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Designing seryl-tRNA synthetase for improved serylation of selenocysteine tRNAs

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Abstract

Selenocysteine (Sec) lacks a cognate aminoacyl-tRNA synthetase. Instead, seryl-tRNA synthetase (SerRS) produces Ser-tRNA^{Sec}, which is subsequently converted by selenocysteine synthase to Sec-tRNA^{Sec}. *Escherichia coli* SerRS serylates tRNA^{Sec} poorly; this may hinder efficient production of designer selenoproteins *in vivo*. Guided by structural modeling and selection for chloramphenicol acetyltransferase activity, we evolved three SerRS variants capable of improved Ser-tRNA^{Sec} synthesis. They display 10-, 8-, and 4-fold increased k_{cat}/K_M values compared to wild-type SerRS using synthetic tRNA^{Sec} species as substrates. The enzyme variants also facilitate *in vivo* read-through of a UAG codon in the position of the critical serine$_{146}$ of chloramphenicol acetyltransferase. These results indicate that the naturally evolved SerRS is capable of further evolution for increased recognition of a specific tRNA isoacceptor.

Keywords

Selenoproteins; synthetic biology; seryl-tRNA synthetase; tRNA; protein engineering

Introduction

Selenocysteine (Sec), genetically encoded by UGA, is the only naturally occurring amino acid that lacks a cognate aminoacyl-tRNA synthetase. Instead, seryl-tRNA synthetase (SerRS) acylates tRNA^{Sec} with serine (Ser); subsequently, the selenocysteine synthase SelA transforms the RNA-bound Ser to Sec. The resulting Sec-tRNA^{Sec} is delivered to the ribosome by the dedicated elongation factor SelB, which in the presence of a specific mRNA structure aids accurate recoding of UGA for Sec insertion into proteins [1]. Thus, *E. coli* tRNA^{Sec} evolved to optimally interact with SerRS, SelA, and SelB, and to reject the general

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elongation factor EF-Tu. Yet, strategies for *in vivo* synthesis of selenoproteins in *E. coli* rely on $tRNA^{Sec}$ variants that interact with EF-Tu and SelA. For this reason a number of $tRNA^{Sec}$ variants were designed [2–4]. Secondary structures and altered nucleotides (compared to $tRNA^{Sec}$) of $tRNA^{UTuX}$, $tRNA^{SecUX}$ and $tRNA^{UTu1}$ are presented in Fig. 1. Significantly, the three base pairs (green) in the center of $tRNA^{Sec}$ are identity elements for SelB recognition and reject EF-Tu. Replacing these base pairs with G7:C66, C/U49:G65 and A/C50:U64 allows these tRNAs to be recognized by EF-Tu. Sec insertion by these variant tRNAs may be feasible for virtually any codon [5].

E. coli SerRS charges five $tRNA^{Ser}$ isoacceptors with equal efficiency [6]. However, compared to the $tRNA^{Ser}$ species, $tRNA^{Sec}$ [7] is serylated 100 times more poorly (as judged by k_{cat}/K_M) [8]. This is plausible; given that there is only one selenoprotein in *E. coli* [9], efficient genome translation requires very much less Sec-$tRNA^{Sec}$ than Ser-$tRNA^{Ser}$ (for translation of >78,000 codons). In light of our synthetic biology efforts to develop an efficient EF-Tu-dependent translation system for UAG directed, site-specific Sec incorporation into proteins [10], we decided to design a variant SerRS enzyme endowed with improved serylation of the $tRNA^{Sec}$ variants $tRNA^{UTuX}$ [3], $tRNA^{UTu1}$ [10]), and $tRNA^{SecUX}$ [4].

We based our *E. coli* SerRS mutagenesis strategy on the examination of *Thermus thermophilus* SerRS•$tRNA^{Ser}$ [11] and *Homo sapiens* SerRS•$tRNA^{Sec}$ [12] co-crystal structures, corresponding library construction, and variant selection by an *in vivo* chloramphenicol acetyltransferase (CAT) assay [13]. This approach led to the discovery of three desired SerRS variants.

Materials and Methods

2.1. Reagents and strains

Pfu Ultra II DNA polymerase was obtained from Agilent Technologies; restriction enzymes, T4 DNA ligase, and the Gibson Assembly mix were from New England Biolabs. In-Fusion cloning kit was from Clontech. The thermosensitive *E. coli* strain KL235 [14] was obtained from the Coli Genetic Stock Center (Yale University).

2.2. Plasmid construction

For *in vivo* serylation assays plasmids pXF113, pXF114 and pXF114 were created by inserting a *lpp*-tRNA-*rrnC* cassette into a modified pACYC184 backbone containing a *cat* gene where an amber stop codon was introduced at position 146 [15]. The cassette and pACYC184 backbone were amplified using primers listed in Table S1 and ligated using In-Fusion cloning. pXF113, pXF114 and pXF115 contained genes from $tRNA^{UTuX}$, $tRNA^{SecUX}$ and $tRNA^{Pyl}$, respectively. Plasmids pXF113 containing coding sequences for $tRNA^{UTu1}$, SupD, and SelC(amb) were created by Gibson Assembly, using sets of primer listed in Table S1. The gene for *E. coli* $tRNA^{Sec}$ with an amber anticodon (selC(amb)) was amplified using a previously published template [2]. To generate pBAD-SerRS plasmids oligonucleotides EcSerSf and EcSerSr (Table S1) were used to amplify the SerRS gene. The product was then digested with *Nco*I and *Kpn*I and inserted into a modified pKTS vector

containing *araC* gene and P_{BAD} promoter. SerRS variants containing S55A mutation or a GGGGS linker were constructed by In-Fusion cloning, using primer sets listed in Table S1. Plasmids constitutively expressing tRNAs and wild-type or SerRS variants were created by inserting a *lpp*-tRNA-*rrnC* cassette into pBAD-SerRS plasmids by In-Fusion cloning (Table S1). pTrc99A-SerRS plasmids were generated by In-Fusion using the primer sets listed in Table S1. To generate pTrc99A-SerRS.V20-A55S the plasmid was mutagenized using primers A55S.F and A55S.R (Table S1). For protein isolation purposes, SerRS variants were cloned into pET15 vector (Novagen) using Gibson Assembly (NEB). The open reading frames of *E. coli* SerRS genes and the pET15 backbone were amplified using primer pairs listed in Table S1. Artificial $tRNA^{Sec}$ genes ($tRNA^{UTuX}$, $tRNA^{SecUX}$, $tRNA^{UTu1}$, [3,4,10]) and wild-type $tRNA^{Ser}_{CGA}$ were amplified using primer pairs listed in Table S1 and cloned using Gibson Assembly (NEB) into a modified pUC18 vector (T7 promotor adjacent to the *Bam*HI restriction site). Construction of pUC19 with wild-type *E. coli* $tRNA^{Sec}$ was described in [16].

2.3. Construction of *E. coli* knock-out strains

Strains DH10B Δ*selAB*::zeo strain and WL30153 were used to test serylation of different tRNAs *in vivo* and for selection purposes. DH10B Δ*selAB*::zeo was created by λ-Red recombination [17] using primers EM7-zeo FW and EM7-zeo RV (Table S1) to amplify the EM7 - zeocin cassette. *E. coli* strain WL30153 (MC4100 Δ*selAB*) was described [18].

2.4. Library creation and chloramphenicol-resistance selection

Four fragments of *serS* gene were amplified by primers listed in Table S1 to generate SerRS coding sequences that are fully randomized at given positions (residues K86, A87, D90, S160 and D228 and T229) by overlap PCR. The mixture of pBAD-SerRS plasmids expressing the wild-type and the S55A variant with 1 to 1 ratio was used as the template to amplify *serRS* gene fragments. Inserts were then digested with *NcoI* and *NdeI* and ligated into pRSF-based vector, under the control of a P_{BAD} promoter. The SerRS library transformation resulted with 8.5×10^8 clones, oversampling the theoretical diversity of 3.5×10^8. For selection purposes, the library was co-transformed with pXF113 into DH10B *ΔselAB*::zeo, and plated onto LB agar supplemented with 55 mg/l Cm.

2.5. Protein purification

E. coli BL21(DE3) cells harboring pET15b-*serS* plasmids were grown to OD_{600} 0.4 at 37 °C, after which 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. Following induction, cells were grown for 3 hr, pelleted and resuspended in buffer A (0.1 M Tris-Cl pH 7.4, 0.1 M NaCl, 5 mM 2-mercaptoethanol, 0.3 mM PMSF). Cells were then lysed by ultrasonic treatment and soluble proteins collected by centrifugation at 12,000 × g. Wild-type SerRS and SerRS variants were then purified by two rounds of anionic exchange, first on HiTrap DEAE FF Sepharose (GE Healthcare) and then HiTrap Q (GE Healthcare). Purification was executed in a linear gradient of salt using buffers A and B (0.1 M Tris-Cl pH 7.4, 1 M NaCl, 5 mM 2-mercaptoethanol). Eluted proteins were concentrated and stored at −80°C in buffer S (0.1 M Tris-Cl pH 7.4, 0.15 M NaCl, 50% glycerol, 5 mM 2-mercaptoethanol). Prior to the aminoacylation assay, proteins were diluted in buffer D (50 mM Tris-Cl pH 8.0, 1 mM 1,4-dithiothreitol (DTT)) and their concentration was measured

according to the theoretical extinction coefficients (68760 M^{-1} cm^{-1} for wild-type and SerRS v.20 and v.22, and 71740 M^{-1} cm^{-1} for SerRS v.8).

2.6. Transcript preparation and purification

pUC18 plasmids containing genes for $tRNA^{Ser}$ and $tRNA^{Sec}$ variants were transformed into *E. coli* DH5α cells, grown overnight and isolated using Plasmid Maxi kit (Qiagen). Following *Bst*NI digestion to generate appropriate 3′-CCA ends, tRNAs were generated by *in vitro* transcription using recombinantly produced T7 RNA polymerase [19]. Transcripts were purified on a HiTrap DEAE FF column (GE Healthcare), as described [20]. Prior use tRNAs were heated to 92°C for 3 min and slowly cooled to room temperature in the presence of 5 mM $MgCl_2$ to allow renaturation. tRNA concentration was estimated according to its absorbance at 260 nm and the percentage of active transcript was obtained from the plateau charging (see below).

2.7. *In vitro* aminoacylation assays

All aminoacylation reactions were performed at 37 °C, essentially as reported [21], and contained 100 mM Tris-Cl (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 10 mM ATP and 0.4 mg/ml bovine serum albumin, 0.05 U thermostable inorganic pyrophosphatase (NEB) and 125 μM [^{14}C]serine (Perkin Elmer). Aliquots of the reaction were removed at given intervals, spotted onto 3 MM filter papers and immersed in 10% trichloroacetic acid to precipitate aminoacyl-tRNA. For the K_M and k_{cat} determination in case of $tRNA^{UTuX}$, tRNA concentration was varied over the range of ~0.1 – 5.6 μM (for SerRS V8 and V20) and ~0.1 – 8.6 μM (for wt and SerRS V22).

In case of $tRNA^{SecUX}$, tRNA concentration was varied over the range of ~0.6 – 11.1 μM and in case of $tRNA^{UTu1}$ over the range of ~0.2 – 12 μM for all SerRS enzymes. To determine the plateau of any selenocysteine tRNA serylation, 2 μM wt SerRS was incubated with of 2 μM transcript for 30 min.

2.8. Complementation assay

A culture of *E. coli* strain KL235 (in which lack of growth at elevated temperatures is a result of a thermosensitive SerRS [14]) was grown to early exponential phase at 30°C. The cells were made electrocompetent and were transformed with plasmids pTrc99A, in which the genes for wild-type and SerRS variants were placed under the control of *trc* promoter. As a negative control, a batch of cells were transformed with pTrc99A lacking the insert. Individual clones of KL235 containing desired plasmids were grown overnight, diluted to OD_{600}=1 and spotted in serial dilutions onto LB plates containing 0.05 and 0.1 mM IPTG. Colonies were then grown at 30, 37 and 42°C for 20 hr.

2.9. Model of the *E. coli* SerRS•$tRNA^{Sec}$

E. coli SerRS was comparatively modeled by Phyre2 (Protein Homology/AnalogY Recognition Engine, [22]. Superposition of the model structure of *E. coli* SerRS onto SerRS•$tRNA^{Sec}$ complex was achieved by UCSF Chimera [23].

Results

Designing a SerRS variant library

SerRS recognizes $tRNA^{Sec}$ and $tRNA^{Ser}$ in a similar manner. The two tRNA species share a set of minor identity elements (discriminator base G73, C72, and acceptor-stem pairs G2:C71, A3:U70, Fig. 1) [24,25]. However, the most important element is the long variable arm, which is recognized by SerRS in a sequence-independent manner; the length and relative orientation have the greatest effect. The binding of the variable arm (of both tRNA species) is accompanied by the movement of the N-terminal tRNA binding domain (NTD) [11,12].

Another idiosyncratic feature of $tRNA^{Sec}$ is the G19:U20:C56 base triplet that defines its tertiary fold [12,26] (Fig. 1). Interestingly, U20 protrudes in the human SerRS•$tRNA^{Sec}$ structure and is solvent accessible [12,26], unlike the corresponding C20 in $TttRNA^{Ser}$ that is buried in the tRNA core [11]. The highly conserved S61 residue presumably allows SerRS to distinguish between $tRNA^{Sec}$ and $tRNA^{Ser}$ by interacting specifically with nucleotide determinant in position 20 [12]. Both S61 (S55 in *E. coli* SerRS) and U20 are present in EcSerRS and $EctRNA^{Sec}$, respectively. Furthermore, all $tRNA^{Sec}$ derivatives ($tRNA^{SecUX}$, $tRNA^{UTu1}$ and $tRNA^{UTuX}$, Fig. 1) retain this uridine. However, our attempts to improve $tRNA^{Sec}$ serylation via S55 mutagenesis, or by increasing NTD flexibility in order to accommodate the longer acceptor-TψC arm of $tRNA^{Sec}$ were unsuccessful (see Supplementary Information for detailed explanations).

Next, we decided to simultaneously target multiple residues that, in the *T. thermophilus* SerRS•$tRNA^{Ser}$ complex, are in close contact (Fig. 2) [11]. We decided to use this structure because $tRNA^{Sec}$ and $tRNA^{Ser}$ share some common features responsible for their recognition by SerRS [25], and because TtSerRS and EcSerRS are more closely related than HsSerRS and EcSerRS [27]. While the discriminator base G73 and the orientation of the variable arm constitute the minimal set of recognition parameters in $tRNA^{Ser}$ and $tRNA^{Sec}$ [8,28,29], the NTD of TtSerRS also appears to interact with the T loop of $tRNA^{Ser}$ (Fig. 2, [11]). Similarly, T loop of $tRNA^{Sec}$ is also in proximity of NTD in the human SerRS•$tRNA^{Sec}$ complex [12]. Given the difference in nucleotide composition of TψC arms of $tRNA^{Ser}$ and $tRNA^{Sec}$, we hypothesized that certain residues in NTD might account for different mode of recognition of these tRNAs. Most closely placed to $tRNA^{Ser}$, charged residues E84, E85, and R88 of *T. thermophilus* SerRS are likely to form direct interactions with the tRNA (Fig. 2). Thus, we decided to randomize the corresponding residues of EcSerRS (K86, A87, and D90) to generate a SerRS variant capable of improved serylation of $tRNA^{Sec}$.

In addition to NTD residues, we wanted to simultaneously target the amino acids located in the catalytic domain of the enzyme. Compared to *T. thermophilus*, *E. coli* SerRS has a much longer motif 1 loop (residues R221-N231) [30]. This exposed motif 1 loop and S160 of EcSerRS are in proximity to the base of the acceptor arm according to our structural model (Fig. 2). Nucleotides in the tRNA that are likely to form interactions with SerRS occupy positions 65 and 66; they differ in $tRNA^{Ser}$ and $tRNA^{Sec}$ ($tRNA^{Ser}$ has C65 and G66 while $tRNA^{Sec}$ contains G65 and A66). Thus, to examine the role of catalytic domain residues in

differential recognition of these two tRNA species we targeted D228 and T229 in the motif 1 loop and S160.

Read-through of UAG codons in chloramphenicol acetyltransferase

The synthetic selenocysteine tRNAs $tRNA^{UTuX}$, $tRNA^{SecUX}$ and $tRNA^{UTu1}$ (Fig. 1) charged with Ser are recognized by EF-Tu, as shown by minor mis-incorporation of Ser instead of Sec into reporter proteins [2,3,10]. Therefore, the capacity of SerRS to aminoacylate these $tRNA^{Sec}$ variants can be monitored *in vivo* via stop-codon read-through in a *selA* deficient strain.

Using the CAT with a TAG stop codon at position 112 as reporter, $tRNA^{UTuX}$ and $tRNA^{SecUX}$ facilitated production of the full-length CAT protein at moderate Cm levels (50 mg/l and 25 mg/l for the respective tRNAs, Fig. S1). Since a number of different amino acids in position 112 lead to CAT activity, we switched to a *cat* gene TAG variant (position 146) that required Ser for activity [31]. Ser-$tRNA^{UTuX}$ facilitated UAG suppression that allowed growth at ≤ 20 mg/l, while no growth was observed with $tRNA^{SecUX}$ (Fig. S2). Both types of assay were conducted in DH10B *ΔselAB*::zeo in which there was no observable near-cognate suppression (Fig. S1).

Selection of desirable variants (a) recognition of $tRNA^{UTuX}$

A SerRS library containing fully randomized positions 84, 85, 88, 160, 228 and 229 was created using both wt and SerRS-S55A coding sequences as templates. SerRS coding sequence was placed under the control of the P_{BAD} promoter to regulate its expression and reduce its toxicity on cell growth. After confirming the library quality, library plasmids were re-transformed into the DH10B *ΔselAB*::zeo strain harboring the selection plasmid (pXF113 that carries the *cat*-146TAG and $tRNA^{UTuX}$ genes). The number of apparent *cat* revertants was determined with pXF115 carrying $tRNA^{Pyl}$ [15] (see above).

To identify SerRS variants with improved activity toward $tRNA^{UTuX}$ we selected those clones that facilitated cell growth at 55 mg/l Cm. About 100 colonies grew on an LB plate supplemented with 55 mg/l Cm when $tRNA^{UTuX}$ was expressed, while only a few colonies appeared when $tRNA^{Pyl}$ was provided. Repeated testing of 22 positives showed that four SerRS variants confer Cm resistance up to 65 mg/l in a $tRNA^{UTuX}$-dependent manner (Fig. S4). After recloning only variants V8, V20 and V22 showed reproducible improvement in CAT read-through assay. Relevant SerRS sequences of these three variants are shown in Fig. 3A. All three variants possess different mutations, except for D228. In all cases D228 is converted to a positively charged K or R, implicating the importance of this motif 1 residue in the interaction with $tRNA^{UTuX}$. Furthermore, S55 is mutated to Ala in SerRS V20. However, this sequence heterogeneity is not accidental. After mutating Ala55 to wild-type serine, SerRS V20 loses its ability to efficiently charge $tRNA^{UTuX}$ and other selenocysteine tRNAs (Fig. S5). Thus, the S55A mutation is advantageous when accompanied by other specific mutations.

Sequencing of the entire plasmids that encode the variants revealed a partial deletion of the *araC* gene. As mutations in the AraC protein may give rise to various induction/repression phenotypes [32] we did not want to take a guess on the SerRS variant expression levels (Fig.

S4 and discussion therein). To unambiguously demonstrate that increased Cm resistance correlates with the activity of evolved SerRS variants, and not caused by unintended mutations in the vector, we recloned the SerRS coding sequences under the control of a *trc* promoter into another low-copy plasmid (Fig. 3B).

The CAT-146TAG read-through assay by the SerRS variants placed under the *trc* inducible promoter revealed that SerRS V8 and V20 outperform wt SerRS and allow growth at higher Cm levels (Fig. 3B). However, high Cm resistance (45 and 60 mg/l) could only be achieved if expression of the variants was induced with 0.05 and 0.1 mM IPTG, presumably allowing only modest level of expression (Fig. 3B). Higher IPTG concentrations led to decreased cell viability suggesting that SerRS overexpression is toxic to the cell [33]. This may explain why AraC deletion variants accumulated during selection of the SerRS library. Our results indicate that the efficiency of CAT stop-codon suppression is related to increased levels of Ser-tRNA^{UTuX}.

To examine the biochemical properties of the evolved variants with tRNA^{UTuX} as substrate, we tested their activity *in vitro* (Table 1). Using an established protocol [29] and similar active enzyme concentrations (determined by active site titration), SerRS V8 and V20 serylate tRNA^{UTuX} 10- and 8-times better than wild-type SerRS (Table 1). SerRS V22 provides a more moderate improvement (about 4-times), yet this is not sufficient to allow higher suppression levels *in vivo* (Fig. 3B). Interestingly, the major contribution to improved activity is the consequence of increased turnover (8- to 19-increased k_{cat} for V20 and V8, respectively), rather than increased affinity (Table 1). Taken together, SerRS variants V8 and V20 aminoacylate tRNA^{UTuX} better than the wild-type enzyme, under *in vitro* and *in vivo* conditions.

Selection of desirable variants (b) recognition of tRNA^{SecUX} and tRNA^{UTu1}

As the different synthetic tRNA^{Sec} species displayed differences in selenoprotein formation we tested the substrate properties of tRNA^{SecUX} and tRNA^{UTu1} (Fig. 1) [4,10,29] with our SerRS variants *in vitro* and *in vivo*. In enzyme-kinetic assays (described above) we determined the steady-state parameters. The values for tRNA^{SecUX} reveal significant serylation improvement by SerRS V8, while the kinetic parameters of SerRS V20 were only modestly improved (Table 2). As was the case with tRNA^{UTuX}, the improvement of SerRS V8 activity arises from a k_{cat} increase (Table 1, 2). This is plausible, as both tRNA^{UTuX} and tRNA^{SecUX} are highly similar in both sequence and structure. Interestingly, SerRS V8 and V20 aminoacylate the more dissimilar tRNA^{UTu1} [10] 11- and 6-times better than wild-type SerRS. Although tRNA^{UTu1}, as allo-tRNA [15], differs significantly from the tRNA^{UTuX} species used in SerRS V8 and V20 evolution, both of these enzyme variants have higher affinity toward tRNA^{UTu1} than the wild-type SerRS. As with tRNA^{UTuX} and tRNA^{SecUX}, both variants have significantly higher turnover numbers (~3 – 6-times for SerRS V20 and V8, respectively, Table 2).

The CAT-146TAG read-through assay with tRNA^{SecUX} and tRNA^{UTu1} corroborated the above *in vitro* data. Both SerRS V8 and V20 confer significantly higher Cm resistance compared to wt SerRS (Fig. 4). In case of tRNA^{SecUX}, both SerRS variants allow growth at 20 mg/l Cm, while the wt enzyme does not. Using the much stronger UAG suppressor

tRNA^{UTu1} [10], SerRS V8 and V20 allow robust growth at Cm 400 mg/l. Wild-type SerRS can also facilitate growth at these Cm levels albeit modestly, and only if expressed at a very low level (0.05 mM IPTG, Fig. 4). Thus, in case of all three selenocysteine tRNAs, the evolved SerRS variants allow growth at higher Cm concentrations but also operate in a larger dynamic range of expression.

SerRS variant V20 supports *E. coli* growth in the absence of other active SerRS enzymes

To examine if the mutations, that gave rise to our SerRS variants, influenced their ability to provide Ser-tRNA^{Ser} sufficient for active cell growth, we determined whether the variant enzymes could restore growth of *E. coli* strain KL235 that carries a temperature-sensitive SerRS [1]. This strain contains a genomic copy of *serS15*, encoding a thermolabile SerRS enzyme; as a result the host strain is unable to grow at 42°C. The SerRS variants were induced with low amounts of IPTG (0.05 and 0.1 mM) for 20 hr at 42°C. Robust growth was detected for KL235 cells expressing either wild-type SerRS or variant V20 (Figure 5). Toxicity of SerRS overexpression was more apparent in case of SerRS V22 that can replace SerS15 only when expressed at a very low level (0.05 mM IPTG). Similarly, SerRS V8, which can (albeit pretty inefficiently) rescue growth when induced with 0.05 mM IPTG, loses this capacity altogether when 0.1 mM IPTG is added. This indicates that SerRS V20 is able to supply sufficient serylated tRNA species for vigorous growth.

Additional information on the serylation properties of SerRS came from a serine-specific CAT-146TAG read-through assay. In order to decode the 146 UAG codon, we used the natural *E. coli* tRNA^{Ser} suppressor SupD [34]. The suppression efficiencies mediated by the SerRS V8•SupD and V20•SupD pairs were comparable, and even higher than in case of wt SerRS. SupD allowed moderate to good growth at 300 mg/l Cm (Supplementary Fig. S6). However, *in vitro* aminoacylation reveals somewhat reduced affinity of SerRS V8 and V20 for SupD; both of these enzymes have about 2-times higher K_M, while k_{cat} is 2–3-times lower, compared to the wt SerRS (Table S2). This suggests that certain mutations improving selenocysteine tRNA recognition may have adverse effects on tRNA^{Ser} aminoacylation by SerRS variants (Figure 5).

Discussion

E. coli SerRS catalyzes serylation of tRNA^{Ser} and tRNA^{Sec} with a 100-fold difference in k_{cat}/K_M [8]. One of the requirements for effective SelA recognition, the 8-bp long acceptor stem of tRNA^{Sec}, negatively influences recognition by SerRS, as demonstrated by the improved activity of tRNA^{Sec} variants lacking the 5A:67A bp [8]. These differences are also reflected by the extent of CAT synthesis *in vivo*, where the conferred resistance matches relative serylation levels of the SupD tRNA suppressor and the synthetic tRNA^{Sec} species (see above). Enzyme-kinetic analyses of these tRNAs with wild-type SerRS reveal tRNA^{UTuX} to be a marginally better substrate than tRNA^{SecUX} (k_{cat}/K_M is 1.5-time higher, Table 1, 2), and that tRNA^{UTu1} is 15-times better aminoacylated than tRNA^{UTuX} (Table 1, 2). However, even tRNA^{UTu1} has about 9-times lower k_{cat}/K_M value than SupD (Table 2, S2). The improved serylation of the synthetic tRNA^{Sec} species by the designed SerRS V8 and V20 variants (Table 1, 2) proves that SerRS can be adapted to better recognize atypical

tRNA^{Sec} structures. Compared to wt SerRS, these variants are inferior in the SupD tRNA^{Ser} acylation (4–5 fold), but superior for synthetic tRNA^{Sec} species (up to 11-fold).

Given the central role of aminoacyl-tRNA synthetases in protein synthesis, the last three decades saw much work directed toward 'improvement' of these enzymes. This effort led to tRNA synthetases endowed with an altered or expanded substrate spectrum for either tRNAs [35] or amino acids [36], or unnatural amino acid substrates for synthetic biology applications [37]. In this work we addressed a different challenge, to design variants of a normal cellular tRNA synthetase that aminoacylates multiple cognate isoacceptors. While wt *E. coli* SerRS serylates its cognate tRNA^{Ser} species 100-fold better than its tRNA^{Sec} isoacceptor [8], we created an enzyme variant with improved serylation properties only for tRNA^{Sec} species; yet this enzyme fully maintains its essential role in providing Ser-tRNA^{Ser} for cellular protein synthesis. This is another illustration of the great plasticity of the translation machinery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

aaRS	aminoacyl-tRNA synthetase
SerRS	seryl-tRNA synthetase
SelA	selenocysteine synthase
CAT	chloramphenicol acetyltransferase
NTD	N-terminal tRNA binding domain

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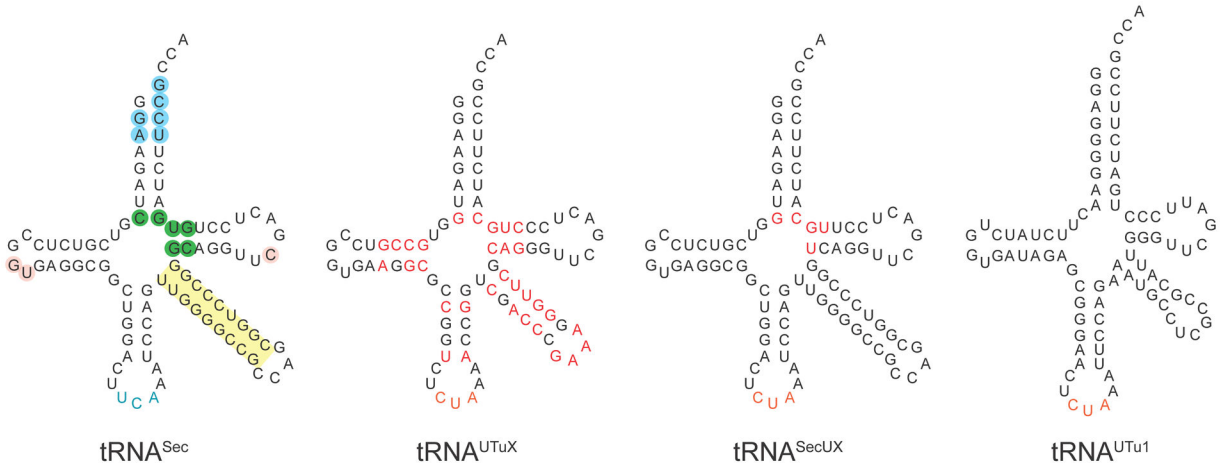

Figure 1.

Wild-type and synthetic selenocysteine tRNAs. Identity nucleotide determinants of wild-type *E. coli* $tRNA^{Sec}$ are shaded blue, and the variable arm, whose length and orientation are specifically recognized by SerRS, is highlighted in yellow. Nucleotides that govern the tertiary fold of $tRNA^{Sec}$ are given in pink. Base pairs acting as EF-Tu antideterminants are shown in green. Synthetic selenocysteine tRNAs employed in this study are given in black, except the amber anticodon (in purple). Mutations introduced into $tRNA^{UTuX}$ and $tRNA^{SecUX}$ are indicated in red. $tRNA^{UTu1}$, derived from a metagenome sequence [10,15] is too dissimilar to *E. coli* $tRNA^{Sec}$ to highlight nucleotide differences.

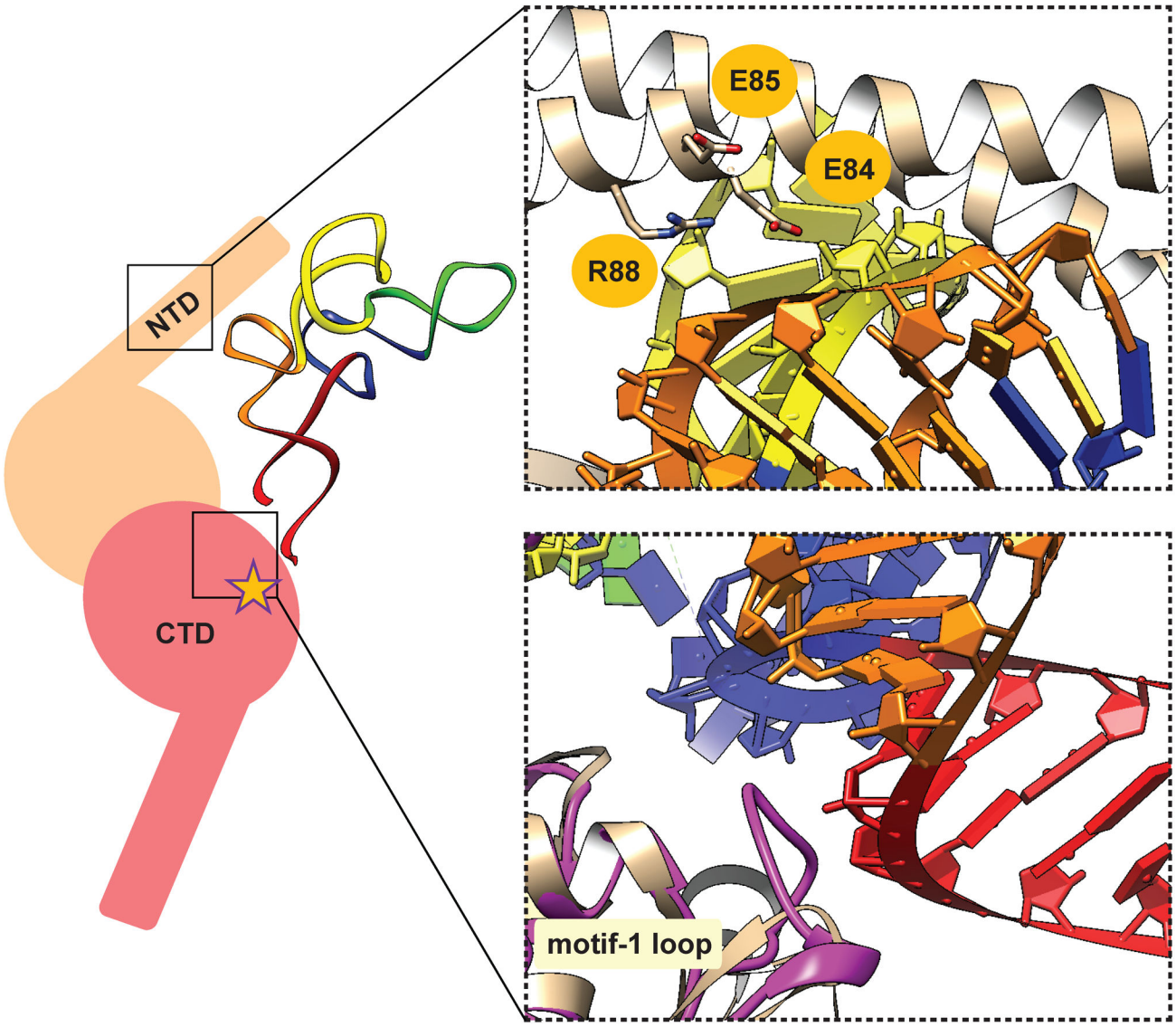

Fig. 2.

Key interactions between SerRS and $tRNA^{Ser}$ as a guide for SerRS engineering. (left) Cartoon representation of SerRS•$tRNA^{Sec}$ productive complex. SerRS is given in two shades of pink, for each monomer of the homodimer. The N-terminal tRNA binding domain (NTD), C-terminal catalytic domain (CTD) and the active site (star) are indicated. Cognate tRNA binds across the dimer. Acceptor, TψC, variable, anticodon, and D arms of $tRNA^{Sec}$ are shown in red, orange, yellow, green and blue, respectively. (right) Magnified view of the two regions targeted for mutagenesis. (up) In the crystal structure of *T. thermophilus* SerRS•$tRNA^{Ser}$ complex (PDB ID: 1SER) NTD residues E84, E85, and R88 are in the vicinity and/or form contacts with the TψC arm. These residues correspond to K86, A87, and D90 of *E. coli* SerRS. (down) Compared to *T. thermophilus* SerRS (light pink), *E. coli* SerRS (purple) has a longer motif 1 loop, which may interact with the base of the acceptor arm.

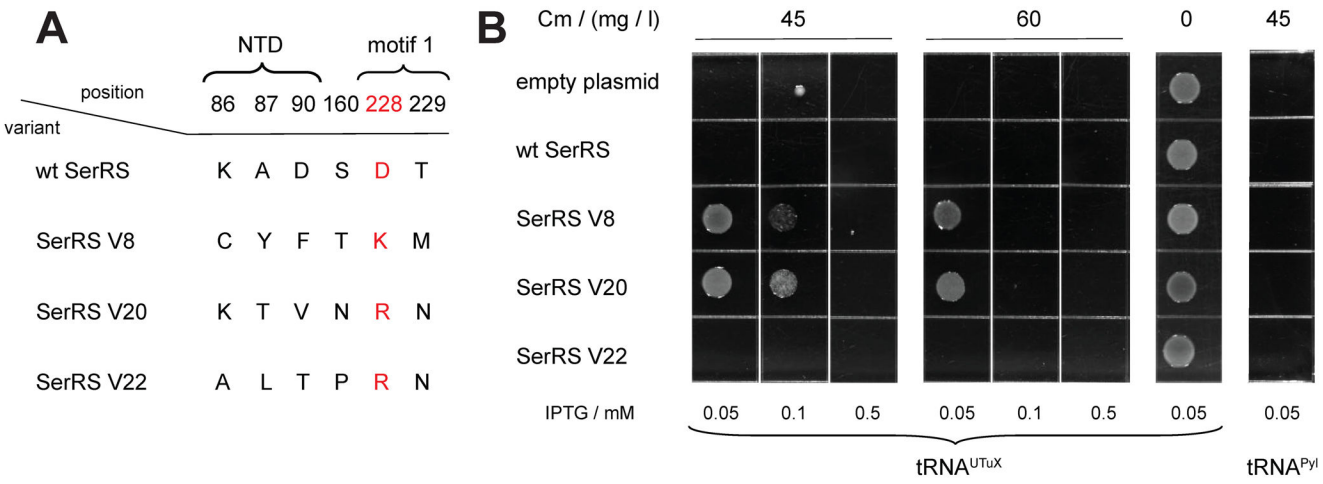

Fig. 3.

SerRS variants improve $tRNA^{UTuX}$-mediated amber stop-codon suppression. (A) Three most proficient SerRS variants contain different mutations, except for D228; this negatively charged motif-1 residue is converted into a basic (K or R) amino acid in all variants (highlighted). Locations of the residues are indicated (NTD or motif 1). In addition to the mutations listed here, SerRS V20 also contains a S55A mutation. (B) Comparison of *in vivo* acylation activity between wt and mutant SerRS for $tRNA^{UTuX}$. *E. coli* cells DH10B *ΔselAB*::zeo expressing $tRNA^{UTuX}$ and SerRS variants were grown on LB plates with different concentration of Cm (0, 45, 60 mg/l). $tRNA^{UTuX}$ was under the control of *lpp* promoter; a CAT gene with a TAG codon at position 146 was on the same plasmid. SerRS expression was controlled by the *trc* promoter on the second plasmid. The read-through facilitated by the variants is $tRNA^{UTuX}$ dependent, as no resistance is conferred in the presence of $tRNA^{Pyl}$.

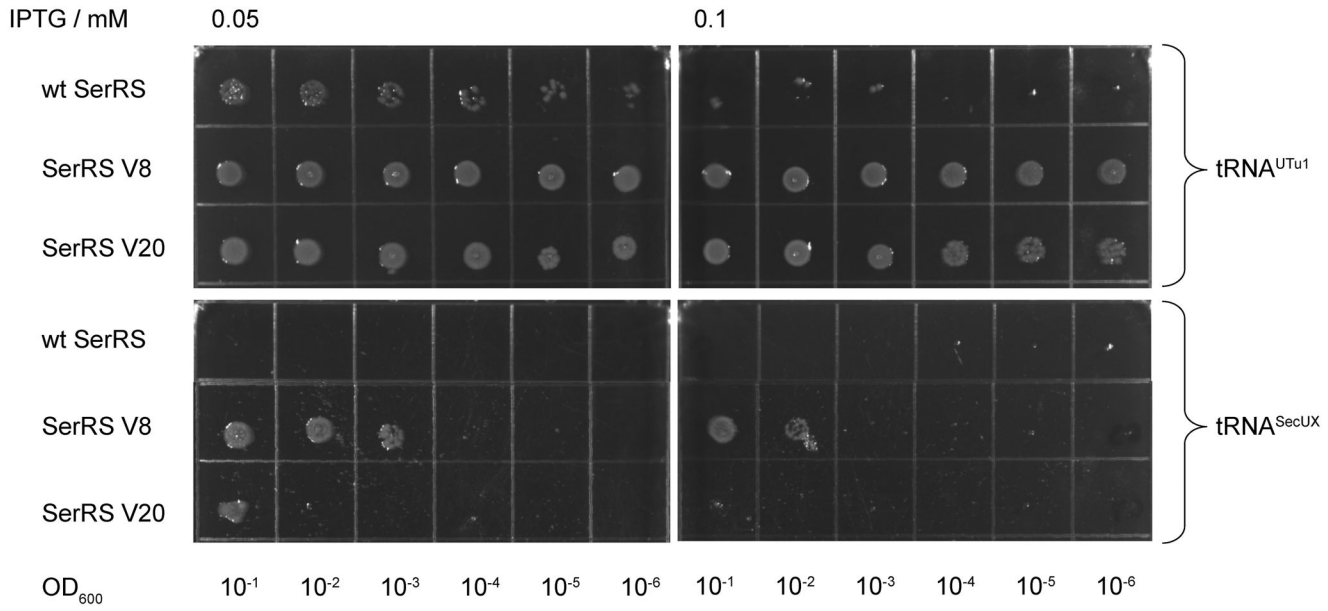

Fig. 4.

In addition to $tRNA^{UTuX}$, SerRS V8 and V20 improve $tRNA^{SecUX}$ and $tRNA^{UTu1}$-mediated TAG codon suppression. WL30153 cells expressing $tRNA^{UTu1}$ and SerRS variants were grown on LB plates with 400 mg/l Cm. The same strain expressing $tRNA^{SecUX}$ and SerRS variants was grown on LB plates with 20 mg/l Cm; both tRNAs were under the control of *lpp* promoter, and the *cat* gene with TAG at codon position 146 was on the same plasmid. SerRS expression was controlled by the *trc* promoter on the second plasmid.

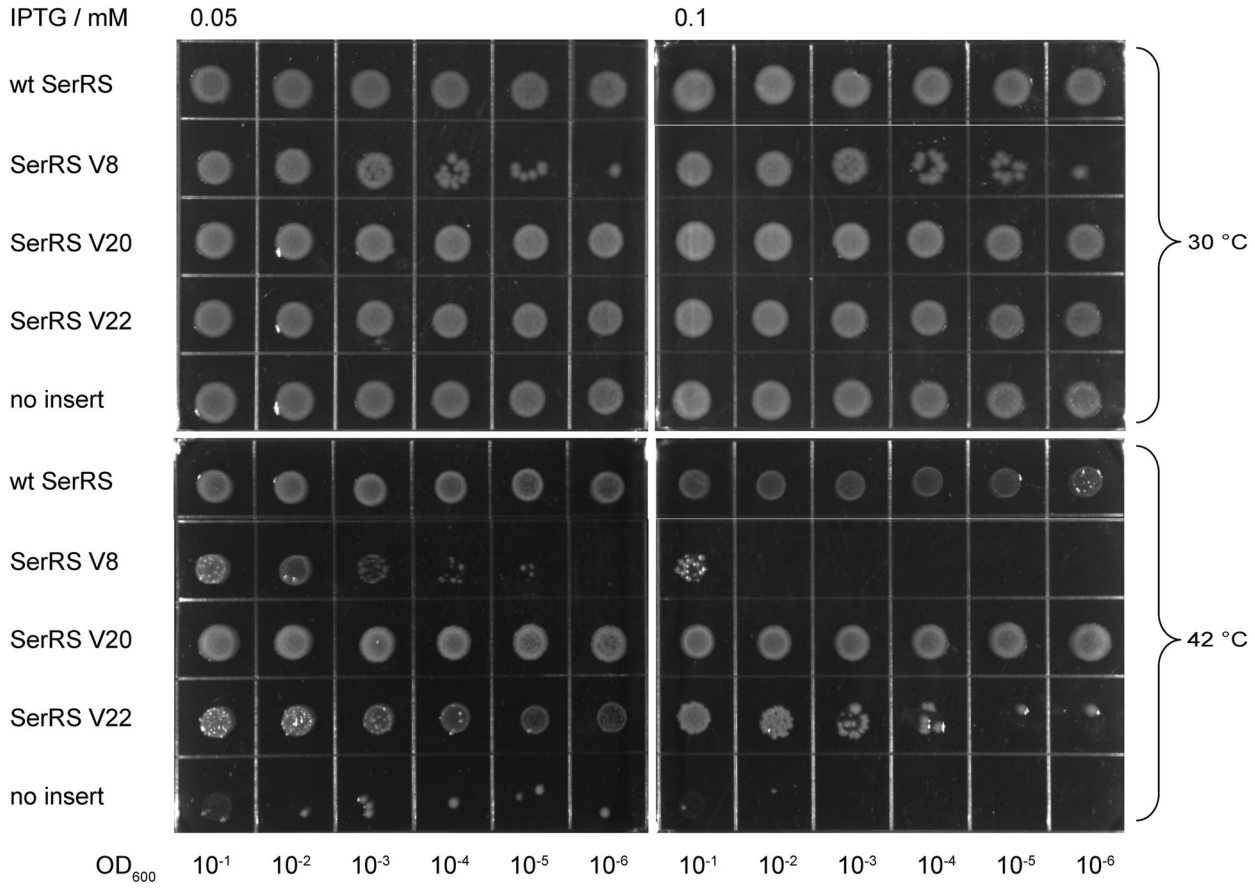

Fig. 5.

SerRS V20 rescues the thermolabile SerRS at nonpermissive temperature. Plasmids pTrc99A containing wt or SerRS variants were transformed into *E. coli* strain KL235 [1]; as a negative control, pTrc99A without an insert was also used. Plates were removed from the incubator after 20 hr at 30°C (upper panels) and 42°C (lower panels).

Table 1.

Steady-state kinetic parameters for $tRNA^{UTuX}$ for wild-type SerRS and evolved variants. The $tRNA^{UTuX}$ concentrations were corrected for plateau charging (48 %). Enzyme concentrations were: 650 nM wild-type SerRS, 187 nM SerRS V8, 92 nM SerRS V20, 224 nM SerRS V22.

enzyme	K_M (μM)	k_{cat} (min^{-1})	k_{cat} / K_M (min^{-1} μM^{-1})	relative improvement
wt	1.3	0.075	0.057	1
V8	2.4	1.4	0.58	10
V20	1.2	0.56	0.48	8.4
V22	7.2	1.5	0.21	3.6

Table 2.

Steady-state kinetic parameters for $tRNA^{SecUX}$ and $tRNA^{UTu1}$ for wild-type SerRS and variants V8 and V20. The $tRNA^{SecUX}$ and $tRNA^{UTu1}$ concentrations were corrected for plateau charging (39 % and 55 %, respectively). For $tRNA^{SecUX}$ serylation the enzyme concentrations were: 500 nM wild-type SerRS, 187 nM SerRS V8, 92 nM SerRS V20; for $tRNA^{UTu1}$ the enzyme concentrations were: 150 nM wild-type SerRS, 34 nM SerRS V8, 34 nM SerRS V20, 224 nM SerRS V22.

enzyme	K_M (μM)	k_{cat} (min^{-1})	k_{cat} / K_M (min^{-1} $μM^{-1}$)	relative improvement	tRNA
wt	3.7	0.14	0.038	1	
V8	4.9	1	0.21	5.5	$tRNA^{SecUX}$
V20	3.7	0.26	0.071	1.9	
wt	5.7	4.9	0.86	1	
V8	3.1	29	9.3	11	$tRNA^{UTu1}$
V20	2.7	13	4.9	5.7	