Quality control despite mistranslation caused by an ambiguous genetic code

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A high level of accuracy during protein synthesis is considered essential for life. Aminoacyl-tRNA synthetases (aaRSs) translate the genetic code by ensuring the correct pairing of amino acids with their cognate tRNAs. Because some aaRSs also produce misacylated aminoacyl-tRNA (aa-tRNA) in vivo, we addressed the question of protein quality within the context of missense suppression by Cys-tRNA^{Pro}, Ser-tRNA^{Thr}, Glu-tRNA^{Gln}, and Asp-tRNA^{Asn}. Suppression of an active-site missense mutation leads to a mixture of inactive mutant protein (from translation with correctly acylated aa-tRNA) and active enzyme indistinguishable from the wild-type protein (from translation with misacylated aa-tRNA). Here, we provide genetic and biochemical evidence that under selective pressure, *Escherichia coli* not only tolerates the presence of misacylated aa-tRNA, but can even require it for growth. Furthermore, by using mass spectrometry of a reporter protein not subject to selection, we show that *E. coli* can survive the ambiguous genetic code imposed by misacylated aa-tRNA tolerating up to 10% of mismade protein. The editing function of aaRSs to hydrolyze misacylated aa-tRNA is not essential for survival, and the EF-Tu barrier against misacylated aa-tRNA is not absolute. Rather, *E. coli* copes with mistranslation by triggering the heat shock response that stimulates nonoptimized polypeptides to achieve a native conformation or to be degraded. In this way, *E. coli* ensures the presence of sufficient functional protein albeit at a considerable energetic cost.

accuracy | aminoacyl-tRNA | fidelity | protein synthesis | missense suppression

Cells have adopted quite dissimilar levels of accuracy for various processes. Although mistakes in metabolism can be substantial (1), extreme precision in replication is needed to avoid mutations (2). Protein synthesis has reached an intermediate level of fidelity with an error rate of 1 in 10,000 (for reviews, see refs. 3 and 4). Several quality control mechanisms exist to safeguard translational fidelity: proofreading performed by the aminoacyl-tRNA synthetases (aaRSs) (5–8), deacylation of mismade tRNA (9), discrimination against misacylated aminoacyl-tRNA (aa-tRNA) by EF-Tu (10), and proper codon–anticodon alignment in the ribosomal A site (11, 12).

The fact that noncanonical amino acids get incorporated into proteins in vivo has been established long ago (for review, see refs. 13 and 14). Renewed more recent interest in the expansion of the standard genetic code via incorporation of a large repertoire of unnatural amino acids has given rise to new technologies (14, 15). The success of such protein-engineering efforts clearly indicates that misincorporation of unnatural amino acids into proteins can be tolerated by both prokaryotes (14) and eukaryotes (16).

The misincorporation of canonical amino acids via noncognate aa-tRNA (e.g., ref. 17) is also more widespread than previously appreciated. The in vivo presence of misacylated aa-tRNA introduces ambiguity into the genetic code and leads to the generation of “statistical” proteins (18), whereby the use

of misacylated in addition to correctly charged aa-tRNA by the ribosome during translation results in a group of related proteins for any given messenger RNA. Such mistranslation may lead to observable cellular and disease pathologies such as cell degeneration and apoptosis in mammalian systems (19) and neurodegeneration and ataxia in mice (20). Moreover, since heritable mutations in human glycyl-tRNA synthetase and tyrosyl-tRNA synthetase genes have been directly associated with the peripheral neuropathy Charcot–Marie–Tooth (21, 22), the role of the aaRSs and their editing function in maintaining translational fidelity have become areas of intense study (23–28).

We were thus prompted to reconsider the quality control mechanisms in protein synthesis from cradle to grave. We use selection-based in vivo missense suppression assays with well-studied *Escherichia coli* model systems to evaluate the overall impact of misacylated aa-tRNA upon the cellular protein pool, and in the process, we determine an estimate of the level of mismade protein that the organism is willing to endure.

Results

In Vivo Misacylation of Four tRNAs Rescues Growth by Missense Suppression. Because several aaRSs are known to misacylate tRNA in vivo (e.g., refs. 17, 23, and 29), missense suppression by misacylated aa-tRNA (30) provides a useful genetic tool to study misincorporation of canonical amino acids into proteins. Thus, we address the question of protein quality within the context of missense suppression by Cys-tRNA^{Pro}, Ser-tRNA^{Thr}, Glu-tRNA^{Gln}, and Asp-tRNA^{Asn}. In each case, *E. coli* growth depends on missense suppression of an altered codon that specifies an active-site residue in an essential enzyme. Only incorporation of the amino acid carried by the misacylated aa-tRNA corrects the defect in the mutant allele to produce a functional enzyme. In contrast, faithful decoding with the cognate aa-tRNA species generates an inactive enzyme and prohibits growth under our selection conditions. For each misacylated aa-tRNA species tested, a different active-site mutation was used as a reporter. Thus, to study the in vivo effects of Cys-tRNA^{Pro}, Ser-tRNA^{Thr}, Glu-tRNA^{Gln}, and Asp-tRNA^{Asn}, we relied on the following four *E. coli* missense suppression systems (Table 1).

Cys-tRNA^{Pro}. For Cys-tRNA^{Pro} incorporation, we constructed the thymidylate synthase (ThyA) missense mutant allele *thyA:146CCA^{C→P}*. This notation indicates the Cys→Pro codon

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Table 1. Genetic systems used for missense suppression

Missense reporter allele	Enzyme	Active-site residue	Mutated active-site codon	Faithful translation	Misacylating aaRS	Missense translation
<i>thyA</i> :146 _{CCA} ^{C→P}	Thymidylate synthase	Cys146	146 _{CCA}	Pro146 (Pro-tRNA ^{Pro})	ProRS	Cys146 (Cys-tRNA ^{Pro})
<i>bla</i> :68 _{ACA} ^{S→T}	β-Lactamase	Ser68	68 _{ACA}	Thr68 (Thr-tRNA ^{Thr})	ΔNThrRS	Ser68 (Ser-tRNA ^{Thr})
<i>trpA</i> :60 _{AAT} ^{D→N}	Tryptophan synthase	Asp60	60 _{AAT}	Asn60 (Asn-tRNA ^{Asn})	ND-AspRS	Asp60 (Asp-tRNA ^{Asn})
<i>TrpA</i> :49 _{CAA} ^{E→Q}	Tryptophan synthase	Glu49	49 _{CAA}	Gln49 (Gln-tRNA ^{Gln})	ND-GluRS	Glu49 (Glu-tRNA ^{Gln})

change at position 146, where the genetically encoded UGC codon for Cys has been mutated to the CCA codon specifying Pro. Thus, translation of the missense mutant allele *thyA*:146_{CCA}^{C→P} with the cognate Pro-tRNA^{Pro} gives rise to an inactive ThyA[146P] enzyme (31) as the active-site Cys at position 146 is replaced by Pro (Fig. 1, upper scheme). However, in the presence of Cys-tRNA^{Pro} [made by *E. coli* prolyl-tRNA synthetase, ProRS (32)] protein with a wild-type amino acid sequence, ThyA[146C], is produced from the mutant mRNA (Fig. 1, lower scheme). ThyA catalyzes the conversion of deoxyuridylate (dUMP) to thymidylate, and an *E. coli* strain that carries a deletion of the endogenous *thyA* gene is a thymine auxotroph (31). Only coexpression of the *thyA*:146_{CCA}^{C→P} missense mutation with a ProRS known to misacylate tRNA^{Pro} with Cys efficiently (32) rescues the thymine auxotrophy of the Δ*thyA* *E. coli* strain (Fig. 2 *Top*). Therefore, missense suppression by Cys-tRNA^{Pro} is sufficient to generate active ThyA[146C] required for growth in the absence of thymine. In contrast, expression of the *thyA*:146_{CCA}^{C→P} allele alone in the Δ*thyA* background leads to production of the inactive ThyA[146P] protein and fails to rescue thymine auxotrophy.

Glu-tRNA^{Gln}. For Glu-tRNA^{Gln}, we used the mutant tryptophan synthase (TrpA) allele *trpA*:49_{CAA}^{E→Q} as the missense reporter. In this case, the genetically encoded GAG Glu codon is mutated to the CAA Gln codon resulting in a Glu→Gln change at position 49. Tryptophan synthase catalyzes the last step in Trp biosynthesis, and the *E. coli* strain KS463 harboring a loss-of-function mutation in *trpA* cannot grow in the absence of Trp (33). Expression of the *trpA*:49_{CAA}^{E→Q} allele in this strain leads to production of the inactive TrpA[49Q] protein and cannot grow

without Trp (Fig. 2 *Middle*). However, coexpression of the *trpA*:49_{CAA}^{E→Q} allele with a nondiscriminating glutamyl-tRNA synthetase (ND-GluRS) that is known to misacylate tRNA^{Gln} with Glu (34, 35) rescued growth of the *trpA*[−] strain in the absence of exogenous Trp, thus implicating Glu-tRNA^{Gln} in missense suppression (Fig. 2 *Middle*).

Ser-tRNA^{Thr}. For Ser-tRNA^{Thr} incorporation, the β-lactamase (Bla) allele *bla*:68_{ACA}^{S→T} was used as the missense reporter. Here, the Ser→Thr change involves mutating the Ser codon AGU at position 68 to ACA coding for Thr. β-Lactamase catalyzes the hydrolysis of the amide bond of the lactam rings of penicillin derivatives (e.g., ampicillin). In the absence of Ser-tRNA^{Thr}-mediated translation, expression of the *bla*:68_{ACA}^{S→T} allele leads to production of the inactive Bla[68T] enzyme that

Fig. 1. Scheme of missense suppression. Misacylating ProRS forms Pro-tRNA^{Pro} and Cys-tRNA^{Pro} (32). When correctly acylated Pro-tRNA^{Pro} decodes the mRNA of *thyA*:146_{CCA}^{C→P}, the resulting inactive ThyA[146P] does not complement the Δ*thyA* strain. In contrast, decoding the mRNA of *thyA*:146_{CCA}^{C→P} with misacylated Cys-tRNA^{Pro} gives a wild-type ThyA[146C] and rescues thymine auxotrophy. In the absence of a misacylating aaRS, the Upper scheme prevails, and suppression is not observed.

Fig. 2. Missense suppression by misacylated aa-tRNAs. (*Top*) Cys-tRNA^{Pro} corrects a nonfunctional *thyA* gene. The *proS* genes from *G. lamblia* (GL), *Borrelia burgdorferi* (BB), or *E. coli* (EC) and the *thyA*:146_{CCA}^{C→P} (*thyA*[−]) allele were expressed in the *E. coli* Δ*thyA* strain at 37 °C on minimal medium (MM) A agar plates with or without thymine (20 μg/ml). (*Middle*) Glu-tRNA^{Gln} corrects a nonfunctional tryptophan synthase gene. The *H. pylori* ND-GluRS (HPgluS2) and the mutant *trpA*:49_{CAA}^{E→Q} (*trpA*[−]) or wild-type (*trpA*) allele were expressed in the *E. coli* *trpA*[−] strain at 37 °C on minimal medium B agar plates with or without tryptophan (20 μg/ml). (*Bottom*) Ser-tRNA^{Thr} corrects a nonfunctional β-lactamase gene. The *thrS* derivatives and the wild-type (*bla*) or mutant *bla*:68_{ACA}^{S→T} (*bla*[−]) allele were expressed in the *E. coli* *thrS*^{ts} strain at 42 °C (nonpermissive temperature) on minimal medium C agar plates with or without ampicillin (20 μg/ml).

Table 2. Analysis of missense-suppressed thymidylate synthase in $\Delta thyA$ background

Reporter allele	Added aaRS	Active ThyA[146C], pmol/mg S100	Total ThyA[146C] + ThyA[146P], pmol/mg S100	Suppression, %*
<i>thyA</i>		800 $\pm$ 80	800	100
$\Delta thyA$		0	0	
<i>thyA:146_{CCA}^{C→P}</i>		3.8 $\pm$ 1	640	0.6
<i>thyA:146_{CCA}^{C→P}</i>	GLproS	137 $\pm$ 13	685	20
<i>thyA:146_{CCA}^{C→P}</i>	ECproS	24.7 $\pm$ 1.6	561	4.4
<i>thyA:146_{CCA}^{C→P}</i>	BBproS	8.7 $\pm$ 1	483	1.8

*Suppression % represents the ratio between the active and total reporter proteins.

is unable to hydrolyze ampicillin (36). Because the endogenous *E. coli* threonyl-tRNA synthetase (ThrRS) possesses an N-terminal editing domain that hydrolyzes misacylated Ser-tRNA^{Thr} formed in vivo (6), it was necessary to use an *E. coli* strain that carries a temperature-sensitive mutant of wild-type ThrRS (37). Indeed, at the nonpermissive temperature (42 °C) no growth of the *thrS^{ts}* *E. coli* strain is observed (Fig. 2 Bottom). Complementation of the *thrS^{ts}* strain with two previously described *thrS* clones (6) that lack the ThrRS N-terminal editing domain ($\Delta N1$ -*thrS* and $\Delta N2$ -*thrS*) allows for translation of an active Bla[68S] enzyme from the *bla:68_{ACA}^{S→T}* mRNA, conferring ampicillin resistance (Fig. 2 Bottom). Coexpression of the wild-type *E. coli* ThrRS with the editing-defective $\Delta N1$ -ThrRS abolishes ampicillin resistance, indicating that formed Ser-tRNA^{Thr} is bound and transcribed by the wild-type ThrRS before its use in protein synthesis (Fig. 2 Bottom).

Asp-tRNA^{Asn}. Finally, for Asp-tRNA^{Asn} we used a different allele of tryptophan synthase, *trpA:60_{AAT}^{D→N}* (Asp→Asn change at position 60 by mutating the wild-type GAU Asp codon to the AAT Asn codon). Expression of a nondiscriminating aspartyl-tRNA synthetase (ND-AspRS) capable of forming Asp-tRNA^{Asn} in an *E. coli* strain that carries a chromosomal copy of *trpA:60_{AAT}^{D→N}* (38) rescues Trp auxotrophy. The results for this system were published in ref. 17.

To support the phenotypic in vivo complementation results in a more quantitative manner, we determined the level of missense suppression present in cells under selection. The presence of functional reporter enzyme was monitored in vitro by measuring the specific activity present in cell extracts of the strains in Fig. 2. Total accumulation of full-length reporter protein (both active and inactive) was estimated from immunoblots (Tables 2–4).

In the Cys-tRNA^{Pro} missense suppression system, overexpression of *E. coli* or *Giardia lamblia* ProRS suppressed the thymine requirement of the *thyA:146_{CCA}^{C→P}* allele in the $\Delta thyA$ strain (Fig. 2 Top). As expected, only in the presence of the misacylating ProRSs did active ThyA[146C] protein represent a significant fraction of the expressed protein (Table 2, 1.8–20% versus 0.6% suppression in the absence of an added synthetase). No ThyA activity was detected in extracts of the $\Delta thyA$ strain (Table 2, second row). In all $\Delta thyA$ derivative strains, expression of

ThyA from the *thyA:146_{CCA}^{C→P}* allele reached a level of up to 80% compared with its expression from the wild-type *thyA* allele (Table 2, 640 vs. 800 pmol/mg of total ThyA protein). Moreover, the differential ability to form Cys-tRNA^{Pro} by the *E. coli* and *G. lamblia* ProRS enzymes (32) is reflected in the disparate suppression levels of the *thyA:146_{CCA}^{C→P}* allele (4.4% vs. 20%, respectively).

In the Ser-tRNA^{Thr} missense suppression system, complementation of the *E. coli* *thrS^{ts}* strain by plasmid-borne editing-defective *thrS* alleles led to suppression of the *bla:68_{ACA}^{S→T}* mutant allele (Fig. 2 Bottom). This suppression correlated with β -lactamase activity measured in Table 3. In the absence of ampicillin, only the strain containing the wild-type *bla* gene produced active β -lactamase (Table 3, compare first row with third and fifth rows). Under selection, coexpression of the *bla:68_{ACA}^{S→T}* allele and the ΔN -*thrS* genes was required to produce substantial amounts of β -lactamase (Table 3, compare second row with fourth and sixth rows). Western blotting showed a 10-fold increase in total β -lactamase protein when these strains were grown under ampicillin selection (9.4 and 6.8 ng/ μ L vs. 0.9 ng/ μ L).

In vivo missense suppression of the *trpA:49_{CAA}^{E→Q}* and the *trpA:60_{AAT}^{D→N}* alleles was brought about by Glu-tRNA^{Gln} and Asp-tRNA^{Asn}, respectively. The nondiscriminating GluRS enzyme from *Helicobacter pylori* allowed an *E. coli* Trp auxotroph cotransformed with the missense reporter *trpA:49_{CAA}^{E→Q}* to grow in the absence of Trp (Fig. 2 Middle). In this case, 4.6% of the total TrpA protein was active compared with wild-type (Table 4 Upper). The presence of the nondiscriminating AspRS enzymes from *Deinococcus radiodurans* (DR) and *Halobacterium salinarum* (HS) allowed the *E. coli* strain harboring the *trpA:60_{AAT}^{D→N}* allele to grow in the absence of Trp (17); suppression levels of up to 28% (compared with wild-type) were measured in the presence of the *H. salinarum* ND-AspRS (Table 4 Lower).

Effective Missense Suppression Relies on Induction of the Heat Shock Response. To assess the cellular response to misfolded proteins made in the presence of misacylated aa-tRNA, we monitored the mRNA levels of the two major *E. coli* chaperones MopA and

Table 3. Analysis of missense-suppressed β -lactamase in *thrS^{ts}* background

Reporter allele	Added aaRS	Ampicillin	Active Bla[68S], units/ μ L medium [†]	Total Bla[68S] + [68T], ng/ μ L medium	Suppression %*
<i>bla</i>	<i>thrS</i>	–	39 $\pm$ 2	0.8	
		+	40 $\pm$ 2	0.9	
<i>bla:68_{ACA}^{S→T}</i>	$\Delta N1$ - <i>thrS</i>	–	0	0.4	0
		+	22 $\pm$ 1	9.4	5.4
<i>bla:68_{ACA}^{S→T}</i>	$\Delta N2$ - <i>thrS</i>	–	0	0.3	0
		+	16 $\pm$ 1	6.8	5.3

*Suppression % was determined by dividing the specific activity of each sample by that of the *bla*, *thrS* strain (47 units/ng).

[†]One unit of Bla enzyme is defined as hydrolyzing 1 nmol of nitrocefin per min using 1 μ L of the test samples.

misincorporation in the indicator DHFR protein both in the presence and absence of Trp selection (Fig. 4), that is $12 \pm 1\%$ vs. $9 \pm 1\%$, respectively. Thus, based on this experiment with one indicator protein, one may conclude that $\approx 10\%$ of the accumulated proteins can differ from the ORF sequence in the presence or absence of nutritional selection in *E. coli*.

Discussion

Using four established missense suppression systems, we show that the misacylating aaRSs, ProRS, Δ N-ThrRS, ND-GluRS, and ND-AspRS generate both misacylated and cognate aa-tRNAs in vivo in *E. coli*. The misacylated aa-tRNA may be rebound and reedited by a second cognate aaRS, as evidenced by the lack of misincorporation of Ser-tRNA^{Thr} when the editing defective Δ N-ThrRS and wild-type ThrRS are both present in the *thrS^{ts}* strain (Fig. 2). This in vivo finding contributes to the discussion of the posttransfer editing mechanism by class II aaRSs and the binding of misacylated aa-tRNA to EF-Tu (42). In the absence of complete tRNA editing, and driven by our missense selection system, the misacylated aa-tRNA is then presented to the ribosome by EF-Tu. This leakage of the EF-Tu barrier (10, 43) allows mistranslation, although the level of misincorporation depends on the concentration and the nature of the misacylated aa-tRNAs in vivo.

Our genetic and biochemical evidence demonstrated that *E. coli* tolerates a significant amount of mistranslation. However, this work does not identify a single editing function (e.g., by the aaRSs, EF-Tu, the ribosome, or the chaperones and proteases) as the most important step in ensuring the production and maintenance of a functional cellular proteome. Our current understanding does not permit us to define the relative contributions of each of the above protein quality control mechanisms to diverse bacterial or eukaryotic growth conditions.

It is intuitively assumed that mistranslation causes reduced growth rate and fitness because it results in altered proteins that may have less overall activity. Indeed, the presence of misacylated aa-tRNA in a protease-deficient strain caused a significant reduction of the growth rate (Fig. 3B). Here, however, we also show that *E. coli* survives the errors of an ambiguous genetic code and does not require a perfectly accurate proteome. An ambiguous code may be beneficial under certain situations (27). The tolerance of mistranslation may be attributed partly to protein plasticity; it is known that many positions in a protein molecule, except the catalytic active-site residues, permit multiple substitutions (44, 45).

Our results illustrate that the presence of $\approx 10\%$ mismatched protein is not detrimental to growing cells. The energetic cost of error correction requires a considerable investment of the cell's resources, and the extra energetic cost of proofreading and protein turnover will tend to reduce the growth rate (46). Thus, the level of mistranslation tolerated is a balance between achieving a functional proteome and an optimal growth rate. The inherent plasticity of the error-minimizing network can buffer against the serious effects of debilitating mutations because it allows for chaperones and proteases to take over when the editing functions of aaRSs are insufficient for error correction. In fact, this is the case for mitochondrial protein synthesis, where three aaRSs inherently lack the editing function (47), and quality control is achieved downstream by degradation of mismatched proteins, leaving only properly folded proteins for functional insertion into the membrane.

Yet, the ways of dealing with mistranslation probably vary greatly in different organisms and distinct outcomes are to be expected. For example, an editing-defective alanyl-tRNA synthetase (AlaRS) is linked to ataxia and neurodegeneration in the mouse. The inability to clear Ser-tRNA^{Ala} and Gly-tRNA^{Ala} causes global misfolding of mistranslated proteins in neurons that lead to the disease phenotype (20). Apart from neurons,

other tissues do not exhibit any dramatic malfunctions because of the presence of misacylated aa-tRNA. Thus, the extent that mistranslation can be tolerated and dealt with by the unfolded protein response varies even within an organism. This allows for very exciting future research on diseases caused by the devastating effects of single amino acid substitutions in a particular protein (e.g., in cystic fibrosis, Alzheimer's disease, cancer). To the degree that mistranslation can be tolerated, expression of editing-defective aaRSs in the affected tissues may correct for the genetic mutation and significantly improve the disease progression.

Materials and Methods

For additional procedures, see [supporting information \(SI\) Text](#).

Strains, Plasmids, and Culture Media. The *E. coli* strain W3110, the Δ thyA strain χ 2913 (31), the *thrS^{ts}* strain EJJ320, the *trpA⁻* strain K5463 (33) and the *trpA34* strain (38) were obtained from the *E. coli* Genetic Stock Center (Yale University). *E. coli* strains MG1655 and its protease-deficient derivative KY2350 [Δ hslIVU, Δ (clpPX-*lon*)] (40) were obtained from M. Kanemori (HSP Research Institute, Kyoto, Japan).

Minimal medium supplemented with 0.4% glucose, 1 μ g/mL thiamine, 20 μ g/mL amino acids, and, if needed, 0.02% arabinose, 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG), 20 μ g/mL ampicillin (Amp), 20 μ g/mL chloramphenicol (Cm), 20 μ g/mL kanamycin (Kan) were used for the in vivo tests. The following media were derived accordingly. Medium A contains 19 aa (10 μ g/ml), in addition to 0.5 mM Cys, 0.008% uracil, 0.02% arabinose, and IPTG. Medium B has only 19 aa (lacking Trp). Medium C contains 19 aa, as well as 1 mM Ser, 0.02% arabinose, and IPTG. Medium D contains 20 aa.

The vectors pCYB1 and pACYC177 were from New England Biolabs; pBad18-Cm and pBad18-Kan were from American Stock Culture Collection. pCB5 and pTech were described in ref. 48. All ORF sequences were amplified by PCR: *trpA*, *thyA*, *thrS*, and *folA* from *E. coli* W3110 genomic DNA, *bla* from pUC19 (GenBank accession no. L09137; the active-site residue is Ser68), *gluS2* from *H. pylori* DNA (49); the *proS* genes (32) and *D. radiodurans aspS2* (17) were described earlier. Mutant genes *thyA*:146_{CCA}^{C→P} (Cys→Pro codon change at position 146), *trpA*:49_{CAA}^{E→Q} (Glu→Gln codon change at position 49), *bla*:68_{ACA}^{S→T} (Ser→Thr codon change at position 68) (36), *folA*:3_{AAT}^{S→N} (Ser→Asn codon change at position 3), Δ N1-*thrS* (removal of amino acids 2–224 of ThrRS), and Δ N2-*thrS* (removal of amino acids 2–241 of ThrRS) (6) were made by PCR mutagenesis with primers containing the corresponding mutations. PCR products were cloned into the pCR 2.1-TOPO vector and then subcloned into desired vectors. The pACYC-Kan vector was constructed by blunt-end ligation of 2 fragments: the replicating fragment of pACYC177 (BstEII-filled, AhdI-filled) and the multiple-cloning sites of pBad18-Cm (ClaI-filled, PvuI-filled). The *thrS*, Δ N1-, and Δ N2-*thrS* genes were cloned into pBAD-Kan under the control of an arabinose promoter. The *aspS2* and *gluS2* genes were cloned into pCB5 and pCYB1 vectors under the control of the *E. coli trpS* promoter and Tac promoter, respectively. The *proS* genes were cloned into pACYC-Kan and pCYB1 vectors under the control of the arabinose and Tac promoter, respectively. The *thyA*:146_{CCA}^{C→P} and C-terminally His₆-tagged *folA*:3_{AAT}^{S→N} were cloned into pCYB1. The *bla*:68_{ACA}^{S→T} and *trpA*:49_{CAA}^{E→Q} genes were cloned into pTech.

Genetic Systems for Missense Suppression. Four *E. coli* systems were used.

Suppression of the *thyA*:146_{CCA}^{C→P} mutation by Cys-tRNA^{Pro}. This system was tested in derivatives of the *E. coli* thymine auxotroph χ 2913 (31). The strain harboring pCYB-*thyA* or *thyA*:146_{CCA}^{C→P} was cotransformed with pACYC-Kan-*proS*. Transformants were grown in medium A plus thymine (1 μ g/mL) to $A_{600} = 0.4$. Cells were harvested, washed with minimal medium, and streaked on medium A agar plates in the presence or absence of thymine and grown at 37 °C.

Suppression of the *trpA*:49_{CAA}^{E→Q} mutation by Glu-tRNA^{Gln}. This system was tested in recombinants of the *E. coli* Trp auxotroph K5463 (33). The strain harboring pTech-*trpA* or *trpA*:49_{CAA}^{E→Q} was cotransformed with pCB5-*gluS2*. Single colonies, streaked on medium B agar plates in the presence or absence of Trp, were grown at 37 °C.

Suppression of the *bla*:68_{ACA}^{S→T} mutation by Ser-tRNA^{Thr}. This system was tested in descendants of the *E. coli thrS^{ts}* strain EJJ320 (37) at 42 °C. Progeny harboring pTech-*bla* or *bla*:68_{ACA}^{S→T} were cotransformed with pBAD-Kan-*thrS*, Δ N1-*thrS*, and Δ N2-*thrS*. Cultures in medium C grown to $A_{600} = 0.4$ were harvested, washed with minimal medium, streaked on medium C agar plates in the presence or absence of Amp (20 μ g/mL), and incubated at 42 °C.

Suppression of the *trpA*:60_{AAAT}^{D→N} mutation by *Asp*-tRNA^{Asn}. This system was tested in the *E. coli* *trpA34* background (17, 38). After transformation with pCBS-*aspS2*, single colonies, streaked on medium B agar plates in the presence or absence of Trp, were incubated at 37 °C.

Quantifying the Heat Shock Response. The transcript levels of *mopA*, *dnaK*, and *gapA* were measured using primers and methods described in ref. 50. The strains (KY2350 or MG1655 alone or containing the *aspS2* plasmid) were grown for 1 h at 37 °C. Then, *aspS2* expression was induced with 1 mM IPTG, whereas cultures of the strains lacking the *aspS2* plasmid were left uninduced. Cells were harvested at $A_{600} = 0.4$ – 0.7 . Total RNA was extracted, reverse transcribed, and quantified by RT-PCR. Expression ratios were generated by the 2^{−ΔΔCt} method (51). The relative abundance of each message in a sample was normalized to *gapA* transcript levels because *gapA* remains constant for strains growing in medium containing glucose.

Growth of Cells Containing Misacylated aa-tRNA. The *E. coli* strain MG1655 and its protease-deficient derivative KY2350 were transformed with pCYB-*GLproS*, pCYB-*DRaspS2*, or pCYB-*HPgluS2*. Overnight cultures of the transformants were used to inoculate medium D. After growth for 1 h at 37 °C, 1 mM IPTG was added to induce expression of the nondiscriminating aaRS genes. Cell growth was monitored (A_{600}) as a function of time.

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