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

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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 in 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.

Occasionally, during the process of translation, ribosomes stall on mRNAs prior to the completion of the polypeptide chain. If not recycled, accumulation of the stalled ribosomes is detrimental to the organism as it sequesters not only the ribosomes but also the tRNAs (as peptidyl-tRNAs) and brings protein synthesis to a halt. In *Escherichia coli* and other eubacteria, a well-known pathway that recycles the stalled ribosomes utilizes tmRNA (SsrA or 10Sa RNA) by a process called *trans*-translation (18, 36). The tmRNA contains a tRNA like domain and an mRNA region encoding a short peptide (AN DENYALAA in *E. coli*) followed by a termination codon (Fig. 1A). The tmRNA is aminoacylated with alanine (in its tRNA-like domain) and recruited to the A-site of the ribosome stalled on a truncated mRNA. The polypeptide from the P-site tRNA is transferred to the alanine on the tRNA-like domain of tmRNA to form (peptidyl)-alanyl-tmRNA. The ribosome then resumes translation (*trans*-translation) decoding the first codon (the "resume codon") and the remaining codons of the short open reading frame in the tmRNA. When the termination codon in the tmRNA reaches the ribosomal A-site, the C-terminally tagged polypeptide is released. Thus, the process of *trans*-translation in *E. coli* appends AANDENYALAA sequence to the incomplete polypeptide and marks it for degradation by cellular proteases. The C-terminal amino acids in the appended sequence are important for recognition by the cellular proteases; and while the tmRNA mutants that encode ANDENYALDD or ANDEHHHHHH rescue the stalled ribosomes, they do not direct the tagged proteins to degradation (9, 11, 17, 25). Interestingly, the first amino acid (Ala) of the short peptide encoded by the resume codon GCN is evolutionarily conserved across the tmRNA sequences from different species. In addition, although the *ssrA* mutants harboring alternate resume codons have been studied (24, 34), the reasons for its high conservation are not understood.

A number of cellular processes result in accumulation of stalled ribosomes (16), and a significant fraction of ribosomes terminate via the tmRNA-mediated pathway (22). The process of *trans*-translation is also known to result in rapid degradation of the truncated mRNA (20, 37). The presence of tmRNA in eubacteria and the eukaryotic organelles (35) highlights the importance of the *trans*-translation for the prokaryotic or the prokaryote-like ribosomes. In fact, in *Neisseria gonorrhoeae*, the *ssrA* gene is essential for the viability of the organism (14). Nevertheless, the fact that *ssrA* is not essential in all organisms suggests that alternate mechanisms to recycle the stalled ribosomes must exist. One such mechanism is mediated by ribosome recycling factor (RRF) in conjunction with elongation factor G (EFG) and initiation factor 3 (IF3) (10, 13, 26, 31) leading to the release of peptidyl-tRNAs. Peptidyl-tRNAs are acted upon by peptidyl-tRNA hydrolase (Pth), an essential protein, which cleaves the ester bond between the tRNA and the peptide to recycle the tRNA (19). There is also evidence of cross talk between Pth and the *trans*-translation pathways. Overproduction of tmRNA rescues the temperature-sensitive allele of *pth* [*pth*(Ts)] in *E. coli*. Conversely, disruption of *ssrA* gene in a *pth*(Ts) strain of *E. coli* confers temperature hypersensitivity to the bacterium (32). Furthermore, in *E. coli*, deficiency of either Pth or SsrA restrains λ*imm*P22 phage replication (28).

We have been interested in understanding the mechanism of recycling of stalled ribosomes. The observation that Ala resume codons (GCN, of which the GCA codon is the most predominant) are highly conserved in tmRNAs points to an important physiological function of the resume codon identity. Here, we show that efficient translatability of the resume codon is a crucial feature for tmRNA function.

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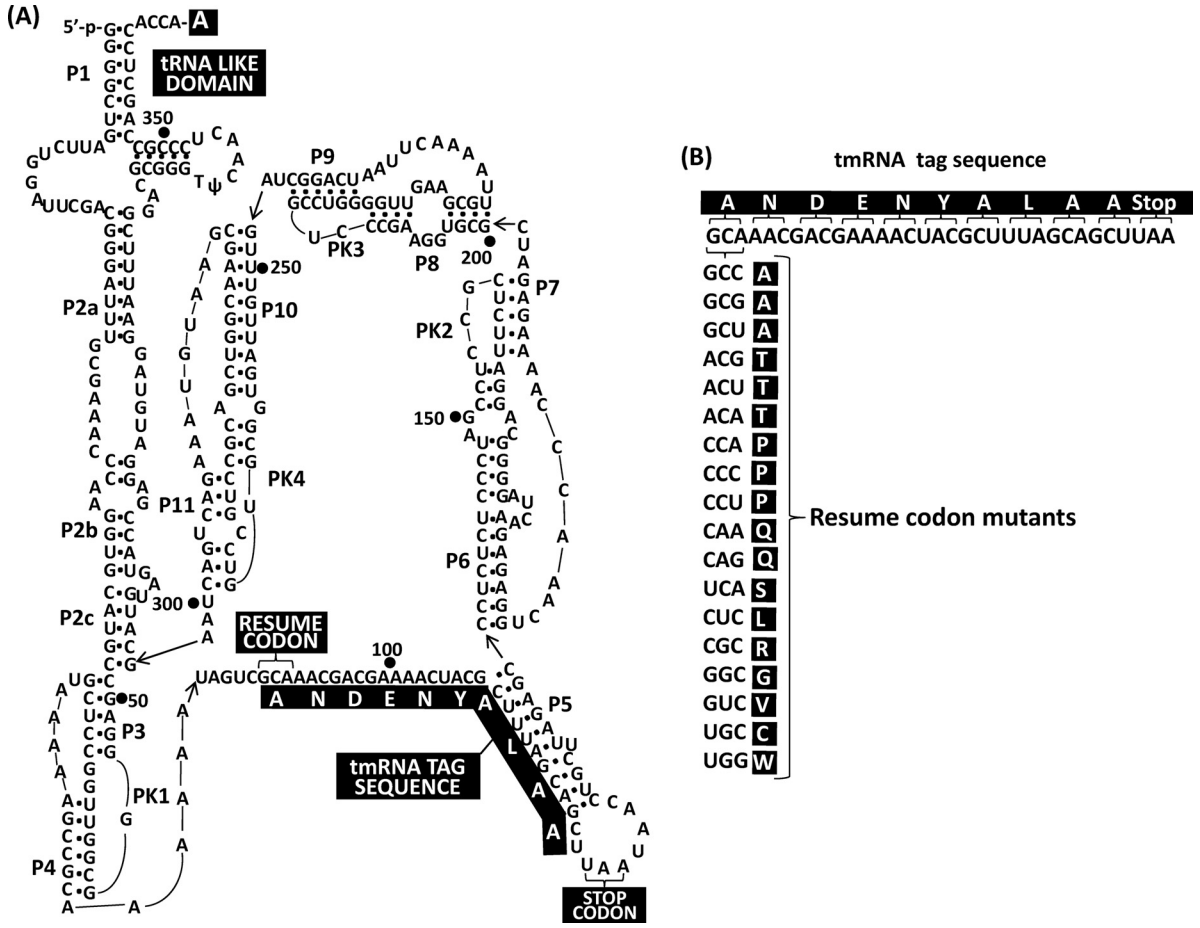

FIG. 1. (A) Secondary structure of *E. coli* tmRNA. Regions corresponding to tRNA-like domain and the short mRNA along with its translated sequence (tmRNA tag sequence) are shown. Encoded amino acids are shown in white type on a black background. Nucleotides have been numbered at intervals of 50 nucleotides. 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.

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 (*pth-1*) of *E. coli* strain AA7852 was tagged with *zch-3060*::Tn*10* (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^r 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. For the phage plating assay, LB medium was supplemented with 0.2% maltose and 10 mM $MgSO_4$, and 0.7% Bacto agar was used for the top agar medium.

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 pTrcRp (5′ CTTTGATCATCCGCCAAAACAGCC 3′) and tmRNA_1stcod random (5′ CCGCAAAAAATAGTCNNNAACGACG 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 (OD_{595}) 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 Na_2EDTA. An equal volume of water-saturated phenol (prewarmed at 65°C) 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 on 1% agarose gel. Equal amounts (~2 μg) of total RNA were electrophoresed on 2% agarose gel, transferred onto nylon membrane using a vacuum

TABLE 1. Relevant characteristics of the plasmids and strains used in this study

Plasmid or *E. coli* strain	Relevant details	Source or reference
Plasmids		
pACYC184 (Tet^r Cam^r)	Cloning vector harboring p15a ori of replication, which is compatible with ColE1 ori in pTrc99C	3
pRARE (Cam^r)	Plasmid harboring p15a ori and genes for rare tRNAs *proL* (reads CCC and CCU), *leuW* (reads UUG), *metT* (reads AUG), *argW* (reads AGG), *thrT* (reads ACC and ACU), *glyT* (reads GGA and GGG), *tyrU* (reads UAC and UAU), *thrU* (reads ACA, ACU, and ACG), *argU* (reads AGA), and *ileX* (reads AUA)	Novagen
pTrc-*ssrA* (Amp^r)	*ssrA* gene (~0.83 kb along with its promoter) amplified from *E. coli* with 5′-GA AAAGCTTATTGGCTATCAC-3′ and 5′-CACTAAGCTTGATTAACAGA G-3′ primers and cloned within HindIII site of pTrc99C	32
pTrc-*ssrA* mutants (Amp^r)	Resume codon in pTrc-*ssrA* mutated to various codons, with constructs named after resume codon of *ssrA* gene [e.g., pTrc-*ssrA*(CCC), pTrc-*ssrA*(ACA), etc.]	This study
pACDH (Tet^r)	Cloning vector harboring p15a ori of replication, which is compatible with ColE1 ori in pTrc99C	27
pACDH-*proL* (Tet^r)	pACDH plasmid harboring *proL* tRNA gene between EcoRI and NcoI sites	This study
pACDH-*thrW* (Tet^r)	pACDH plasmid harboring *thrW* tRNA gene between EcoRI and NcoI sites	This study
Strains		
CAG12016 (Tet^r)	*E. coli* (F$^-$ λ$^-$ *rph-1 zch-3060*::Tn*10*)	23, 30
AA7852	F$^-$ Arg$^-$ Leu$^-$ Thr$^-$ His$^-$ thiamine$^-$ *relA*$^+$ T1 *pth-1*	1
AA7852 *pth*(Ts) *ssrA*::*kan* (Kan^r)	AA7852 containing *pth-1* and disruption of *ssrA* gene with Kan^r marker	32
DY330 *ssrA*::*kan* (Kan^r)	W3110 Δ*lacU169 gal490* [λ*c*1857Δ(*cro-bioA*)] *ssrA*::*kan*	32
TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F$^-$ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	29
MG1655	F$^-$ λ$^-$ *rph-1*	2
MG1655 *pth*(Ts) (Tet^r)	MG1655 containing *pth-1* (linked with Tet^r)	This study
MG1655 *pth*(Ts) *ssrA*::*kan* (Tet^r Kan^r)	MG1655 containing *pth-1* (linked with Tet^r) and disruption of *ssrA* gene with Kan^r marker	This study

blot apparatus, and subjected to hybridization (32) using 5′-^{32}P-labeled DNA oligomers (5′ GTTTCGCAAATCCCGTCGAAT 3′ and 5′ TACCATCGGCG CTACGGCGTTTC 3′) complementary to regions of tmRNA and 5S rRNA, respectively, and subjected to BioImage analysis (FLA2000, Fuji).

λ*imm*P22 hybrid phage plating assay:. The λ*imm*P22 hybrid phage was kindly provided by D. I. Friedman, University of Michigan. Cultures of *E. coli* MG1655 *ssrA*::*kan* harboring various pTrc-*ssrA* plasmids (wild-type and mutant *ssrA*) were infected with 0.1 ml of a 10^{-6} dilution of a fresh lysate of the phage and plated (31). Plates were incubated for 8 h at 37°C. The PFU were counted and also pictured by a gel documentation machine.

Rescue of the temperature-hypersensitive phenotype by pRARE plasmid. The pRARE plasmid (p15a ori compatible with the ColE1 ori plasmids) (Table 1) harboring genes for rare tRNAs or a vector-alone plasmid pACYC184 (p15a ori) (Table 1) was introduced into *E. coli* MG1655 *pth*(Ts) *ssrA*::*kan* along with the pTrc-*ssrA* plasmids (harboring CCC or ACA resume codon mutants). The transformants were selected on Amp and Cam plates, grown to saturation at 30°C, streaked on LB agar (Amp and Cam) plates, and incubated at 30°C and 37°C for 12 h.

Rescue of the temperature-hypersensitive phenotype by overexpression of specific tRNA genes. Elongator tRNA genes *proL* and *thrW*, decoding CCU (Pro) and ACG (Thr), respectively, were PCR amplified from *E. coli* genomic DNA with following DNA oligomers. For the *proL* gene, 5′ CTCCCCATGGA CGAGAAGCGTTTTATC 3′ and 5′ CGTTGAATTCACAGAAAATAAAC AGGC 3′ and for the *thrW* gene 5′ CTCCCCATGGTGCAAAAGTAGCCA 3′ and 5′ TGTTGAATTCGATTGATCTTCGT TG 3′ were used as forward and reverse primers, respectively, in a 50-μl volume containing 3 U of *Pfu* DNA polymerase, 200 μM dNTPs, and 20 pmol each of forward and reverse primers with 30 cycles of incubation at 95°C for 1 min, 60°C for 45 s, and 72°C for 1 min 30 s. The PCR product was then cloned into pACDH (Tet^r) using EcoRI and NcoI. Positive clones (pACDH-*proL*, and pACDH-*thrW*) were confirmed by sequencing and introduced into *E. coli* AA7852 *pth*(Ts) *ssrA*::*kan* carrying the pTrc-*ssrA* plasmids (harboring CCU or ACG resume codon mutants). The transformants were selected on Amp and Tet plates, grown to saturation at 30°C, streaked on LB agar (Amp and Tet) plates along with suitable controls, and incubated at 30 and 37°C for 18 h.

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 GGC (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 Pth to avoid any selection bias (see Materials and Methods). Sequence analysis of over 100 miniplasmid preparations identified the 18 mutants shown in Fig. 1B.

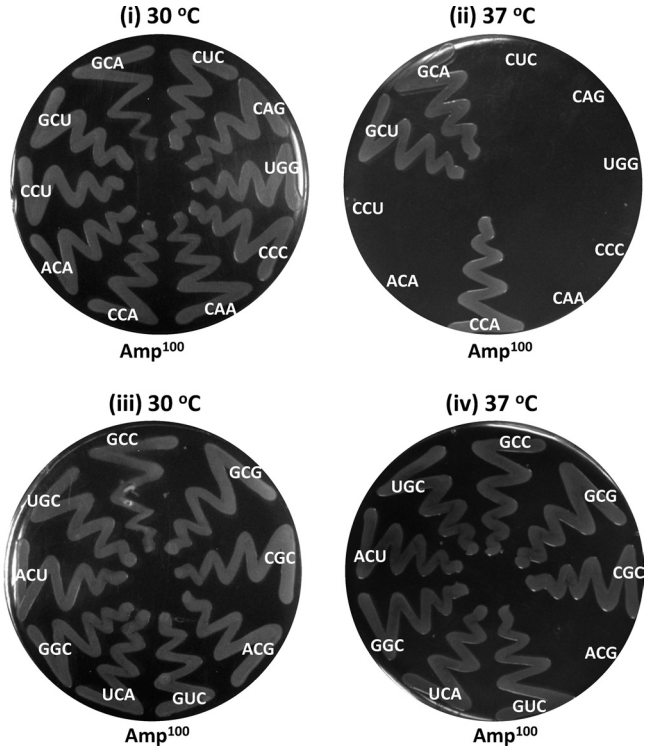

FIG. 2. Rescue of the temperature-hypersensitive phenotype of *E. coli* MG1655 *pth*(Ts) *ssrA*::*kan*. The strain was transformed with wild-type *ssrA* or various *ssrA* resume codon mutants. Transformants were grown to saturation in liquid cultures at 30°C, streaked on LB agar (Amp), and incubated 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-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, GCG, 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 5S rRNA. As shown in Fig. 3, the relative levels of tmRNA to 5S 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 λ*imm*P22 hybrid phage. Earlier studies have shown that λ*imm*P22 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 cor-

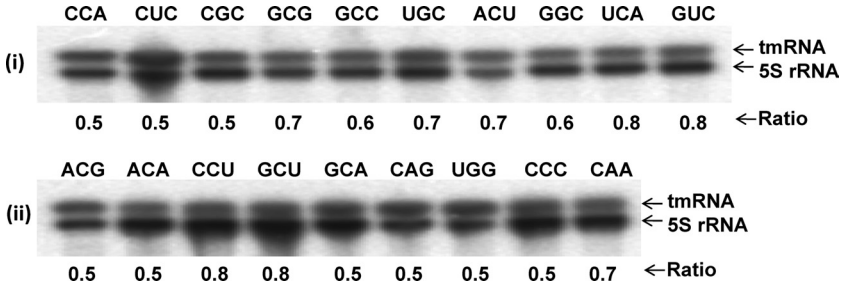

FIG. 3. Northern blot analysis of tmRNA and 5S rRNA. Total RNA was isolated from *E. coli* MG1655 *pth*(Ts) *ssrA*::*kan* harboring wild-type or mutant *ssrA* constructs, separated on 2% agarose gel, processed for Northern blot analysis using tmRNA- and 5S rRNA-specific probes, and analyzed using a BioImage analyzer (FLA2000; Fuji). Bands corresponding to tmRNA and 5S rRNA are indicated. "Ratio" indicates the ratio of pixels in the tmRNA band to pixels in the 5S rRNA band.

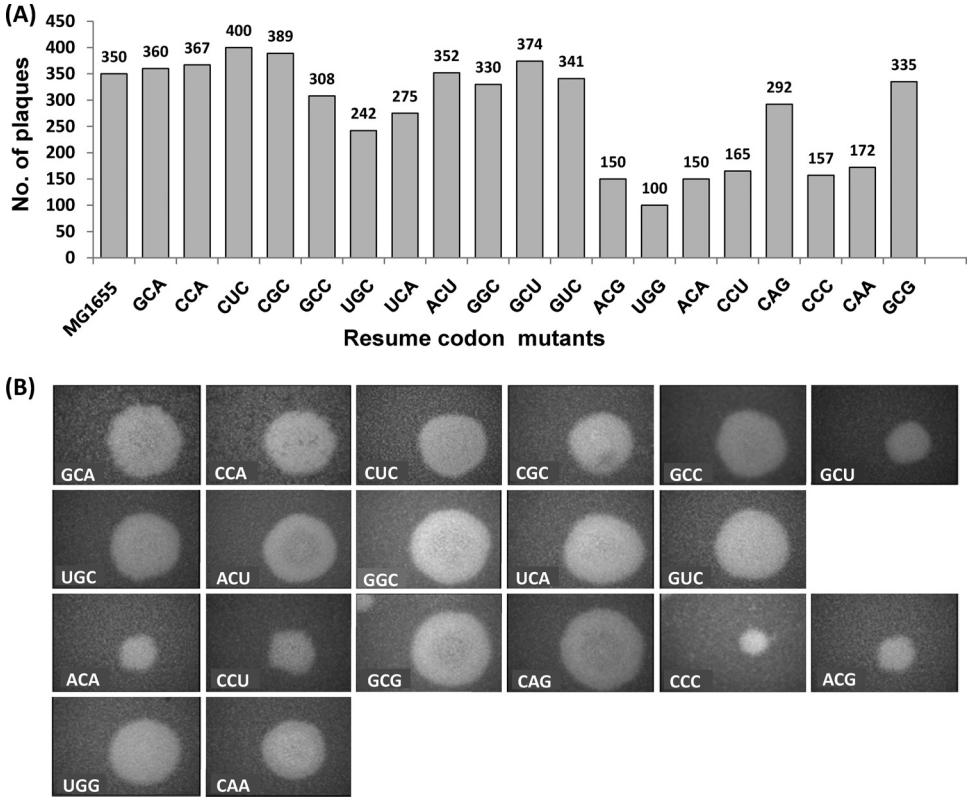

FIG. 4. Hybrid phage λ*imm*P22 plating assay. (A) *E. coli* MG1655 *ssrA*::*kan* cells harboring wild-type or mutant *ssrA* constructs were used as hosts for infection with 0.1 ml of a 10^{-6} dilution of a fresh lysate of λ*imm*P22hybrid phage and plated. Plates were incubated for 8 h at 37°C. The PFU were counted and also pictured using a gel documentation machine. Data from a representative experiment are shown. The pTrc-*ssrA* construct present within the *E. coli* MG1655 *ssrA*::*kan* host is indicated by the resume codon name. For example, pTrc-*ssrA* (GCA) is indicated by "GCA." Numbers above the bars indicate the numbers of PFU on the plate. MG1655 is a control for wild-type *E. coli*. Of these strains, the *ssrA* mutants with resume codons CCU, ACA, CAA, CCC, UGG, CAG, CUC, and ACG were seen not to rescue the temperature-hypersensitive phenotype of the *pth*(Ts) *ssrA*::*kan* strain (Fig. 2) (B) Plates were imaged using a gel documentation system. Uniformly enlarged (computationally) images of the plaques (enlarged ~8×) are shown in white type.

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-

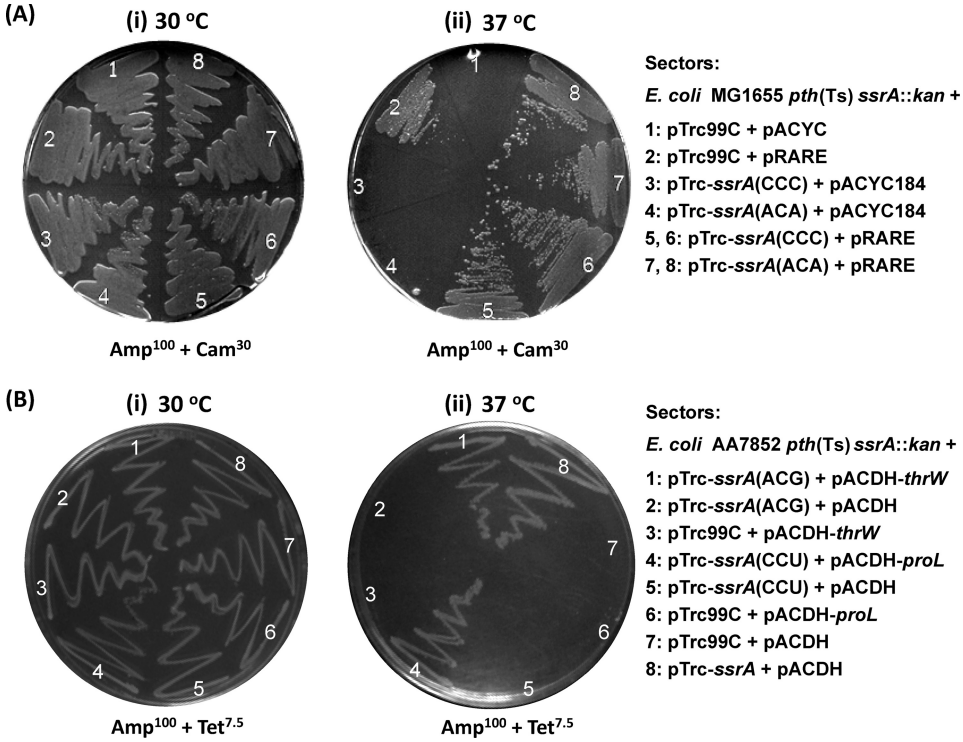

FIG. 5. Rescue of the temperature-hypersensitive phenotype of *E. coli* MG1655 *pth*(Ts) *ssrA*::*kan* in the presence of overproduced tRNAs. (A) *E. coli* MG1655 *pth*(Ts) *ssrA*::*kan* cells harboring pTrc99C, pTrc-*ssrA* (ACA), or pTrc-*ssrA* (CCC) were transformed with pACYC184 or the pRARE plasmid. Transformants were grown in liquid cultures at 30°C, streaked on LB agar (Amp and Cam), incubated at 30°C (panel i) or 37°C (panel ii) for 12 h, and documented. (B) *E. coli* AA7852 *pth*(Ts) *ssrA*::*kan* cells harboring pTrc99C, pTrc-*ssrA*(CCU), or pTrc-*ssrA*(ACG) were transformed with pACDH (vector alone), pACDH-*proL*, or pACDH-*thrW*. Transformants were grown in liquid cultures at 30°C, streaked on LB agar (Amp and Tet), incubated at 30°C (panel i) or 37°C (panel ii) for 18 h, and documented. The plasmids present in the *pth*(Ts) *ssrA*::*kan* strains streaked in various sectors are listed to the right.

lowed 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 $<0.5\%$ (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

lead to scarcity of the tRNA decoding UGG. Hence, the lack of rescue by the UGG resume codon *ssrA* mutant may be an indirect effect of sequestration of the tRNA that decodes UGG. Likewise, neither CAG nor CAA is a rare codon in *E. coli*, yet when present as resume codons in tmRNA, CAG and CAA rendered the *pth*(Ts) *ssrA*::*kan* strain hypersensitive at 37°C (Fig. 2). It was predicted (8) that the charging levels of Gln isoacceptors decreased uniformly to zero as the supply of Gln approached zero. Hence, it is quite likely that CAG and CAA would fail to be efficiently translated during the conditions that lead to increased stalling and thus would not serve as good resume codons. Furthermore, the observations in Fig. 2 suggest that the CUC (Leu) and ACG (Thr) codons are also likely to be quite unfavorable for translation. In fact, overproduction of *thrW*, which decodes ACG, does rescue the temperature-hypersensitivity phenotype of the tmRNA mutant containing the ACG resume codon (Fig. 5B). Furthermore, an earlier study showed that the minigenes carrying CAG, CAA, UGG, and ACG codons were toxic in the *pth*(Ts) background (5), suggesting a deficiency of the tRNAs that decode them. This observation also supports our finding that the mutants carrying these codons as resume codons do not rescue the temperature-hypersensitive phenotype of the *pth*(Ts) strain. Other codons like GUC (Val), UCA (Ser), GGC (Gly), ACU (Thr), UGC (Cys), GCC (Ala), CGC (Arg), CCA (Pro), GCA (Ala, found in *E. coli ssrA*), GCG (Ala), and GCU (Ala) showed an efficient rescue of the *pth*(Ts) *ssrA*::*kan* strain. All of these codons belong to a category of codons decoded by tRNA isoacceptors abundant in *E. coli.*

When *E. coli* cells are subjected to severe amino acid starvation, 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 concentrations of isoacceptors and how often their codons are encountered during protein synthesis. In fact, this study supports 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 isoacceptors that read four codons of Ala, and it has been shown that the residual charging level of tRNA Ala1(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 variations 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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AUTHOR CORRECTION

Correction for Kapoor et al., "Functional Significance of an Evolutionarily Conserved Alanine (GCA) Resume Codon in tmRNA in *Escherichia coli*"

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Volume 193, no. 14, p. 3569–3576, 2011, https://doi.org/10.1128/JB.01446-10. Page 3573, Fig. 4B: The two panels showing individual plaques with the ACA and ACG codons are identical; it appears that the same image was downloaded twice by mistake during assembly of the figure. Plaques for both the ACA and ACG codons were of similar small sizes and obtained on two distinct agar plates (not shown). The figure error does not in any way alter the interpretations or the conclusions drawn in the paper.

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