The Long D-stem of the Selenocysteine tRNA Provides Resilience at the Expense of Maximal Function

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The D-stem of the selenocysteine tRNA (tRNA\textsuperscript{Sec}) contains 2 additional base pairs, which replace tertiary interactions 8-14 and 15-48 universally present in all other cytosolic tRNAs. To study the role of these additional base pairs in the tRNA\textsuperscript{Sec} function, we used the instant evolution approach. In vivo screening of six combinatorial gene libraries provided 158 functional variants of the Escherichia coli tRNA\textsuperscript{Sec}. Analysis of these variants showed that the additional base pairs in the D-stem were not required for the tRNA\textsuperscript{Sec} function. Moreover, at lower temperatures, these base pairs notably harmed the tRNA\textsuperscript{Sec} activity. However, at elevated temperatures, these base pairs became essential as they made the tRNA structure more stable. The alternative way to stabilize the structure through formation of the standard tertiary interactions was not an option for tRNA\textsuperscript{Sec} variants, which suggests that the absence of these interactions and the resulting flexibility of the tertiary structure are essential for tRNA\textsuperscript{Sec} function.

Selenocysteine (Sec)\textsuperscript{2} is the 21st amino acid co-translationally inserted into the nascent polypeptide chain. The insertion takes place at the UGA codon when the latter is accompanied by a particular downstream mRNA secondary structure termed the selenocysteine insertion sequence (1). Selenocysteine is delivered to the ribosome by a special selenocysteine-tRNA (tRNA\textsuperscript{Sec}) in the ternary complex with GTP and a special elongation factor, SelB (analog of elongation factor Tu) (1). Among all cytosolic tRNAs, the tRNA\textsuperscript{Sec} is distinguished by its unusual secondary structure of which the most documented feature is its long acceptor stem (2, 3). Although in all other cytosolic tRNAs the acceptor stem universally has 7 base pairs, in the tRNA\textsuperscript{Sec}, it has either 8 (in bacteria) or 9 (in archaea and eukaryotes) base pairs. Although the presence of the additional base pairs in the acceptor stem of the tRNA\textsuperscript{Sec} has been clearly established (2, 4–6), it remains unclear how a tRNA with such an unusual feature is able to share the ribosomal sites with all other tRNAs having the invariable 7-base pair acceptor stem.

Another abnormal feature of the tRNA\textsuperscript{Sec} is the long D-stem, which has at least 6 base pairs instead of the normal 4. Although such an abnormal length of the D-stem is unprecedented among tRNAs, it is not expected to create major problems for the tRNA\textsuperscript{Sec} function as the 2 additional base pairs in the D-stem replace the universal tertiary base pairs 8-14 (Watson-Crick/Watson-Crick base pair) without changing the Watson-Crick/Hoogsteen base pair U8-A14) and 15-48 (trans-Watson-Crick/Hoogsteen base pair U8-A14) and 15-48 (trans-Watson-Crick/Watson-Crick base pair) without changing the overall shape of the molecule. However, it remains unclear how such an extension of the D-stem can be beneficial for the function of the tRNA\textsuperscript{Sec}. In the archaean and eukaryotic tRNAs\textsuperscript{Sec}, the long D-stem may be important for the phosphorylation of the seryl moiety by O-phosphoseryl-tRNA kinase, an intermediate step required for the successful delivery of the amino acid selenocysteine (7, 8). However, in bacteria, this reaction does not exist (9, 10), and yet the D-stem of all bacterial tRNA\textsuperscript{Sec} contains 6 base pairs. Thus, the particular role of the extended stem and how critical it is for the bacterial tRNA\textsuperscript{Sec} function remain unknown.

Here, based on the analysis of 158 in vivo screened functional variants of the Escherichia coli tRNA\textsuperscript{Sec}, we show that the presence of 6 base pairs in the D-stem is not a prerequisite for the tRNA\textsuperscript{Sec} function. Some tRNA\textsuperscript{Sec} variants with only 5 or 4 base pairs in the D-stem had robust activity in vivo. Surprisingly, when the activity was measured at a lower temperature, some variants with a shortened D-stem significantly outperformed the wild-type tRNA\textsuperscript{Sec} (WT). Therefore, the long D-stem is not only dispensable but can even be inhibitory for the tRNA\textsuperscript{Sec} function.

**Experimental Procedures**

**Bacterial Strains**—The E. coli strains WL81460 (Δ(argF-lac)U169 rpsL150, rpsL\textsuperscript{+} rpsE13, Δ(srl-recA)306::Tn10, Δ(selC)400::Kan) (11) with a deletion in the gene coding for the tRNA\textsuperscript{Sec} (SelC) and WL81300 (Δ(argF-lac)U169 rpsL150, rpsL\textsuperscript{+} rpsE13, Δ(srl-recA)306::Tn10, Δ(selB)300::Kan) (12) with a deletion in the gene coding for the elongation factor SelB were used in this study.
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Combinatorial Library Designs and Cloning for Instant Evolution—Oligonucleotides used for all six libraries (see Fig. 1C) and for the primers used for the library amplification were ordered from Bio-Corp Inc. (Montreal, Canada). The nucleotide sequences of all combinatorial libraries are listed in the supplemental information. Each library was PCR-amplified using the two primers 5′-CGGAATTCCGAAGATC-3′ and 5′-TTCAATGTCAATGGCTGAGTGCCGAAGATCATCACAGGAGTCCAGTGC-3′ and was cloned into plasmid pGFIB-1 using restriction sites EcoRI and PstI as described before (13).

In Vivo Screening of Active tRNA^Sec Variants—Active colonies were screened after plating the ligation reaction onto MacConkey nitrate agar plates (14) and incubation for 48 h at 37 °C under anaerobic conditions. MacConkey nitrate agar contained (per liter) 40 g of MacConkey agar base, 0.5 g of sodium formate, 10 g of potassium nitrate, and 0.5 g of sodium formate. Bright white colonies were picked and sequenced.

Preparation of Samples for the Formate Dehydrogenase H Assay—The formate dehydrogenase H (FDH-H) assay was performed as described elsewhere (15) with some modifications. Plasmids of selected clones were transformed into the E. coli bacterial strain devoid of the tRNASec (11). Due to the nature of this approach, the appearance of each single functioning variant can be considered as an isolated genetic event independent of other screened variants and of the will of the researcher. Such independence would allow us to further analyze the screened clones using statistical methods. In fact, as one can see below, the bioinformatics analysis of the obtained sequences comprised a significant part of the results.

RESULTS

General Approach—For analysis of the structure-function relationships in the E. coli tRNA^Sec, we used the approach known as instant evolution (13, 17). In our case, it consisted of the in vivo screening of active E. coli tRNA^Sec variants originating from several combinatorial gene libraries expressed in the bacterial strain devoid of the tRNA^Sec (11). Due to the nature of this approach, the appearance of each single functioning variant can be considered as an isolated genetic event independent of other screened variants and of the will of the researcher. Such independence would allow us to further analyze the screened clones using statistical methods. In fact, as one can see below, the bioinformatics analysis of the obtained sequences comprised a significant part of the results.

In total, we explored six combinatorial gene libraries (F1–F6), the designs of which are shown in Fig. 1. The designs of all libraries except F1 contained additional nucleotide positions in the acceptor stem (Fig. 1, library F1). The expression independence would allow us to further analyze the screened clones using statistical methods. In fact, as one can see below, the bioinformatics analysis of the obtained sequences comprised a significant part of the results.

For some selected variants, the level of activity was measured using the assay for another selenoprotein, FDH^-45, which is able to reduce benzyl viologen. By following the rate of the benzyl viologen reduction, we quantitated the level of FDH^-45 in the cell (15) (see “Experimental Procedures”).

In both proteins FDH^-45 and FDH^-45, whose activities were used for assessment of the performance of tRNA^Sec variants, the selenocysteine is a key amino acid of the catalytic center. We thus assume that all tRNA^Sec variants screened and measured in this research have been able to successfully pass through all intermediate steps required for the proper delivery of selenocysteine. Therefore, the activities of both selenoproteins directly reflect the functionality of the tRNA^Sec variant.

Library F1—To assess whether the 5th and 6th base pairs of the D-stem are required for the tRNA^Sec function, we designed a combinatorial library in which the 4 nucleotides composing these base pairs as well as the unpaired nucleotides between the acceptor and D-stems (nucleotides 8 and 9; Connector 1) and between the extra arm and the T-stem (nucleotide 48; Connector 2) were fully randomized (Fig. 1, library F1). The expression of this library provided 63 variants different from WT (supplemental Table S1). The number of the screened colonies exceeded the number of unique nucleotide sequences generated by this library (4^7 = 16,384).

For some tRNA^Sec variants from library F1, their steady state levels inside the cell were verified by Northern blot (Fig. 2). All tested variants showed about the same presence in the cell and about the same level of aminoacylation as WT (Fig. 2). The function of all tested variants required SeB as none of them...
provided white colonies when expressed in the bacterial strain WL81300 lacking SelB (data not shown). Analysis of the selected F1 variants showed that the 5th and 6th base pairs were Watson-Crick (WC) in 28 (44%) and 14 (22%) variants, respectively. Only in three cases (5%) were the 5th and 6th base pairs simultaneously WC. The WC identities of the 5th and 6th base pairs were shown in the WT tRNA^Sec* and variants F1-24, F1-54, and F1-62 at 30 °C (lanes 3–10) and 37 °C (lanes 11–18). The negative control containing the void vector is shown in lanes 1 and 2. Northern blot hybridization was performed using a specific DNA probe complementary to the extra arm of the tRNA^Sec*. For each variant checked, the presence in the cell at 30 and 37 °C is comparable. The samples in the even lanes were deacylated by incubation with Tris, pH 9.0, whereas the samples in the odd lanes were not. The fact that the deacylated form migrated more slowly than the deacylated form allowed evaluation of the level of aminoacylation. Each variant existed in the cell predominantly in the aminoacylated form. An additional probe specific to 5 S rRNA was used to monitor the amount of total RNA in the samples (indicated by arrow A). For each sample, the identity of the 5th base pair of the D-stem (base pair 14-21) is provided. The upper part of the figure showing the 5 S rRNA was taken from a short exposure of the Northern blot, whereas the lower part showing tRNA^Sec* variants was taken from a longer exposure (the original blots with the short and long exposure are shown in supplemental Fig. S1). The relative amounts of tRNA are shown for each variant as a percentage of the WT tRNA^Sec* at 30 °C (indicated by arrow C; lane 4), which was taken to be 100%. The aminoacylation levels for each variant was calculated by dividing the aminoacylated band density (indicated by arrow B; odd lanes) by the total tRNA density (indicated by arrow C; even lanes). All density calculations were normalized to the amount of 5 S rRNA (indicated by arrow A).
pairs varied between the clones; in different F1 clones, the 5th base pair had all WC identities except G14-C21, whereas the 6th base pair had all four possible identities.

If the GU dinucleotide combination was included in the definition of a base pair, the 5th and 6th base pairs would then be formed in 34 (54%) and 20 (32%) clones, respectively. Even with this expanded definition, only 11 F1 variants (17%) contained both base pairs, whereas 20 variants (32%) had none of them. Although in most variants 1 or 2 base pairs were missing in the D-stem, there was no indication for a compensatory formation of the standard tertiary interactions 8-14 or 15-48. The identities of the nucleotides composing the connector regions varied randomly without any obvious relationship to the identities of the corresponding base pairs.

In the selected F1 clones, the 5th base pair was formed notably more often than the 6th base pair. Moreover, a co-variation analysis showed that the number of clones in which the 5th base pair is WC (28 clones) is substantially higher than the number expected if both nucleotide positions varied independently (18.4 clones; supplemental Table S7) all details are provided in the supplemental Extended Experimental Procedure). The same analysis performed for the 6th base pair showed that the number of clones in which this base pair is WC (14 clones) is only marginally higher than one would expect if both nucleotide positions varied independently (12.9 clones). Therefore, the formation of the 5th base pair seemed to be more important for tRNA_ASP function than the formation of the 6th base pair.

To determine whether the existence of 1 base pair could influence the formation of the other base pair, we divided all F1 clones into two groups: those variants with (group 1) and without (group 2) a WC dinucleotide combination at the 5th base pair. Analysis showed that the chance of having the 6th base pair WC was notably higher in group 2 than in group 1 (33 versus 17%). In other words, the probability for formation of the 6th base pair became twice as high in the absence of the 5th base pair compared with the situation when it existed.

In a similar way, when the 6th base pair did not form, the probability for the 5th base pair to be formed was higher than in the clones having the 6th base pair (51 versus 40%). Comparable results were obtained when the definition for a base pair was expanded to include the GU dinucleotide combination. These findings suggest the existence of a compensatory cross-talk between the 5th and 6th base pairs; if one of them does not exist, its absence has a tendency to be compensated by the presence of the other base pair. The existence of such a cross-talk is more evident when the 5th base pair is unpaired compared with the alternative situation when the 6th base pair is unpaired.

**Libraries F2 and F3**—We next modified the design of the F1 library by extending both connector regions by 1 nucleotides 9a and 48a; library F2; Fig. 1, B and C) and 2 (nucleotides 9a and 9b and nucleotides 48a and 48b; library F3) randomized nucleotide positions. Library F2 resulted in the screening of 33 functional variants shown in supplemental Table S2. We noticed that during the screening of library F2 the frequency of appearance of positive clones was substantially lower than during the screening of library F1. Compared with F1 clones, the proportion of F2 clones having base pairs in both the 5th and 6th base pairs of the D-stem became notably higher. In particular, among all 33 F2 clones, the 5th and the 6th base pairs were WC in 30 (91%) and 23 (70%) clones, respectively. If the GU combination is also considered as a base pair, the corresponding numbers would increase to 31 (94%) and 26 (79%) clones, respectively (Fig. 3). Again, as observed among F1 clones, the 5th base pair in F2 clones occurred more frequently than the 6th base pair, whereas the nucleotide sequences of the connector regions varied randomly without any notable relation to the nucleotides corresponding to the 5th and 6th base pairs.

The increased tendency for the 5th and 6th base pairs to be formed when the connector regions were extended was even more pronounced among the seven selected F3 variants as all of them had a WC or GU base pair in the 5th layer, whereas all variants except one had a WC or GU base pair in the 6th layer (Fig. 3 and supplemental Table S3). Again, the frequency of appearance of positive F3 clones was substantially lower than that of positive F2 clones.

**Libraries F4, F5, and F6**—As discussed above, analysis of the nucleotide sequences of the obtained F1 clones suggested the existence of a cross-talk between the 5th and 6th base pairs. When either the 5th or 6th base pair did not form, the other base pair had a greater tendency to form as WC or GU. To further verify the existence of such relationship, we designed three more combinatorial libraries, libraries F4–F6. In each of these libraries, we took the design of library F2 as a prototype into which we forcefully introduced a purine-purine or pyrimidine-pyrimidine mismatch at the place of either the 5th or 6th base pair of the D-stem (Fig. 1C). Analysis of the screened variants showed that, compared with F2 clones, the clones screened from libraries F4–F6 were characterized by a very limited set of dinucleotide combinations in the other base pair, whose nucleotide identities were not limited during the experiment. Thus, the introduction of a pyrimidine-pyrimidine combination at the 5th base pair (library F5; supplemental Table S4) made the 6th base pair predominantly C15-G20. The exceptions happened in seven of the 27 collected variants and consisted of base pairs with the D-stem...
pairs U15-A20 (one clone), G15-C20 (three clones), and mismatch G15-G20 (three clones). Also, the introduction of a purine-purine combination at the same place (library F4; supplemental Table S5) made the 5th and 6th base pairs predominantly A14-G21 and C15-G20 with exceptions happening in three of the 11 obtained clones. Finally, the introduction of a pyrimidine-pyrimidine combination at the 6th base pair (library F6; supplemental Table S6) made the 5th base pair almost exclusively C14-G21 or A14-G21, and exceptions happened only in two of 17 obtained variants. These data additionally support the existence of a cross-talk between the 5th and 6th base pairs of the D-stem so that a particular type of mismatch in 1 base pair strongly limits the set of acceptable dinucleotide combinations in the other base pair.

The Extended D-stem as a Factor for the tRNA\textsubscript{Sec} Stability—
The results obtained so far clearly demonstrate that although the 5th and 6th WC base pairs are uniquely present in the tRNA\textsubscript{Sec} neither of them plays a specific role in the tRNA\textsubscript{Sec} function. In different variants of the tRNA\textsubscript{Sec}, each of the 2 base pairs assumes all possible WC identities, AU, UA, GC, and CG. Moreover, both base pairs can have extended identities, GU and UG, or contain mismatches. The absence of distinct requirements imposed on the structure of these base pairs makes improbable their involvement in specific interactions with factors assisting the tRNA\textsubscript{Sec} functioning.

Although neither the 5th nor the 6th base pair in the D-stem was required for the tRNA\textsubscript{Sec} function, both of them played a positive role: the presence of the 5th base pair strongly increased the chance for a tRNA\textsubscript{Sec} variant to be selected in our procedure, and in the absence of the 5th base pair, a similar effect was observed for the 6th base pair. Also, additional perturbing elements introduced into the nucleotide sequence of the tRNA\textsubscript{Sec} strongly favored the selection of variants containing these base pairs. Such perturbing elements included 1 (library F2) or 2 (library F3) extra nucleotides in the connector regions as well as a mismatch at the place of either the 5th (libraries F4 and F5) or 6th base pair (library F6) of the D-stem. Introduction of each of these elements sharply increased the chance for selection of clones with a D-stem containing more than 4 base pairs. The results of the screening of libraries F4–F6 also confirm the initial observation made based on analysis of the F1 library that the 2 base pairs are involved in a cross-talk between themselves. The absence of either the 5th or the 6th base pair notably increased the chance for the other base pair to be formed.

Although the designs of libraries F2–F6 were different, they all shared the same feature consisting of creation of obstacles for formation of the normal secondary structure. The obstacles consisted of the extension of the connector regions and/or the introduction of mismatches into the D-stem. The introduction of mismatches into the D-stem would destabilize the standard secondary structure, whereas the extension of the connector regions by randomized nucleotides would increase the probability for alternative secondary structures. This general conclusion was also checked by folding simulations using the program Mfold (18). These simulations demonstrated that indeed both the extension of the connector regions and the introduction of mismatches into the D-stem notably decreased the probability for formation of the proper cloverleaf secondary structure (not shown). Therefore, the observed elevated level of base pairings at the end of the D-stem compared with that observed among F1 clones (Fig. 3) should be seen as a compensatory effect able to restore the stability of the variants. This in turn leads us to the general conclusion that the major role of the additional 5th and 6th base pairs in the D-stem of the tRNA\textsubscript{Sec} consists in a non-specific stabilization of the D-stem and through this of the whole tRNA\textsubscript{Sec} secondary structure.

Thermal Stability of tRNA\textsubscript{Sec} Variants—
To further verify the stabilizing role played by the additional base pairs in the D-stem, we measured activities for some of the variants screened from library F1 that represented the spectrum of base pairing strength found within the D-stem. In total, we studied 12 molecules, including WT. Among them, the 5th base pair had identities UA (WT and three variants) as well as CG, UG, and AG (two variants for each combination). The identities of the 6th base pair were CG (WT and one variant), UA (one variant), AU (two variants), AA (five variants), UU (one variant), and GA (one variant). For all variants analyzed, the activities were measured at 30 and at 37 °C (Table 1), whereas for WT and for variant F1-24 (in F1-24, the 5th and the 6th base pairs were CA and UA, respectively), the activities were also measured at 33 and 42 °C (Fig. 4).

As anticipated, at 37 °C, WT was the most active among all measured variants. Interestingly, when measured at 30 °C, WT performed better than at 37 °C. This fact, however, was expected and essentially repeated the result of a previous study (15). All other variants followed the same pattern, having a higher activity at 30 °C than at 37 °C. Unexpectedly, a few variants performed at 30 °C significantly better than WT. In particular, clone F1-24 at 30 °C demonstrated an activity 2-fold (243%) greater than WT. Three more variants, F1-4, F1-51, and F1-11, had activities that ranged from 158 to 184% of WT. When the temperature was raised from 30 to 37 °C, all these clones experienced a substantial drop in activity to become no better than other clones. The same tendency continued to 42 °C with a further substantial drop in activity as shown for clone F1-24 (Fig. 4). For the other clones that did not have such robust

### Table 1

| Clones | 30°C (n=9) | Relative Activity (A) | 37°C (n=9) | Relative Activity (B) | 5th Position (n=11) | 6th Position (n=13) | 1°C (n=8) | 2°C (n=11) |
|--------|------------|-----------------------|------------|-----------------------|---------------------|---------------------|-----------|-----------|
| FI-50  | 35%        | 31%                   | 1.1        | FI-50                 | G                   | C                   | A         | A         |
| FI-18  | 36%        | 23%                   | 1.5        | FI-18                 | A                   | G                   | C         | C         |
| FI-62  | 76%        | 38%                   | 2.0        | FI-62                 | C                   | A                   | A         | A         |
| FI-31  | 86%        | 53%                   | 1.6        | FI-31                 | A                   | A                   | A         | A         |
| WT     | 100%       | 66%                   | 1.5        | WT                    | C                   | G                   | C         | C         |
| FI-21  | 102%       | 49%                   | 2.1        | FI-21                 | A                   | G                   | C         | C         |
| FI-28  | 101%       | 21%                   | 4.9        | FI-28                 | G                   | A                   | A         | A         |
| FI-54  | 114%       | 31%                   | 3.6        | FI-54                 | U                   | G                   | U         | U         |
| FI-4   | 158%       | 40%                   | 4.0        | FI-4                  | A                   | G                   | C         | C         |
| FI-51  | 177%       | 36%                   | 4.9        | FI-51                 | A                   | G                   | A         | A         |
| FI-11  | 184%       | 44%                   | 4.2        | FI-11                 | A                   | C                   | A         | A         |
| FI-24  | 243%       | 55%                   | 4.4        | FI-24                 | A                   | A                   | U         | G         |

Relative FDH\textsubscript{a} activities of selected variants performed at 30 and 37 °C

The activities of selected variants performed at 30 °C (parameter A) and 37 °C are shown with the WT activity at 30 °C being 100%. Parameter B is the ratio between the activity at 30 °C divided by the activity of 37 °C. The corresponding nucleotide identities for the 5th base pair, 6th base pair, first connector, and second connector are shown. In the 5th base pair column, variants are boxed on the base pairing combinations that form either WC, GU, or non-WC base pairs. nt(s), nucleotide(s).
activity at 30 °C, the drop in the activity associated with the increase in temperature was substantially more modest than for the first mentioned clones F1-24, F1-4, F1-51, and F1-11.

The difference in the behavior of the measured clones did not relate to their presence in the cytosol. At 30 °C, clone F1-24 performed better than WT, although its level in the cytosol was no higher than that of the latter (Fig. 2, compare lanes 5 and 6 with lanes 7 and 8). Conversely, at 37 °C, WT performed better than F1-24 despite having an equal or even slightly lower presence in the cell (Fig. 2, compare lanes 11 and 12 with lanes 13 and 14). This observation allows us to make a general suggestion that the differences in the activities of the measured clones are somehow linked to particular features of their structure.

To reveal such linkage, we assigned for each variant two parameters. The first parameter (parameter A; Table 1) represented the activity of the variant at 30 °C normalized by the corresponding activity of WT. Given that all clones demonstrated the highest activity at 30 °C, parameter A would thus represent the maximal activity measured for each clone. The second parameter (parameter B; Table 1) was calculated as the ratio between the activities at 30 and 37 °C and thus represented the loss in activity caused by the increase in temperature.

On the plot of B versus A (Fig. 5), all measured clones form three distinct groups. Group 1 is composed of six clones, including WT, that had a low to average activity at 30 °C and a small decline in activity when temperature was elevated (low values of both A and B). Group 2 consists of the four above mentioned clones, F1-24, F1-4, F1-51, and F1-11, and is characterized by a high maximal activity and a significant decrease in activity caused by the increase in the temperature (high values of both A and B). Finally, group 3 included two clones, F1-28 and F1-54, with a low value of A and a high value of B. In other words, clones F1-28 and F1-54 had at 30 °C activities similar to that of WT but that dropped as sharply as in clones of group 2 when the temperature was raised from 30 to 37 °C.

Analysis of the nucleotide sequences of the measured clones revealed a clear link between the position of a clone on the A/B plot and the identity of the 5th base pair in the D-stem. In all clones of group 1, this base pair was WC, being either UA (three clones and WT) or CG (two clones). In group 2, this base pair does not exist, being replaced by either the AG (two clones) or CA (two clones) combination. Finally, in both variants of group 3, the 5th base pair is UG. Although each variant has 5 more variable nucleotides, which in principle could affect their level of activity, we do not think that the role of these nucleotides is essential. Between the clones, the identities of these 5 nucleotides vary widely and without any visible relation to the function of the clones. This makes the identity of the 5th base pair of the D-stem the major if not the only factor that determines the differences in the functional behavior of the measured clones.

How exactly do the identities in the 5th base pair of the D-stem determine the functionality of the tRNA^Sec variants? The presence of a WC base pair at this position (group 1) is linked to a relatively low level of activity loss when the temperature increases from 30 to 37 °C. In other words, if the 5th base pair is WC, then it provides the tRNA with a higher resistance to heat (a low value of parameter B in group 1). Conversely, the absence of a WC base pair is associated with a substantial decline in activity when the temperature was increased from 30 to 37 °C or with a lower resistance to heat (high value of B in groups 2 and 3). Therefore, a WC base pair in the 5th position of the D-stem appears to be the major factor for the tRNA stability when the temperature rose from 30 to 37 °C.

Surprisingly, at 30 °C, the presence of either a WC or UG base pair in the 5th position of the D-stem, despite its stabilizing effect, makes a tRNA^Sec variant substantially less active (low values of A in groups 1 and 3; Fig. 5) compared with the variants that do not have such a base pair (group 2; Fig. 5). This means that at 30 °C the additional stabilization of the tRNA structure caused by the presence of a WC or UG base pair is harmful for the tRNA^Sec function.

Thus, at lower temperatures, a higher activity of the tRNA^Sec is associated with the additional conformational flexibility in the D-stem that can be achieved through the absence of the 5th base pair. At higher temperatures, the stability of the tRNA^Sec...
becomes critical, thus requiring that this base pair be formed. The UG base pair behaves in a peculiar way. At 30 °C, it behaves like a WC base pair, making the tRNA less effective (low level of parameter A). However, when the temperature is elevated, the UG base pair behaves as if it is inexistent (high level of parameter B). Such a dual behavior is consistent with the known mediocrе stability of the UG base pair.

**DISCUSSION**

In this study, we analyzed the role of the extended D-stem in the function of the *E. coli* tRNA sec. For this purpose, we used the instant evolution approach consisting of *in vivo* screening of a large number of functional variants originating from several combinatorial gene libraries. By focusing on positive variants, this approach is able to overcome the general problem usually associated with *in vivo* studies of attributing a poor functioning of a given variant to the particular aspect of the synthesis, maturation, or functioning of a molecule. As we screened only highly efficient clones, all of them should have had no major problems at any of these steps. Also, due to the inherent combinatorial nature of the approach, it allowed further statistical analysis of the nucleotide sequences of the screened clones.

The results presented in this study clearly show that the 5th and 6th base pairs of the D-stem that are uniquely present in the tRNA sec do not play any specific functional role and are not required for formation of any essential interaction of this molecule with other elements of the Sec-incorporating machinery in *E. coli*. A tRNA can be functional even if neither of the 2 base pairs is formed. Moreover, the formation of the 5th base pair as WC or UG can even hamper the functionality of the tRNA sec as was demonstrated by the lower activity at 30 °C of the variants containing these base pairs compared with variants having a shorter D-stem. Only at higher temperatures did the presence of this base pair become essential for the tRNA sec function.

The presented data allow us to conclude that the major role of the additional base pairs in the D-stem of the tRNA sec consists in providing for sufficient stability of the tRNA structure. This conclusion is based on three sets of experiments. First, analysis of the screened variants of the tRNA sec revealed the existence of a cross-talk between the 5th and 6th base pairs in the D-stem so that the absence of one of base pairs has a tendency to be compensated by the presence of the other base pair. Second, each time when the design of a combinatorial library contained additional obstacles for formation of the proper secondary structure, the percentage of the screened variants having these base pairs notably increased. Finally, the measurement of the activity of several tRNA sec variants demonstrated that the 5th base pair in the D-stem is required only at an elevated temperature, whereas at a lower temperature its formation can be harmful for the tRNA sec function. A reasonable question would concern the reasons of why the stability of the tRNA sec requires the presence of the base pairs that do not exist in any other tRNA. We think that the requirement for these base pairs relates to the fact that although all other tRNAs contain universal tertiary interactions 8-14 and 15-48 in the tRNA sec these interactions do not exist. The formation of the additional base pairs in the D-stem of the tRNA sec should thus be considered as a compensatory measure that would stabilize the tRNA structure in the absence of the standard tertiary interactions.

If the major aspect limiting functionality of tRNA sec variants pertains to their stability, can the latter be restored through formation of the normal tertiary interactions existing in other tRNAs? Inspection of the selected clones shows that of the 63 F1 clones only in three, F1-14, F1-36, and F1-1, do the identities of nucleotides 8, 14, 15, and 48 allow the formation of the 2 canonical tertiary base pairs 8-14 and 15-48. However, in two clones, F1-14 and F1-1, the formation of base pair U8-A14 has to compete with the 5th base pair of the D-stem, A14-U21. Whether in these exceptional cases the tertiary interactions 8-14 and 15-48 indeed form is unknown. It is clear, however, that of the two options of structural stabilization, either through extension of the D-stem or through formation of tertiary interactions 8-14 and 15-48, F1 clones demonstrate a strong preference toward the first option. Such preference is even more pronounced among clones selected from libraries F2 and F3. In the latter clones, the stabilization of the tRNA sec structure always proceeds through the extension of the D-stem, whereas the identities of the nucleotides of the connector regions do not provide even a theoretical opportunity for formation of the tertiary base pairs.

Based on the fact that between the two options for stabilization of the tRNA structure either through restoration of the long D-stem or through formation of tertiary interactions 8-14 and 15-48 the selected variants systematically choose the first option, we suggest that the tertiary interactions are harmful for the tRNA sec function. The absence of these interactions makes the tRNA sec conformation more flexible compared with that of other cytosolic tRNAs. The existence of such flexibility has already been noticed when the available x-ray conformations of the tRNA sec were compared with each other (5, 8).

The flexibility provided by the absence of standard tertiary interactions seems to be essential for the tRNA sec function. Moreover, the efficiency of WT can even be improved if one introduced additional flexibility to its structure by disrupting the last base pairs of the D-stem. Such strategy, however, works only at low temperatures when the modified tRNA is still able to maintain its secondary structure. At higher temperatures, when the stability of the whole tRNA sec structure diminishes, the additional base pairs in the D-stem become essential. This result shows that as long as the integrity of the tRNA sec secondary structure is not compromised a higher conformational flexibility would correspond to a higher selenocysteine-incorporating activity. For the *E. coli* tRNA sec, which normally functions at 37 °C, the formation of the 5th and 6th base pairs in the D-stem seems to be the best compromise between flexibility and integrity.

The presented data strongly suggest that the observed conformational flexibility of the tRNA sec is required for some unique aspects of the tRNA sec function that this molecule does not share with other tRNAs. In particular, it may be linked to another unusual feature of the tRNA sec, the long acceptor stem. In the normal tRNAs, the standard tertiary interactions 8-14 and 15-48 result in the acceptor/T helical domain being rigidly attached to the D/anticodon domain. If the tRNA sec contained the standard tertiary interactions and thus was as rigid as other...
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tRNAs, then the unusually long acceptor stem would have juxtaposed the CCA terminus and the anticodon loop differently compared with other tRNAs. This in turn would create problems for the proper accommodation of the tRNA\textsuperscript{Sec} at the standard ribosomal sites. However, due to the absence of the standard tertiary interactions in the D stem-loop, the juxtaposition of the two functional centers of the tRNA becomes rather flexible. Thus, such flexibility would be helpful for the simultaneous accommodation of the anticodon loop and of the CCA terminus at the proper places on the ribosome surface.

At the same time, the flexible characteristic of the tRNA\textsuperscript{Sec} may also be related to its unusual functional pattern consisting in the theft of the UGA stop codon in response to the selenocysteine insertion sequence (19), the downstream secondary structure element essential for selenocysteine incorporation. How exactly the absence of the tertiary interactions in the tRNA\textsuperscript{Sec} relates to its function will require further analysis.

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