Quality control despite mistranslation caused by an ambiguous genetic code

Benfang Ruan1, Sotiria Palioura2, Jeffrey Sabina, Laure Marvin-Guy, Sunil Kochhar, Robert A. LaRossa, and Dieter Söll1,4,5,6,7

Departments of 1Molecular Biophysics and Biochemistry, and 2Chemistry, Yale University, New Haven, CT 06520-8114; 3Nestlé Research Center, Nestec Ltd., Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland; and 4Biological Science and Engineering, Central Research and Development, DuPont Co., Wilmington, DE 19880-0173

Contributed by Dieter Söll, September 14, 2008 (sent for review September 2, 2008)

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-tRNAPro, Ser-tRNAThr, Glu-tRNAGln, and Asp-tRNAAsn. 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 mismatched protein. The editing function of aaRSs to hydrolize 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.

Cytosine 1466CCA is 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 glycyld-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 mismatched protein that the organism is willing to endure.

Results

In Vivo Misaclylation 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-tRNAPro, Ser-tRNAThr, Glu-tRNAGln, and Asp-tRNAAsn. 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-tRNAPro, Ser-tRNAThr, Glu-tRNAGln, and Asp-tRNAAsn, we relied on the following four E. coli missense suppression systems (Table 1).

Cys-tRNAPro. For Cys-tRNAPro incorporation, we constructed the thymidylate synthase (ThyA) misense mutant allele thyA1466CCA. This notation indicates the Cys→Pro codon


The authors declare no conflict of interest.

1B.F. and S.P. contributed equally to this work.

2To whom correspondence should be addressed: Department of Molecular Biophysics and Biochemistry, Yale University, P.O. Box 208114, 266 Whitney Avenue, New Haven, CT 06520-8114. E-mail: dieter.soll@yale.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0809179105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA

PNAS | October 28, 2008 | vol. 105 | no. 43
16502–16507
www.pnas.org/cgi/doi/10.1073/pnas.0809179105
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:146CCAC is reported with the cognate Pro-tRNAPro 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-tRNAPro [made by E. coli prolly-tRNA synthetase, ProRS (32)] protein with a wild-type amino acid sequence, ThyA[146C], is produced from the mutant mRNA thyA expression of the requered for growth in the absence of thymine. In contrast, thyA:146CCAC Cys-tRNAPro (dUMP) to thymidylate, and an (Fig. 1, lower scheme). ThyA catalyzes the conversion of deoxyuridylate (dUMP) to thymidylate, and 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-tRNAPro [made by E. coli prolly-tRNA synthetase, ProRS (32)] protein with a wild-type amino acid sequence, ThyA[146C], is produced from the mutant mRNA.

Missense reporter allele | Enzyme | Active-site residue | Mutated active-site codon | Faithful translation | Misacylating aaRS | Missense translation
---|---|---|---|---|---|---
thyA:146CCAC | Thymidylate synthase | Cys146 | 146CCAC | Pro146 (Pro-tRNAPro) | ProRS | Cys146 (Cys-tRNAPro)
bla:68ACAS | β-Lactamase | Ser68 | 68ACAS | Thr68 (Thr-tRNAThr) | ND-ThrRS | Ser68 (Ser-tRNAThr)
trpA:49CAAE | Tryptophan synthase | Asp60 | 49CAAE | Asn60 (Asn-tRNAAsn) | ND-AspRS | Asp60 (Asp-tRNAAsn)
TrpA:49CAAE | Tryptophan synthase | Glu49 | 49CAAE | Gin49 (Gln-tRNAGln) | ND-GluRS | Glu49 (Glu-tRNAGln)

Glu-tRNAGln. For Glu-tRNAGln, we used the mutant tryptophan synthase (TrpA) allele trpA:49CAAE as the missense reporter. In this case, the genetically encoded GAG Glu codon is mutated to the CCA 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:49CAAE 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:49CAAE allele with a nondiscriminating glutamyl-tRNA synthetase (ND-GluRS) that is known to misacylate tRNAPro with Glu (34, 35) rescued growth of the trpA- strain in the absence of exogenous Trp, thus implicating Glu-tRNAGln in missense suppression (Fig. 2 Middle).

Ser-tRNAThr. For Ser-tRNAThr incorporation, the β-lactamase (Bla) allele bla:68ACAS 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-tRNAGln-mediated translation, expression of the bla:68ACAS allele leads to production of the inactive Bla[68T] enzyme that
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 E. coli strain is observed (Fig. 2 Bottom). Complementation of the thrS strain with two previously described thrS clones (6) that lack the ThrRS N-terminal editing domain (∆N1-thrS and ∆N2-thrS) allows for translation of an active Bla[68S] enzyme from the bla:68ASCA^{6–7} mRNA, conferring ampicillin resistance (Fig. 2 Bottom). Coexpression of the wild-type E. coli ThrRS with the editing-defective ∆N1-ThrRS abolishes ampicillin resistance, indicating that formed Ser-tRNA^{Thr} is bound and transedited 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:60AAT^{D–N} (Asp→Asn change at position 60 by mutating the wild-type GUA Asp codon to the GAT 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:60AAT^{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:146CCA^{C–P} allele in the ΔthyA strain (Fig. 2 Top). As expected, only in the presence of the missacylating ProRS 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 ΔthyA strain (Table 2, second row). In all ΔthyA derivative strains, expression of ThyA from the thyA:146CCA^{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:146CCA^{C–P} allele (4.4% vs. 20%, respectively).

In the Ser-tRNA^{Thr} missense suppression system, complementation of the E. coli thrS strain by plasmid-borne editing-defective thrS alleles led to suppression of the bla:68ASCA^{6–7} 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:68ASCA^{6–7} allele and the ∆N-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 6.5 ng/μL vs. 0.9 ng/μL).

In vivo missense suppression of the trpA:49CAU^{E–Q} and the trpA:60AAT^{P–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:49CAU^{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:60AAT^{P–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^E background

<table>
<thead>
<tr>
<th>Reporter allele</th>
<th>Added aaRS</th>
<th>Ampicillin</th>
<th>Active Bla[68S], units/μL medium*</th>
<th>Total Bla[68S] + [68T], ng/μL medium</th>
<th>Suppression %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>bla</td>
<td>thrS</td>
<td>–</td>
<td>39 ± 2</td>
<td>0.8</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>40 ± 2</td>
<td>0.9</td>
<td>6</td>
</tr>
<tr>
<td>bla:68ASCA^{E–T}</td>
<td>∆N1-thrS</td>
<td>–</td>
<td>0</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>22 ± 1</td>
<td>9.4</td>
<td>5.4</td>
</tr>
<tr>
<td>bla:68ASCA^{E–T}</td>
<td>∆N2-thrS</td>
<td>–</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>16 ± 1</td>
<td>6.8</td>
<td>5.3</td>
</tr>
</tbody>
</table>

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

1One unit of Bla enzyme is defined as hydrolyzing 1 nmol of nitrocefin per min using 1 μL of the test samples.
Table 4. Analysis of missense-suppressed tryptophan synthase in trpA* background

<table>
<thead>
<tr>
<th>Reporter Allele</th>
<th>Added aaRS*</th>
<th>Active TrpA</th>
<th>Total TrpA</th>
<th>Suppression %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TrpA</td>
<td>HPGluS2</td>
<td>1.6 ± 0.2</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>trpA:49CA[6]→Q</td>
<td>HPGluS2</td>
<td>0.08 ± 0.01</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>trpA:49CA[60]→Q</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>trpA:60A[60D]</td>
<td>TrpA[60D]</td>
<td>5.5 ± 0.2</td>
<td>2 ± 0.2</td>
<td>NA</td>
</tr>
<tr>
<td>trpA:60A[60N]</td>
<td>TrpA[60N]</td>
<td>1.2 ± 0.1</td>
<td>2.8 ± 0.3</td>
<td>16</td>
</tr>
<tr>
<td>trpA:60A[60N]</td>
<td>TrpA[60N]</td>
<td>2.1 ± 0.2</td>
<td>2.7 ± 0.3</td>
<td>28</td>
</tr>
<tr>
<td>trpA:60A[60N]</td>
<td>TrpA[60N]</td>
<td>0</td>
<td>0.74 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

*Units are units/mg S100. One unit of TrpA enzyme is defined as producing 0.1 μmol of tryptophan in 20 min at 37 °C using 1 mg of protein.

NA, not applicable.

Suppression % was determined by dividing the activity seen in the second row (0.08 units/mg) by the activity in the presence of the wild-type trpA allele (1.6 units/mg).

Fig. 3. Proteases are essential for coping with mismade proteins. (Left) Growth curves of protease-replete derivatives. The E. coli strain MG1655 (□) was transformed with the nondiscriminating HPGluS2 (●), the GLproS (□), or the nondiscriminating DRaspS2 (△) and grown at 37 °C in minimal medium. (Right) Growth curves for the protease-deficient strain derivatives. The isogenic strain KY2350 was transformed with the same set of misacylating aaRSs and grown under the same conditions as described above.

Fig. 4. Asp-tRNA\(^{\text{Asn}}\) caused misincorporation in the reporter protein DHFR. The folA:3A[A]\(^{\text{Ser}}\) strain containing the nondiscriminating DRaspS2 with (Upper) or without (Lower) tryptophan. Tryptic digests of both DHFR protein preparations were methylated before MALDI-TOF MS analysis. Two N-terminal fragments of interest are shown: MINUAAAVD*R (monoisotopic mass, M\(^+\) = 1,327.78) and MI D\(^{\text{Asp}}\)AAAV D\(^{\text{R}}\) (monoisotopic mass M\(^+\) = 1,342.78). The ratio of absolute intensities of peaks at m/z 1,327.78 and 1,342.78 corresponds to the Asp/Asn ratio of the amino acid at position 3.

Surviving Mistranslation. We next sought to quantify more rigorously the degree of misincorporation caused by the presence of misacylated aa-tRNA in a polypeptide that is not subject to selection. We chose the E. coli dihydrofolate reductase (DHFR) protein as a nonselected, missense indicator. The third codon, specifying Ser in the wild-type folA gene was changed to AAT (denoted folA:3A[A]\(^{\text{Ser}}\)) that is decoded by tRNA\(^{\text{Asn}}\). This is a permissive site for mutations in this protein since the presence of amino acid residues other than the wild-type Ser at position 3 does not affect the structure and function of the mature DHFR protein (41). Thus, in the absence of misacylated aa-tRNA, expression of the folA:3A[A]\(^{\text{Ser}}\) strain will produce DHFR with a Ser→Asn substitution at position 3. The folA:3A[A]\(^{\text{Ser}}\) allele was coexpressed with the D. radiodurans ND-AspRS in the E. coli strain harboring a chromosomal trpA:60A[60D] allele in minimal media in the absence of exogenous Trp (selecting for TrpA[60D] function as in Fig. 2) or in minimal media supplemented with Trp. An analysis of the aminoacyl-tRNA pool by acid gel electrophoresis followed with tRNA\(^{\text{Asn}}\)-specific probing (data not shown) indicated that both Asp-tRNA\(^{\text{Asn}}\) (made by D. radiodurans ND-AspRS) and the cognate Asn-tRNA\(^{\text{Asn}}\) were present. In the presence of selection (i.e., without Trp), equal amounts of Asp-tRNA and Asn-tRNA were observed, whereas there was less Asp-tRNA in the presence of Trp. This presence of Asp-tRNA\(^{\text{Asn}}\) in addition to the cognate Asn-tRNA\(^{\text{Asn}}\) should lead to a mixture of Asp and Asn at position 3 of the DHFR protein. After growth to late log phase, the two DHFR protein samples were extensively purified and subjected to MALDI-MS analysis. The activity of ND-AspRS caused similar levels of
misincorporation in the indicator DHFR protein both in the presence and absence of Trp selection (Fig. 4), that is 12 ± 1% vs. 9 ± 1%, respectively. Thus, based on this experiment with one indicator protein, one may conclude that ≈10% of the accumulated misincorporated 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, ΔN-ThrRS, ND-GluRS, and ND-AspRS generate both misacylated and cognate aa-tRNAs in vivo in E. coli. The misacylated aa-tRNA may be reloaded and recycled by a second cognate aaRS, as evidenced by the lack of misincorporation of Ser-tRNA^Thr^ when the editing defective ΔN-ThrRS and wild-type ThrRS are both present in the thr^Str^ 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 ≈10% mismade 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 ΔthyA strain χ2913 (31), the thr^Str^ strain EJJ320, the trpA^A^ strain KS463 (33) and the trpA^Str^ strain (38) were obtained from the E. coli Genetic Stock Center (Yale University). E. coli strains MG1655 and protease-deficient derivative KY2395 [ΔphlpX-lon] (40) were obtained from M. Kanemori (HSP Research Institute, Kyoto, Japan).

Minimal medium supplemented with 0.4% glucose, 1 μg/mL thiamine, 20 μg/mL amino acids, and, if needed, 0.02% arabinose, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 20 μg/mL ampicillin (Amp), 20 μg/mL chloramphenicol (Cm), 20 μg/mL kanamycin (Kan) were used for the in vivo tests. The bacterial strains were grown in the following media: Medium A contains 10 μM Ser, 0.02% arabinose, and IPTG. 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 Type Culture Collection, pCBS and pTech were described in ref. 48. All ORF sequences were amplified by PCR, trpA^A^, thyA^A^, thrS, and foIA 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 aspS (17) were described earlier. Mutant genes thyA^146CCAC^→^A^ (Cys→Pro codon change at position 146), trpA^49CAAE^→^Q^ (Glu→Gln codon change at position 49), blα^68ACA^→^T^ (Ser→Thr codon change at position 68) (36), foIA^3AATS^→^A^ (Ser→Asn codon change at position 3), ΔN1-thrs (removal of amino acids 2–224 of ThrRS), and ΔN2-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 (Clal-filled, PvuII-filled). The thrS, ΔN1-, and ΔN2-thrs genes were cloned into pBad-Kan under the control of proS. The αs2 and gluS2 genes were cloned into pCBS and pCYB1 vectors under the control of the E. coli trpP promoter and Tac promoter, respectively. The proS genes were cloned into pACYC-Kan and pACYC vectors under the control of the arabinose and Tac promoter, respectively. The thyA^146CCAC^→^A^ and C-terminally His-tagged foIA^3AATS^→^Q^ were cloned into pACYC1. The blα^68ACA^→^T^ and trpA^49CAAE^→^Q^ genes were cloned into pTech.

Genetic Systems for Missense Suppression. Four E. coli systems were used. Suppression of the thyA^146CCAC^→^A^ mutation by proS-ΔN1Pc. This system was tested in derivatives of the E. coli thymine auxotroph χ2913 (31). The strain harboring pACYC-thyA or thyA^146CCAC^→^A^ was cotransformed with pACYC-Kan-proS. Transformants were grown in medium A plus thymine (1 μg/mL) to A^S^ = 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. Colonies were scored by the trpA^49CAAE^→^Q^ system. This system was tested in recombinants of the E. coli Trp auxotroph KS463 (33). The strain harboring pTech-trpA or trpA^49CAAE^→^Q^ were cotransformed with pCBS-gluS2. Single colonies, streaked on medium B agar plates in the presence or absence of Trp, were grown at 37 °C.

Suppression of the blα^68ACA^→^T^ mutation by Ser-tRNA^Ala^, E. coli thrSts strain EJJ320 (37) at 42 °C. Progeny harboring the ΔN1-thrs strain KS463 (33) and ΔN2-thrs strains MG1655 were cotransformed with pBad-Kan-thrs, ΔN1-thrs, and ΔN2-thrs. Cultures in medium C grown to A^S^ = 0.4 were harvested, washed with minimal medium, streaked on medium C agar plates in the presence or absence of Amp (20 μg/mL), and incubated at 42 °C.
Suppression of the trpA60GluDp/3 mutation by Asp-tRNA\(^{80}\). This system was tested in the E. coli strain MG1655 and its protease-deficient derivative KY2350 were transformed with pCJB-GluSp5, pCJB-Drasp2, or pCJB-HpgU2. 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 (A260) as a function of time.

ACKNOWLEDGMENTS. We thank R. A. Bonono (Louis Stokes Veterans Affairs Medical Center, Cleveland, OH), G. Chin, L. Fay, L. Feng, M. Ibbá, M. Kanemori (HSP Research Institute, Kyoto, Japan), S. G. Kreft, J. Ling, K. B. Low, B. Min, S. Namamoto, A. Pfiefer, J. Pober, J. Salazar, D. Steege, M. Springer (Institut de Biologie Physico-Chimique, Paris, France), P. van Bladeren, and C. Yanofsky (Stanford University, Stanford, CA) for gifts of materials, advice, and encouragement. This work was supported by grants from the National Institute of General Medical Sciences and the National Science Foundation (to D.S.).


Thymidylate Synthase, β-Lactamase, and Tryptophan Synthase Levels.

For each reporter, both enzyme activity and polypeptide quantity were determined. The relevant strains were grown to the late-log phase; cells were harvested by sonication, and a clarified S-100 supernatant was prepared by high-speed centrifugation. Triplicate enzyme preparations were assayed for each system.

Thymidylate synthase activity was determined by [3H]FdUMP titration (1). Each lysate was incubated with [3H]FdUMP and 5,10-methylenetetrahydrofolate. Retention of radioactivity on nitrocellulose filters, indicating formation of a 1:1:1 ternary complex, was used to quantify thymidylate synthase activity.

Tryptophan synthase activity in S-100 supernatants was determined by monitoring conversion of [3H]Ser and indole-3-glycerol phosphate to [3H]Trp (2). Amino acids were separated by TLC on PEI-cellulose plates and visualized by PhosphorImaging with [14C]Trp as a standard. Excised radioactive spots were analyzed with the corresponding secondary antibody. The protein bands were visualized using the Opti-4CN substrate and detection kit and quantified by using ImageQuant software. The intensity of each band, after background subtraction, was compared with a standard curve generated from Western blotting of a dilution series of purified proteins. Our data are based on dilutions that fell within the linear portion of the standard curve.

Analysis of Asp/Asn Ratio in Recombinant Dihydrofolate Reductase (DHFR) Proteins. A trpA34 transformant, harboring f0lA:A3AAT7-N encoding a C-terminal His6-tagged DHFR, was transformed with the Deinococcus radiodurans asp52 plasmid and grown in medium B with or without Trp. Cells were harvested at late-log phase, and the DHFR were proteins purified by sequential Ni-NTA agarose, methotrexate (4) affinity, and size-exclusion FPLC chromatography. The resultant preparations yielded a single band corresponding to DHFR on a silver-stained SDS/polyacrylamide gel. Samples were digested overnight with trypsin (1:1-tyrosylamido-2-phenylethyl chloromethyl ketone-treated; Promega) in 100 mM NH4HCO3 at 37°C at an enzyme to substrate ratio of 1:100. The tryptic peptides (10 μL; 100 pmol) were treated with 3 N methanolic HCl (50 μL) for 2 h at room temperature to esterify the Asp side chain to increase the molecular mass difference between Asp- and Asn-containing peptides by 14 Da (5). The derivatized peptides were desalted on a ZipTip C18 (Millipore) and dried under vacuum. The mass spectra of the derivatized peptides were recorded on an Autoflex (Bruker Daltonics) MALDI-TOF mass spectrometer operating in delayed extraction reflectron positive ion mode. Ions formed upon irradiation by a pulsed UV laser beam (nitrogen laser, 337 nm) were accelerated at 19 kV. α-Cyano-4-hydroxycinnamic acid (saturated solution in acetone and 0.1% trifluoroacetic acid, 97:3, vol/vol) was used as the matrix. Typically, 2 μL of the saturated matrix was deposited on the Anchorchip (Bruker Daltonics) target followed by 2 μL of the peptide mixture and allowed to dry at room temperature. External calibration was performed with a peptide mixture.