber codons had also been shown to be enhanced *in vitro*, when the translation extract was prepared from $RF1^{ts}$ *E. coli* strain and heat-treated at a nonpermissive temperature to inactivate

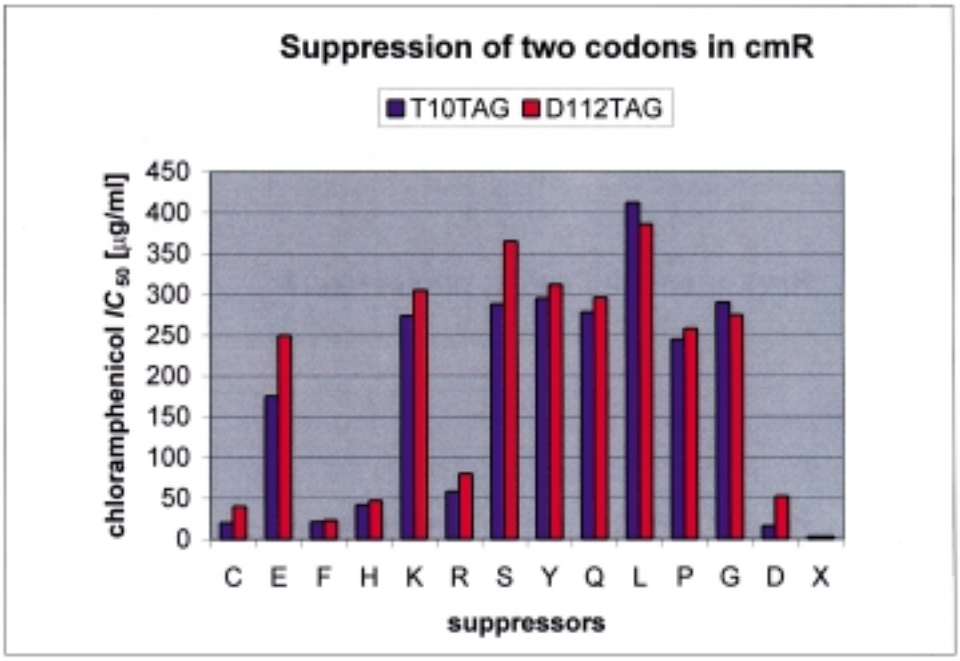

Fig. 2. *Suppression of two different amber codons in CAT.* The listed amino-acids represent individual suppressor strains transformed with pACM(T10TAG) and pACM(D112TAG). The 'D' suppressor strain is a release-factor-1-deficient strain containing pTAK-DRS^{E188K}-YD$_{CUA}$ (see *Fig. 3.*). 'X' is a control strain without a suppressor tRNA.

most of the RF1 protein [18]. A comparison of the two antibiotic selections in the presence and absence of RF1 is presented in *Fig. 3.* Indeed, diminution of competition between RF1 and the suppressor tRNA results in significantly better selection performance, further improving the sensitivity of both systems. The only limitation to use of the MRA8 strain for selections is its very low transformation efficiency. Thus, we are now moving the *prtA1^{ts}* marker into a general cloning strain, which is more suitable for selections.

Conclusion. – We have shown that the yeast AspRS(E188K) together with the yeast $tRNA^{Asp}_{CUA}$ constitute a functional orthogonal pair *in vitro* and in *E. coli*. The performance of this orthogonal pair in two antibiotic selections has been optimized, and its selection properties were compared with those of two other orthogonal pairs. The inactivation of RF1 *in vivo* increased the sensitivity of both selections. We are currently generating large naive and targeted libraries of mutant yeast AspRS enzymes, which will be subjected to the selections described here in presence of unnatural amino-acids [4] in order to isolate synthetases with novel amino-acid specificities.

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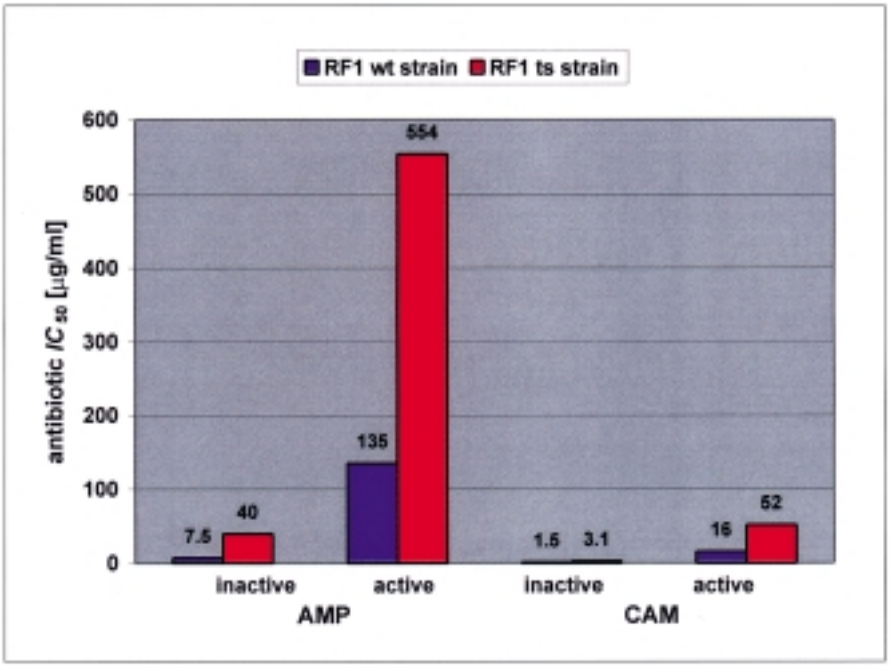

Fig. 3. *Effect of the RF1 on the selections.* The wild-type RF1 strain is DH10B, and the temperature-sensitive RF1 strain is MRA8. Plasmid pTAK-DRS^{E188K}-YD$_{CUA}$ containing either the active or inactive (Ala_5) AspRS was co-transformed with the appropriate reporter (pACBLA^{C3TAG} or pACM(D112TAG)) into each strain. The IC_{50} values were determined as described (*Sect. 3.4*).

strain CS89. We would like to thank *John Anderson* (TSRI) for unpublished construct, and *Lei Wang* (TSRI) for unpublished results. We thank Dr. *David Liu* for helpful discussions and *John Anderson* for critical reading of the manuscript. *T. J. M.* is supported by an *NSF Predoctoral Fellowship*. This research was supported by the *U.S. Department of Army.*

Experimental Part

1. *Strains, Plasmids, and Reagents.* Strains DH10B, CS89 [14], BL21λDE3, and MRA8 [22] were obtained from *GIBCO/BRL* (Grand Island, NY, USA), *Garry Sharples* (University of Nottingham, Nottingham, United Kingdom), *Novagen* (Madison, WI, USA), and *Jeannie Barrett* (University of California, Berkeley, CA, USA), resp. pTG908-APS plasmid encoding wild-type yeast AspRS gene [23] was obtained from *Gilbert Eriani* (CNRS, Strasbourg, France). Plasmid for runoff transcription of suppressor tRNA was derived from pYPhe2 [24] as described. *In vivo* expression vectors bearing suppressor tRNAs were derived from pAC123 [3] as described below. L-[2,3-$^3H_2$]aspartic acid was obtained from *NEN* (Boston, MA, USA), restriction enzymes from *New England Biolabs* (Beverly, MA, USA), shrimp alkaline phosphatase, crude tRNA from yeast, and *E. coli*, rNTPs, and dNTPs from *Roche Molecular Biochemicals* (Indianapolis, IN, USA), and Turbo *Pfu* DNA polymerase from *Stratagene* (La Jolla, CA, USA).

2. *Construction of Synthetase and Suppressor tRNA Expression Vectors.* The gene encoding the yeast AspRS was amplified from pTG908-APS [23] by two successive PCRs (restoring the wild-type N-terminal residues and introducing N-terminal histidine tag), and subcloned between the *Nde*I and *Xba*I sites of pTAK-HYQRS to afford pTAK-DRS. Plasmid pTAK-HYQRS was derived from pHYQRS by replacing the *E. coli* GlnRS promoter between *Nde*I and *Ava*I sites with the *tac* promoter and *lacI^q* gene from pMAL-p2x (*New England Biolabs*). Plasmid pHYQRS was generated by ligating the *Ava*I/*BspH*I fragment from pBLAM-HYQRS [3] with the kanamycin resistance gene from pACYC177 (*New England Biolabs*). The E188K mutation in yeast

AspRS was introduced using mutagenic oligonucleotides as described [25] to yield pTAK-DRS^{E188K}. The Ala_5-inactive version of DRS^{E188K} was made similarly by mutating five active-site residues crucial for AspRS activity [10][19–21]. The vector pET-DRS^{E188K} for synthetase overexpression and purification was generated by inserting the *NdeI*/*Spe*I DRS^{E188K} fragment between the *Nde*I/*Nhe*I sites of plasmid pET25b (*Novagen*, Madison, WI, USA). The suppressor tRNA expression cassette was amplified by PCR from pACYD$_{CUA}$ and inserted between the *Pac*I and *Age*I sites of pTAK-DRS^{E188K} to yield pTAK-DRS^{E188K}-YD$_{CUA}$. Genes encoding tRNAs for *in vivo* expression were constructed from overlapping oligonucleotides (*Operon*, Alameda, CA, USA) and inserted between the *Pst*I and *Eco*RI sites in pAC123 [3] to afford pACYD$_{CUA}$. Oligonucleotide sequences were as follows, with the tRNA sequence underlined, and the differences from the wild-type yeast tRNA^{Asp} sequence in small letters: 5′-CGGAATTCgCCGTGATAGTTTAATGGTCAGAATGGGCGCTTcTaGCGTGCCAGATC-3′, and 5′-AAACTGCAGTGGCgCCGCGACGGGGAATTGAACCCCGATCTGGCACGCtAgAAGC-3′. The tRNA gene for runoff transcription was similarly constructed and inserted between the *Kpn*I and *Hind*III sites of pYPhe2 as described in [24]. Runoff transcription of the tRNA was carried out with T7 RNA polymerase as described in [26], and the identity of each tRNA was confirmed by MALDI mass spectrometry and denaturing PAGE.

3. In Vitro *Assays of AspRS(E188K) Enzyme.* The AspRS(E188K) enzyme was expressed in the *E. coli* BL21λDE3 strain from the vector pET-DRS^{E188K}, and purified from the $(NH_4)SO_4$-precipitated S-100 fraction using the *Bio-Scale CHT5-I* column (*Bio-Rad*, Hercules, CA, USA). The enzyme concentration was determined using *SDS-PAGE* (*Bio-Rad*) and staining with *Coomassie Blue R250* (*Sigma*, St. Louis, MO, USA), followed by comparison with known concentrations of bovine serum albumin (BSA; *Sigma*). Activity assays contained 10 mM ATP, 0.1 mM Asp, 8 μM tRNA, 3 mM glutathione, 0.1 mg/ml BSA, 20 mM $MgCl_2$, 30 mM KCl and 50 mM *Tris*-Cl, pH 7.5, and were performed at 37° as described in [27].

4. *β-Lactamase Selection.* Vector pACBLA-YD$_{CUA}$ was constructed by inserting the *Sph*I/*Eco*RV fragment containing the β-lactamase gene from plasmid pBR322-*SX* between the same sites in pACYD$_{CUA}$. Plasmid pBR322-*SX* is plasmid pBR322 (*New England Biolabs*), containing a unique *Spe*I site between the β-lactamase leader sequence and the sequence coding for mature polypeptide, and a unique *Xma*I site at the end of the gene (*Chris Anderson*, TSRI). Various forms of β-lactamase (*C3*^{N6TAG}-BLA, BLA^{A184TAG}, and *C3*^{N6TAG}-BLA^{A184TAG}), with appended histidine tags at the C-termini, were constructed by PCR as described in [25], and subcloned between the *Spe*I and *Xma*I sites in pACBLA-YD$_{CUA}$ to generate the plasmids described in *Table 2*. The sequence of the peptide appended to the N-terminus of mature β-lactamase is HPETLVGPASTTXKDKLGL, with the *C3*^{N6TAG} sequence underlined. The suppressor tRNA constructs in *Table 3* were generated by replacing the orthogonal tRNA *Nco*I/*Sph*I cassette in pACBLA^{C3TAG}-YD$_{CUA}$ with orthogonal tRNA$^{Gln}_{CUA}$ cassette from pACYsupA38 [4], or the orthogonal tRNA$^{Tyr}_{CUA}$ cassette from pAC-JY$_{CUA}$ [5]. The ampicillin IC_{50} values were determined by plotting the percentage of cells surviving on 2xYT plates containing a wide range of ampicillin concentrations after 16 h of growth at 37° [4].

5. *CAT Selection.* Plasmid pACM(D112TAG) contains an amber mutation at residue D112 of CAT, and was constructed from pACYC184 using mutagenic oligonucleotides as described in [25]. Plasmid pACM(T10TAG) provided with the *Interchange System* (*Promega*) is identical to the pACM(D112TAG) plasmid, except that the amber mutation is at T10 of CAT. Electrocompetent cells made from 12 suppressor strains plus a control strain in the kit were transformed with pACM plasmids, and the chloramphenicol IC_{50} values were determined similarly to those for ampicillin.

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