Functional Significance of an Evolutionarily Conserved Alanine (GCA) Resume Codon in tmRNA in *Escherichia coli*V

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Occasionally, ribosomes stall on mRNAs prior to the completion of the polypeptide chain. In *Escherichia coli* and other eubacteria, tmRNA-mediated trans-translation is a major mechanism that recycles the stalled ribosomes. The tmRNA possesses a tRNA-like domain and a short mRNA region encoding a short peptide (ANDENYALAA in *E. coli*) followed by a termination codon. The first amino acid (Ala) of this peptide encoded by the resume codon (GCN) is highly conserved in tmRNAs from different species. However, reasons for the high evolutionary conservation of the resume codon identity have remained unclear. In this study, we show that changing the E. coli tmRNA resume codon to other efficiently translatable codons retains efficient functioning of the tmRNA. However, when the resume codon was replaced with the low-usage codons, its function was adversely affected. Interestingly, expression of tRNAs decoding the low-usage codon from plasmid-borne gene copies restored efficient utilization of tmRNA. We discuss why in *E. coli*, the GCA (Ala) is one of the best codons and why all codons in the short mRNA of the tmRNA are decoded by the abundant tRNAs.

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Materials and Methods

Plasmids, bacterial strains, and growth conditions. A list of the plasmids and E. coli strains used in this study is provided in Table 1. Derivatives of E. coli MG1655 were generated by standard genetic methods (21). Briefly, the pth(Ts) allele of E. coli strain AA7852 was tagged with zch-306:TsTn10 (linked to pth-1) using P1 phage raised on E. coli strain CAG12016. Subsequently, the tagged pth(Ts) allele was moved into E. coli MG1655 to derive E. coli MG1655 pth(Ts) (Tet' and temperature sensitive for growth at 42°C). This was followed by introduction of ssrA:kan allele into this strain by another transduction using P1 phage raised on E. coli DY330 ssrA::kan to generate E. coli MG1655 pth(Ts) ssrA::kan. Bacterial cultures were grown in Luria-Bertani (LB) medium (29) supplemented with kanamycin (Kan; 25 µg/ml), ampicillin (Amp; 100 µg/ml), tetracycline (Tet; 7.5 µg/ml), or chloramphenicol (Cam; 30 µg/ml) as required. For growth on solid support, 1.5% Bacto agar (Difco) was included in the medium. Cells were grown to saturation at 30°C. Loopfuls of the cultures were streaked on LB agar (Amp) plates, and the plates were incubated at 30 and 37°C for 12 h.

Generation of the resume codon mutants in tmRNA. The ssrA gene cloned in plasmid pTrc99C (pTrc-ssrA; 200 ng) was amplified by PCR in a 50-µl volume containing 3 U of Pfu DNA polymerase, 200 µM deoxynucleoside triphosphates (dNTPs), 20 pmol each of pTrc-ssrA (5' CTCTCGATATCAGCGAAAACG 3') and tmRNA-A lected random (5' CCACCCAAAATAGTCCNNAAGCAG AAAACTAC 3') DNA oligomers in Pfu buffer, with 30 cycles of incubation at 95°C for 1 min, 40°C for 45 s, and 72°C for 1 min 30 s. The amplicon of ~0.62 kb (~200 ng) so obtained was used as megaprimer in a reaction mixture (50 µl) containing pTrc-ssrA (200 ng), 3 U of Pfu DNA polymerase, and 200 µM dNTPs in Pfu buffer. The reaction mixture was subjected to 19 cycles of incubation at 95°C for 1 min, 45°C for 1 min, 55°C for 30 s, and 72°C for 12 min, followed by treatment with DpnI to degrade the original wild-type template. An aliquot (10 µl) from the DpnI-treated reaction mixture was used to transform E. coli TG1 competent cells. Plasmid minipreparations from the transformants were sequenced (Macrogen, South Korea) to identify resume codon mutants.

Complementation analysis using E. coli MG1655 pth(Ts) ssrA::kan. Wild-type and resume codon mutant ssrA gene constructs were introduced into E. coli MG1655 pth(Ts) ssrA::kan by transformation. Minicultures of the transformants were grown to saturation at 30°C. Loopfuls of the cultures were streaked on LB agar (Amp) plates, and the plates were incubated at 30 and 37°C for 12 h.

Total RNA isolation and Northern blot analysis of tmRNA and 5S rRNA. E. coli MG1655 pth(Ts) ssrA::kan cells harboring wild-type or mutant ssrA constructs were grown to an optical density at 595 nm (OD595) of ~0.8 in 5-ml cultures at 30°C and chilled on ice for ~5 min. Cells were harvested by centrifugation at 6,000 rpm (Sorvall SS34 rotor) at 4°C for 5 min and suspended in 0.5 ml 300 mM sodium acetate buffer (pH 5.0) containing 10 mM Na2EDTA. An equal volume of water-saturated phenol (pH 6.5) was added, and the mixture was vortexed twice for 1 min each and centrifuged at 12,000 rpm at 4°C for 10 min in a microcentrifuge. The aqueous layer was separated and extracted once more with an equal volume of phenol, followed by a single extraction with an equal volume of chloroform, and centrifuged. The aqueous layer was taken out, mixed with 2.5 volumes of double-distilled alcohol, and stored at ~20°C for 2 h to precipitate nucleic acids. The precipitate was recovered by centrifugation at 12,000 rpm at 4°C for 15 min in a microcentrifuge, air-dried, dissolved in 30 µl 100 mM sodium acetate (pH 5.0), and estimated by loading 2 µl of the preparation onto 1% agarose gel. Equal amounts (~2 µg) of total RNA were electrophoresed on 2% agarose gel, transferred onto nylon membrane using a vacuum

FIG. 1. (A) Secondary structure of E. coli tmRNA. Regions corresponding to tRNA-like domain and the short mRNA along with its translated sequence (tmRNA tag sequence) are shown. Encoded amino acids are shown in white type on a black background. Nucleotides have been numbered at intervals of 50 nucleotides. Various paired regions (P1 to P11) and pseudoknot structures (PK1 to PK4) are as indicated. (B) The tmRNA tag sequence and the various derivatives containing mutations at the resume codon position are shown.
TABLE 1. Relevant characteristics of the plasmids and strains used in this study

<table>
<thead>
<tr>
<th>Plasmid or E. coli strain</th>
<th>Relevant details</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>pACYC184 (Tet&lt;sup&gt;+&lt;/sup&gt; Cam&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Cloning vector harboring p15a ori of replication, which is compatible with ColE1 ori in pTrc99C</td>
<td>3</td>
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<tr>
<td>pRARE (Cam&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Plasmid harboring p15a ori and genes for rare tRNAs proL (reads CCC and CUC), tRNA&lt;sup&gt;Leu&lt;/sup&gt; (reads UUG), tRNA&lt;sup&gt;Met&lt;/sup&gt; (reads AUG), tRNA&lt;sup&gt;Arg&lt;/sup&gt; (reads AGG), tRNA&lt;sup&gt;Thr&lt;/sup&gt; (reads ACC and ACU), tRNA&lt;sup&gt;Glu&lt;/sup&gt; (reads GGA and GGG), tRNA&lt;sup&gt;Thr&lt;/sup&gt; (reads UAC and UAU), tRNA&lt;sup&gt;His&lt;/sup&gt; (reads ACA, ACU, and ACC), tRNA&lt;sup&gt;Val&lt;/sup&gt; (reads AGA), and tRNA&lt;sup&gt;Ile&lt;/sup&gt; (reads AUA)</td>
<td>Novagen</td>
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<tr>
<td>pTrc-ssrA (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>ssrA gene (~0.83 kb along with its promoter) amplified from E. coli with 5'-GA AAAGCTTATGGGCTATAC-3' and 5'-CACAAGTCCGTTAACAAGCA G-3' primers and cloned into HindIII site of pTrc99C</td>
<td>32</td>
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<tr>
<td>pTrc-ssrA mutants (Amp&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>ssrA gene with various proL and thrW, decoding GGA (along with GGG), Asp1 decoding GAU and GAC, Thr decoding ACC and ACU, Leu decoding CUG and UUG, Arg decoding AGG and AGU, Ile decoding AUG, and Val decoding GUA and GUU.</td>
<td>This study</td>
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<tr>
<td>pACDH (Tet&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Cloning vector harboring p15a ori of replication, which is compatible with ColE1 ori in pTrc99C</td>
<td>27</td>
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<tr>
<td>pACDH-proL (Tet&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>pACDH plasmid harboring proL tRNA gene between EcoRI and NcoI sites</td>
<td>This study</td>
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<tr>
<td>pACDH-thrW (Tet&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>pACDH plasmid harboring thrW tRNA gene between EcoRI and NcoI sites</td>
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<td>Strains</td>
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<td>CAG12106 (Tet&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>E. coli (F&lt;sup&gt;+&lt;/sup&gt; λ&lt;sup&gt;+&lt;/sup&gt; rph&lt;sup&gt;-&lt;/sup&gt; 1 zch-3060::Tn10)</td>
<td>23, 30</td>
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<td>AA7852</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; Arg&lt;sup&gt;−&lt;/sup&gt; Leu&lt;sup&gt;−&lt;/sup&gt; Thr&lt;sup&gt;−&lt;/sup&gt; His&lt;sup&gt;−&lt;/sup&gt; thiamine&lt;sup&gt;−&lt;/sup&gt; relA&lt;sup&gt;−&lt;/sup&gt; T1 rph&lt;sup&gt;-&lt;/sup&gt;</td>
<td>1</td>
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<td>AA7852 ph(Ts) ssrA::kan (Kan&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>AA7852 containing ph&lt;sup&gt;-&lt;/sup&gt; and disruption of ssrA gene with Kan&lt;sup&gt;+&lt;/sup&gt; marker</td>
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<td>DY330 ssrA::kan (Kan&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>W3110 ΔlacU169 gal490 [lac&lt;sup&gt;1857Δ(cro-bioA)]] ssrA::kan</td>
<td>32</td>
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<td>TG1</td>
<td>supE hsd&lt;sup&gt;+&lt;/sup&gt; Δlac&lt;sup&gt;−&lt;/sup&gt; Δ(pro&lt;sup&gt;-&lt;/sup&gt;proAB) &lt;sup&gt;F&lt;sup&gt;+&lt;/sup&gt; [traD&lt;sup&gt;−&lt;/sup&gt; proAB&lt;sup&gt;−&lt;/sup&gt; lacI&lt;sup&gt;−&lt;/sup&gt; lacZ&lt;sup&gt;−&lt;/sup&gt;]M&lt;sup&gt;15&lt;/sup&gt;</td>
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<td>MG1655</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; λ&lt;sup&gt;−&lt;/sup&gt; rph&lt;sup&gt;-&lt;/sup&gt;</td>
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<td>MG1655 ph(Ts) (Tet&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>MG1655 containing ph&lt;sup&gt;-&lt;/sup&gt; (linked with Tet&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>This study</td>
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<tr>
<td>MG1655 ph(Ts) ssrA::kan (Tet&lt;sup&gt;+&lt;/sup&gt; Kan&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>MG1655 containing ph&lt;sup&gt;-&lt;/sup&gt; (linked with Tet&lt;sup&gt;+&lt;/sup&gt;) and disruption of ssrA gene with Kan&lt;sup&gt;+&lt;/sup&gt; marker</td>
<td>This study</td>
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RESULTS

Conservation of the resume codon in ssrA. Analysis of the resume codons of over 600 tmRNA sequences in the database (35) revealed that ~87% of these encode Ala (GCA, ~50%; GCC, ~25%; GCU, ~7%; and GCG, ~4.5%). The resume codons in the remaining sequences (~13%) are represented by Gly (GGC, ~9%; and GGA, ~2%), Asp (GAC and GAU, ~1.5%), and Val (GUA, ~0.5%), as well as a single occurrence of Ile (AUU). Thus, Ala and Gly are the two most preferred amino acids encoded by the resume codons in various tmRNAs. In E. coli, the GCC codon which is read by Ala2 tRNA occurs at 0.95% of the level of the total tRNA. However, the remainder of these codons are read by tRNAs that occur at higher levels. For example, Ala1B decoding GCA, GCU, and GCG, Gly3 decoding GCC (along with GGU), Gly2 decoding GGA (along with GGG), Asp1 decoding GAU and GAC, Val1 decoding GUA (along with GUG and GUU), and Ile1 decoding AUU (along with AUC) occur at 5.96, 6.76, 3.31, 3.72, 5.96, and 5.39% of the levels of the total cellular tRNA, respectively (7).

Mutagenesis of the resume codon in ssrA. To investigate the importance of the highly conserved Ala (GCA) resume codon in E. coli tmRNA (Fig. 1A), it was mutated by using a synthetic DNA oligomer containing a random sequence (NNN) in place of GCA, and the mutants were selected from an E. coli TG1 strain with wild-type tmRNA and Pho to avoid any selection bias (see Materials and Methods). Sequence analysis of over 100 miniplasmid preparations identified the 18 mutants shown in Fig. 1B.
borne wild-type ssrA gene in these strains rescues them not only for the ssrA:kan-mediated temperature hypersensitivity but also for the Pth deficiency (32). Thus, to test the function of the resume codon mutants of ssrA, we introduced plasmid-borne copies of the ssrA mutants into E. coli MG1655 pth(Ts) ssrA::kan. As shown in Fig. 2 (panels i and iii), all transformants harboring the wild-type or the mutant ssrA genes grow well at 30°C. At 37°C (panels ii and iv), the growth of many transformants harboring the resume codon mutants (GCU, CCA, CGC, GGC, GCC, UGC, ACU, GGC, UCA, and GUC) appeared at par with the strain complemented with the wild-type ssrA (GCA), suggesting that these resume codons functionally substituted for the native GCA resume codon. However, the other resume codon mutants (CCU, ACA, CAA, CCC, UGG, CAG, CUC, and ACG) failed to rescue the temperature hypersensitivity of the MG1655 pth(Ts) ssrA::kan strain at 37°C, suggesting that these resume codons rendered the ssrA mutants deficient in their function.

To rule out that differences in phenotypes observed in Fig. 2 were a consequence of differences in the levels of tmRNA expression, total RNA from transformants (grown at the permissive temperature of 30°C) were analyzed by Northern blot analysis for the abundance of tmRNA, together with an internal (chromosomally encoded) control of SS rRNA. As shown in Fig. 3, the relative levels of tmRNA to SS rRNA signals in all samples were found to be fairly uniform. This observation suggests that the deficiency of some of the resume codons in supporting tmRNA function is their intrinsic property.

**Plating efficiency of KimmP22 hybrid phage.** Earlier studies have shown that KimmP22 hybrid phage, due to the presence of translatable sequences called minigenes in its immunity region, does not multiply within the sip (ssrA) and rap (pth) mutants of E. coli and fails to form plaques on these strains (12, 28). As expected from these observations, in our experiments also, the hybrid phage did not form plaques on E. coli pth(Ts) strains. Also, the phage was unable to grow on the pth(Ts) ssrA::kan strain when complemented with wild-type ssrA (pTrc-ssrA) alone. However, the phage was able to form plaques on the ssrA::kan strain when complemented with wild-type ssrA (pTrc-ssrA). Thus, to analyze the activity of the ssrA mutants, we carried out phage plating assays by infecting E. coli MG1655 ssrA::kan harboring different resume codon mutants of ssrA under identical conditions and scored for differences in numbers and sizes of plaques. Although there is a discernible co-

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**FIG. 2. Rescue of the temperature-hypersensitive phenotype of E. coli MG1655 pth(Ts) ssrA::kan.** The strain was transformed with wild-type ssrA or various ssrA resume codon mutants. Transformants were grown to saturation in liquid cultures at 30°C, streaked on LB agar (Amp), and incubated at 30°C (panels i and iii) or 37°C (panels ii and iv) for 12 h and documented. The pTrc-ssrA construct in the strains streaked in various sectors are indicated by the resume codon. For example, pTrc-ssrA (GCA) is indicated by “GCA.”

**Functional analysis of the resume codon mutants of ssrA.** We had earlier shown that disruption of ssrA (ssrA::kan) in the pth(Ts) background [pth(Ts) ssrA::kan] confers temperature hypersensitivity to E. coli. The pth(Ts) strain grows at 37°C, but it is sensitive to 42°C. However, the growth of the pth(Ts) ssrA::kan strain is sensitive to even 37°C. It may be that the increased levels of stalled ribosomes in this strain sequester a significant fraction of the available tRNAs, making them (especially those occurring in lower abundance) unavailable for further rounds of translation (32). Introduction of a plasmid-

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**FIG. 3. Northern blot analysis of tmRNA and SS rRNA.** Total RNA was isolated from E. coli MG1655 pth(Ts) ssrA::kan harboring wild-type or mutant ssrA constructs, separated on 2% agarose gel, processed for Northern blot analysis using tmRNA- and SS rRNA-specific probes, and analyzed using a BioImage analyzer (FLA2000; Fuji). Bands corresponding to tmRNA and SS rRNA are indicated. “Ratio” indicates the ratio of pixels in the tmRNA band to pixels in the SS rRNA band.
relation between the efficiency of the resume codon mutant function as observed in Fig. 2 and the PFU obtained in the strains supported by various ssrA mutants (Fig. 4A), differences in the plaque numbers are very small. A similar observation was made earlier (24), indicating that the phage plating assay is insensitive at discerning the in vivo function of the tmRNA mutants. Nevertheless, at least in the cases of the ACA, CCU, CCC, and ACG resume codon mutants, which failed to rescue the MG1655 pth(Ts) ssrA::kan strain for its temperature-hypersensitive growth (Fig. 2), we noted that the plates were populated with smaller plaques (Fig. 4B).

Increased levels of tRNAs decoding rare or low-usage codons facilitate rescue of E. coli pth(Ts) ssrA::kan by ssrA mutants. We noted that some of the ssrA mutants that were poor or unable to rescue the growth defect of the MG1655 pth(Ts) ssrA::kan strain (Fig. 2) possessed resume codons decoded by tRNAs of low abundance in the cell. Hence, we investigated if the growth defect could be rescued by increasing levels of tRNAs that read low-usage codons. The pRARE plasmid is routinely used to increase levels of the low-abundance tRNAs in E. coli for overproduction of heterologous gene products. Of the tRNA genes this plasmid harbors, two decode the CCC and ACA codons. (Both of these codons did not function well as resume codons [Fig. 2].) Hence, we utilized this plasmid to analyze its effect on the function of the CCC (encoding a rare subset of Pro codon) and ACA (a low-usage Thr codon) resume codon mutants of ssrA. As shown in Fig. 5A, all derivatives of pth(Ts) ssrA::kan strains (E. coli MG1655) harboring vector alone or the ssrA mutants (CCC or ACA) grow well at 30°C (panel i). Also, as expected, at 37°C (panel ii), neither the empty-vector-alone strain derivatives (sector 1) nor those harboring pTrc-ssrA (CCC) or pTrc-ssrA (ACA) along with the pACYC184 (sectors 3 and 4, respectively) show any growth. Interestingly, the presence of pRARE plasmid supported the growth of the strain at 37°C by both the CCC and ACA resume codon mutants (compare sectors 5 and 6 with sector 3 and sectors 7 and 8 with sector 4). However, it may also be noted that the transformant harboring pRARE plasmid alone (along with pTrc99C) showed some rescue of the strain growth at 37°C (sector 2). Considering that partial rescue of pth(Ts) strains upon overexpression of rare tRNAs has been observed before (33), this was an expected effect of the introduction of the pRARE plasmid in the pth(Ts) ssrA::kan strain. To further validate our analysis, we subcloned individual tRNA genes (proL and thrW). Introduction of these genes into the pth(Ts) ssrA::kan strain (E. coli AA7852) al-
allowed both the CCU and ACG resume codon-containing ssrA genes to rescue the temperature-hypersensitive phenotype of the host at 37°C (Fig. 5B, panel ii, compare sector 5 with sector 4 and sector 2 with sector 1), as well as the wild-type ssrA gene (sector 8). As a control, introduction of tRNA genes alone (along with pTrc99C) did not rescue the temperature-hypersensitive phenotype of the strain to any significant level (Fig. 5B, panel ii, sectors 6 and 3). Finally, at the permissive temperature of 30°C, all strains showed growth (Fig. 5B, panel i).

**DISCUSSION**

Alanine codons (GCN) are the most frequently used resume codons in tmRNAs. Replacement of the *E. coli* tmRNA resume codon (GCA) with other proficiently translated codons (irrespective of whether they code for Ala or other amino acids) allows efficient rescue of the *E. coli* MG1655 pth(Ts) ssrA::kan strain with the tmRNA mutants. However, replacement of the resume codon with a rare or a low-usage codon (4) rendered the tmRNA mutants deficient in their function in *E. coli* (Fig. 2).

Rare codons, infrequent codons, and minor codons are not only used rarely or infrequently in a genome but are also decoded by rare/low-abundance tRNAs. The translational rate of a rare codon is much lower than that of a common (major) codon. In *E. coli*, there are about 30 low-usage sense codons (4). However, 20 of them have been determined to be rare codons. Of these, seven (AGG, AGA, CGA, CUA, AUA, CCC, and CGG) are used at a frequency of $\leq 0.5\%$ (group I) and the remaining 13 (ACA, CCU, UCA, GGA, AGU, UCG, CCA, UCC, GGG, CUC, CUU, UCU, and UUA) are used at a frequency of $>0.5\%$ (group II) of the total codons analyzed from the database (4). Studies have shown that all rare codons in group I and the first six in group II can be unfavorable for translation in *E. coli*. In addition, although UGU and UGC codons for Cys, ACU and ACG codons for Thr, or CAC and CAU codons for His are less frequently used, they are not defined as rare codons (4). Furthermore, it has been shown that the levels of charged tRNA determine if a particular codon would be translated efficiently; and the concentration of tRNA isoacceptors is often positively correlated with the frequency of the occurrence of the cognate codon(s) they read (7, 15).

The complementation assay of the ssrA mutants (Fig. 2) shows that CUC, CAG, UGG, CCC, CAA, ACA, CCU, and ACG did not rescue the pth(Ts) ssrA::kan strain, suggesting that these codons are not efficient for translation of the short mRNA region of the tmRNA. Of these, CCC (Pro), ACA (Thr), and CCU (Pro) are already known to be unfavorable for translation in *E. coli*. The UGG (Trp) codon is not a rare codon. However, it is a nondegenerate codon decoded by a single tRNA. Expression of a gene rich in UGG codon may
showed an efficient rescue of the ssrA ports the selection of GCA (Ala) as the best resume codon in encountered during protein synthesis. In fact, this study supercharging of the tRNA isoacceptors is determined by the con-
zero, whereas for some others they remain high. The selective
vation, they retain high residual charging levels for several isoacceptors abundant in
rare codon of
E. coli
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When
E. coli
 cells are subjected to severe amino acid star-
vation, they retain high residual charging levels for several tRNA isoacceptor families (38). In an elegant computational study (8), it was shown that when an amino acid becomes growth limiting, the charged levels of some tRNAs approach zero, whereas for some others they remain high. The selective charging of the tRNA isoacceptors is determined by the con-
centrations of isoacceptors and how often their codons are encountered during protein synthesis. In fact, this study sup-
ports the selection of GCA (Ala) as the best resume codon in ssrA in
E. coli
 as follows. (i) GCA is neither a low-usage nor rare codon of
E. coli
, nor is the tRNA (Ala1) reading this codon limiting in the cell. (ii) There are two different isoac-
ceptors that read four codons of Ala, and it has been shown that the residual charging level of tRNA Alal(GCA) remains high (at ~18%) even when the supply level of the amino acid approaches near zero (8). (iii) Determination of the charged levels of various tRNAs showed that even when the alanine isoacceptors were charged to a lower level (compared to the other tRNAs) before starvation, it increased about 2-fold after starvation (6).

In conclusion, our study suggests that the presence of low-
usage codons (including the rare codons) at the resume codon position affects the efficiency of resumption of trans-translation by tmRNA in
E. coli
. In fact, the codons selected in the entire short mRNA region of the tmRNA do not belong to the category of low-usage codons, but are decoded by tRNAs that are abundant in the cell. In this context, it is important to mention that the tmRNA mutants harboring CCC, CCU, ACA, and ACG resume codons function well in
E. coli
 strains supplied with extra copies of the tRNA genes that decode these codons (Fig. 5). A crucial feature of a tmRNA is to ensure efficient release of the stalled ribosomal complexes to avoid toxicity. Thus, it may well be that the characteristic vari-
atations in the codon usage in different organisms are the reason for the naturally occurring distinctions in the short mRNA region codons of their tmRNAs (e.g., including the alternate resume codons encoding Ala or another amino acid).

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