Recognition of pyrrolysine tRNA by the Desulfitobacterium hafniense pyrrolysyl-tRNA synthetase

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ABSTRACT

Pyrrolysine (Pyl), the 22nd co-translationally inserted amino acid, is incorporated in response to a UAG amber stop codon. Pyrrolysyl-tRNA synthetase (PylRS) attaches Pyl to its cognate tRNA, the special amber suppressor tRNAPyl. The genes for tRNAPyl (pylT) and PylRS (pylS) are found in all members of the archaeal family Methanosarcinaceae, and in Desulfitobacterium hafniense. The activation and aminoacylation properties of D. hafniense PylRS and the nature of the tRNAPyl identity elements were determined by measuring the ability of 24 mutant tRNA Pyl species to be aminoacylated with the pyrrolysine analog N-e-cyclopentyloxycarbonyl-L-lysine. The discriminator base G73 and the first base pair (G1/C15/C72) in the acceptor stem were found to be major identity elements. Footprinting analysis showed that PylRS binds tRNA Pyl predominantly along the phosphate backbone of the T-loop, the D-stem and the acceptor stem. Significant contacts with the anticodon arm were not observed. The tRNA Pyl structure contains the highly conserved T-loop contact U54/C55A56 and position 57 is conserved as a purine, but the canonical T- to D-loop contact between positions 18 and 56 was not present. Unlike most tRNAs, the tRNA Pyl anticodon was shown not to be important for recognition by bacterial PylRS.

INTRODUCTION

Every known member of the archaeal family Methanosarcinaceae is able to utilize methylamines as an energy source, a virtually exclusive feature of this family. Crucial to this process are the methylamine methyltransferase enzymes, the successful biosynthesis of which requires suppression of an in-frame UAG codon (1,2). The amino acid incorporated at the UAG position is the 22nd genetically encoded amino acid pyrrolysine (Pyl) (3). Pyl is charged specifically onto the amber suppressor tRNA by the pyrrolysyl-tRNA synthetase (PylRS) (4–6). PylRS and SepRS are the only two examples to date of aminoacyl-tRNA synthetases specific for a modified amino acid. The genes for tRNA Pyl (pylT) and PylRS (pylS) are found in every known member of the archaeal family Methanosarcinaceae and in the bacterium Desulfitobacterium hafniense (5). As D. hafniense also encodes the machinery for selenocysteine incorporation, it is the only known organism that potentially uses all 22 amino acids in protein translation. While the archaeal pylT and pylS genes are highly conserved throughout the members of the Methanosarcinaceae family, the bacterial genes differ more markedly from their archaeal counterparts. The archaeal tRNA Pyl sequences are highly conserved with only 7 nucleotide changes among the 6 known isoacceptors, while the D. hafniense tRNAPyl has 23 unique residues relative to the archaeal consensus. In addition, in both the archaea and bacteria, the amber suppressor tRNAPyl has several unique structural features when compared to canonical tRNAs. These include (a) an anticodon stem of 6 base pairs instead of 5, (b) a short variable loop of only 3 bases, (c) a single base separating the acceptor and D stems, rather than 2, (d) a small D-loop with only 5 bases, (e) the absence of the almost universally conserved G18G19 sequence in the D-loop and T54C55C56 sequence in the T-Loop and (f) only two modified bases have been proposed in Methanosarcina. barkeri Fusaro tRNA Pyl: 4-thiouridine at position 8 and 1-methyl-pseudouridine at position 54 (4).

The archaeal PylRS enzymes have ~55–75% amino acid identity, while the D. hafniense PylRS shares
Purification of the vector (Novagen). The overexpression protocol was performed (DE3)-RIL (Stratagene) was transformed with the result of PylRS in D. hafniense may be a product of horizontal gene transfer from an archaeal progenitor (7).

The D. hafniense PylRS also has an approximately 100 amino acid amino-terminal truncation when compared to the archael PylRS. The absence of these 100 amino acids brings into question whether D. hafniense PylRS can charge tRNA Pyl. In this study we investigate whether the D. hafniense PylRS is able to aminocylate tRNA Pyl in vitro. Furthermore, as Pyl likely plays an essential role in methyamine methyltransferase activity, the accurate formation of Pyl-tRNA Pyl is crucial. Thus, we embarked on determining the molecular recognition of tRNA Pyl by PylRS in D. hafniense.

MATERIALS AND METHODS

General

Oligonucleotides synthesis and DNA sequencing were performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University. Uniformly labeled sodium [32P]pyrophosphate [1–60 Ci/mmol (1 Ci = 37 GBq)], and [z-32P]ATP (10 mCi/ml) was from Amersham Biosciences. N-z-cyclopentanyloxycarbonyl-lysine (Cyc) was purchased from Sigma.

Purification of D. hafniense PylRS

The D. hafniense pylS gene was cloned into the pET15b vector (Novagen). Escherichia coli BL21-CodonPlus (DE3)-RIL (Stratagene) was transformed with the resulting construct. The overexpression protocol was performed as described (8). Briefly, cells were grown overnight at 37°C until a density of A600 = 6.0 was reached in Luria Broth supplemented with 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, 50 × 52 buffer (0.5% glycerol, 0.05% glucose, 0.2% z-lactose), NPS (50 mM Na2HPO4, 50 mM KH2PO4 and 25 mM (NH4)2SO4) and 1 mM MgSO4. PylRS was purified by Ni-NTA technology according to the manufacturer’s protocols (Qiagen).

Cloning and in vitro transcription of the tRNA Pyl substrates

Desulfotubacterium hafniense wild type and mutant tRNA Pyl were prepared as previously reported for the M. barkeri Fusaro tRNA Pyl (9). tRNA genes for in vitro T7 RNA polymerase transcription were cloned by annealing two DNA oligonucleotides and direct ligation to the pUC18 plasmid using BamHI and HindIII restriction sites. The E. coli DH5α was transformed with the pYT containing plasmids. The 3'-end of the transcription template was generated by digestion overnight with BstNI at 60°C. In vitro T7 RNA polymerase run off transcription was conducted according to standard procedures (10). tRNA Pyl transcripts were gel purified, then 3'-end-labeled with [z-32P]ATP using the E. coli CCA-adding enzyme (11,12). 3'-labeled tRNAs were gel purified, and the concentration determined by measuring A260.

ATP-[32P]PPi exchange

The assay was used to measure PylRS activation of amino acid substrates, and performed as previously described (13). Reactions (200 µl final volume) were performed at 37°C in 100 mM Na-HEPES, pH 7.2, 10 mM MgCl2, 50 mM KCl, 5 mM DTT, 2 mM KF, 2 mM ATP, 2 mM [z-32P]PPi (1.6 cpm/pmol), 0.5 µM PylRS enzyme and either 1 mM lysine or 10 mM Cyc. [32P]ATP formation was followed by specific absorption on acid-washed Norit [0.2 ml of a 1% suspension (wt/vol) of Norit in a solution of 0.4 M sodium pyrophosphate solution containing 15% (vol/vol) perchloric acid), filtration with Whatman GF/C fiber glass filter disks (Millipore), washed with 15 ml ethanol and 2 times 15 ml water, dried and liquid-scintillation-counted (Beckman Coulter).

Aminoacylation and determination of kinetics parameters

Aminoacylation assays were adapted from a recently described procedure (11). Aminoacylation reactions (10 µl) were carried out at 37°C in 100 mM Na-Hepes, pH 7.2, 25 mM MgCl2, 60 mM NaCl, 5 mM ATP, 1 mM DTT, 10 mM Cyc (14) and 3'-[z-32P]ATP-labeled tRNA ranging in concentration from 0.25- to 5-fold Km. PylRS concentrations ranged from 5 to 90 nM. Reactions were stopped by removing 1 µl reaction mix, adding it to 3 µl of 2.5 units/µl of nuclease P1 (American Bioanalytical) in 0.1 M sodium citrate (pH 4.5) at 25°C for 30 min, thus releasing [z-32P]AMP from uncharged tRNA and Cyc-[z-32P]AMP from charged tRNA. [z-32P]AMP and Cyc-[z-32P]AMP were separated by thin layer chromatography (TLC) on PEI cellulose plates (Baker) in 0.1 M sodium acetate and 5% acetic acid (15). [z-32P]AMP and Cyc-[z-32P]AMP were exposed to phosphoimager plates (FujiFilms), visualized using a Molecular Dynamics Storm 860 scanner (Amersham) and quantified using ImagQuan® software. Kinetic parameters were calculated using nonlinear regression plots of the initial velocity (v) and represent the results of at least three independent experiments.

tRNA Pyl footprinting with SI and T1 nucleases

Partial digestions of D. hafniense tRNA Pyl 3'-labeled transcripts with nuclease S1 and T1 were carried out in buffer containing 100 mM NaCl, 50 mM Na-Hepes, pH 7.2, 20 mM MgCl2 and 0.5 µg/µl cold yeast RNA (Ambion) (with 1 mM ZnCl2 for RNase S1 digest only). Each reaction (10 µl) contained 0.25 µM 3' labeled tRNA Pyl (~100 000 Cerenkov counts), and either 50 U of RNase S1 or 1 U of RNase T1 in the absence or presence of 26 µM D. hafniense PylRS. Reactions were preincubated on ice for 10 min, followed by the addition of RNase and 5-min digestion at 25°C. Reactions were stopped by phenol and chloroform extraction and ethanol precipitation. Samples were washed with 70% ethanol, dried, re-suspended in gel loading buffer (95% formamide, 10 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol), then quantified using a LS 6500 multi-purpose

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liquid scintillation counter (Beckman Coulter). An equal number of cpm's per sample were analyzed by electrophoresis on an 8% denaturing polyacrylamide gel. Gels were exposed to a phosphoimaging plate (FujiFilms) for 12 h, visualized using a Molecular Dynamics Software.

**tRNA footprinting with phosphorothioate incorporated tRNA**

Phosphorothioates were randomly incorporated into the tRNA by inclusion of either 0.05 mM CTP [sS], GTP [sS], ATP [sS] or UTP [sS] (Sp isomers, Amersham & Invitrogen) as described previously (16,17). After transcription, tRNAs were gel purified, and 3' labeled as described above. tRNAs were co-incubated with PyrRS enzyme as described above for the RNase footprinting reactions, only in the absence of any DTT. Digestions were performed by adding 1 µl of 10 mM iodine to the binding reaction for 60 s on ice. Iodine cleavage was stopped by adding 20 µl of stop buffer (100 mM DTT and 1 mg/ml yeast RNA (Ambion), followed by ethanol precipitation. Digests were analyzed by gel electrophoresis as described previously for the RNase footprinting.

**RESULTS**

*D. hafniense* PyrRS aminoacylates tRNA<sup>Pyl</sup>

Pyrrolysine is not a commercially available substrate, and its synthesis has proven both difficult and expensive (4,14,18). A pyrrolysine analog, N-e-cyclopentylxycarbonyl-L-lysine (Cyc), was recently shown to be a reasonable substrate for *M. barkeri* PylRS; Cyc can be aminoacylated onto tRNAPyl both *in vitro* and *in vivo* (14).

The ability of Cyc to promote the *D. hafniense* PylRS catalyzed ATP-[32P]PP exchange reaction was assayed. PylRS was able to activate Cyc but not lysine (Figure 1A).

Subsequently Cyc was used to assay PylRS ability to aminoacylate tRNA<sup>Pyl</sup> *in vitro* (Figure 1B). The tRNA<sup>Pyl</sup> transcripts was labeled at its 3'-terminal adenosine with [z-32P]ATP by the *E. coli* CCA-adding enzyme (11) and used in the aminoacylation reaction. At designated time points, aliquots of the aminoacylation reaction were digested with nuclease P1 at acidic pH in order to prevent deacylation of any Cyc-tRNA<sup>Pyl</sup> formed. Nuclease P1 treatment released [32P]AMP from uncharged tRNAs, and Cyc-[32P]AMP from charged tRNAs. These two species were then separated by thin layer chromatography (TLC), [32P]AMP was visualized using a phosphoimager plate (Figure 1B, insert), and the fraction of charged versus uncharged tRNA was quantified as described in the Materials and Methods section. No charging was observed in the no amino acid control (Figure 1B).

In the presence of excess PylRS enzyme, 80–90% of the tRNA<sup>Pyl</sup> transcript could be aminoacylated with Cyc (data not shown).

**Identity elements of *D. hafniense* tRNA<sup>Pyl</sup>**

Extensive studies of tRNA identity elements have revealed that for most tRNAs the anticodon, the first base pair of the acceptor stem and the discriminator base N73 are important for tRNA aminoacylation by the cognate synthetase (19). To investigate the role of these standard identity elements in recognition of tRNA<sup>Pyl</sup> by PylRS, a series of *D. hafniense* tRNA<sup>Pyl</sup> variants with mutations at these positions were cloned and transcribed *in vitro*. The *D. hafniense* tRNA<sup>Pyl</sup> mutants were assayed for their ability to be charged by *D. hafniense* PylRS with Cyc and k<sub>cat</sub> and K<sub>m</sub> were measured. The loss of aminoacylation efficiency relative to wild type tRNA<sup>Pyl</sup> (L) was determined (L = [k<sub>cat</sub>/K<sub>m</sub>mutant]/[k<sub>cat</sub>/K<sub>m</sub>wild type]) (Table 1).

Surprisingly, the tRNA<sup>Pyl</sup> anticodon, one of the most common identity elements in tRNAs, is not recognized by the PylRS. Changing the tRNA<sup>Pyl</sup> anticodon from...
Table 1. Kinetic parameters of pyrolysis of wild-type and mutant D. hafniense tRNA^Pyl by D. hafniense PylRS

| tRNA       | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) $\times 10^{-2}$ | $k_{cat}/K_m$ $\times 10^{-3}$ | $L^\alpha$ |
|------------|------------|-------------------------------------|--------------------------------|------------|
| WT         | 2.9 ± 1.5  | 1.9 ± 0.45                          | 6.4                            | 1          |
| Anticodon  | 5.3 ± 2.3  | 1.8 ± 0.61                          | 3.5                            | 1.8        |
| CUA → UUU  | 2.1 ± 0.96 | 0.39 ± 0.098                        | 1.9                            | 3.4        |
| Anticodon  | 3.7 ± 0.68 | 0.15 ± 0.023                        | 3.9                            | 1.6        |

$L^\alpha$ refers to the loss in aminoacylation efficiency compared to the wildtype; $L$ is calculated as the ratio $[k_{cat}(\text{mutant})/K_m(\text{mutant})]/[k_{cat}(\text{wild type})/K_m(\text{wild type})]$. NA: not determined because below detection.

CUA to UUU, CUU or AAA did not decrease the aminoacylation efficiency (Table 1).

The discriminator base proved to be an important identity element, as changing G73 to U and C decreased aminoacylation efficiency (Table 1). The simultaneous changes of both base pairs further impacted aminoacylation when present in conjunction with the top of the acceptor stem is an important part of the RNA–protein interface.

Footprinting of D. hafniense tRNA^Pyl with D. hafniense PylRS

In order to search for other possible identity elements, and to look more generally at the PylRS–tRNA^Pyl complex, the D. hafniense tRNA^Pyl was probed in the presence or absence of PylRS using two methods of analysis: RNase digestion and iodine cleavage of phosphorothioate-containing transcripts.

RNase footprinting of tRNA^Pyl

The structure of tRNA^Pyl was probed with RNase S1 (digests at single-stranded RNA) and RNase T1 (digests at single-stranded G) in the presence and absence of D. hafniense PylRS (Figure 2A). Under the experimental conditions for the RNase footprinting, approximately 90% of the tRNA was confirmed to be in the bound state by a nitrocellulose filter binding assay (data not shown). Due to intrinsic RNA instability, there is non-specific degradation in the anticodon loop, and in the variable loop region (Figure 2A). In the absence of PylRS, RNase T1 digested tRNA^Pyl at G5, G8, G14, G22, G30, G42, G44, G48 and G55. Other than G5, which is wobble paired to U68, no cleavage is observed in the acceptor stem as G1 to G4 and G6 are G-C paired and not accessible to RNase T1. The G30-U40 wobble pair in the acceptor stem is also disrupted, while G29-C41 is not. Cleavage at G22 indicates the G22-C13 pair at the end of the D-stem is subject to opening. Relative to the other positions, there is enhanced cleavage at G44, but no change in intensity in the presence or absence of PylRS. In the presence of PylRS, tRNA^Pyl was protected from RNase T1 digestion at nucleotides G14 in the D-loop and G48 in the variable loop. In the PylRS-tRNA^Pyl complex the anticodon loop was not protected from RNase S1 digestion as expected since the tRNA^Pyl anticodon sequence is not important for aminoacylation by PylRS. RNase S1 and T1 probing of the bacterial tRNA^Pyl agreed with the proposed secondary structure of the M. barkeri tRNA^Pyl (20).

Iodine probing of phosphorothioate-containing tRNA^Pyl

Transcripts of tRNA^Pyl with approximately 5% of the nucleotides statistically substituted with phosphorothioates were probed with iodine in the presence and absence of D. hafniense PylRS (Figure 3A). Phosphorothioate-containing transcripts were digested with iodine in the presence of 7, 14 and 28 μM PylRS, and band intensities were quantified and compared to look for changes in cleavage intensity that were dependent on the PylRS concentration (Figure 2B). The cleaved phosphates are located 5’ to the nucleotides listed. Changes in cleavage intensities in the presence of PylRS relative to the no enzyme control were labeled weak if they were less than 2-fold, moderate if they were 2- to 5-fold, and strong if they were greater than 5-fold (17).

Strong and moderate protection from iodine cleavage was observed in the D-stem (G10, A11, U12, C13, U24, C25), D-Loop (G14, U20), T-loop (G55, A57, A58, C59,
U60), one base in the T-stem (U65) and the acceptor stem (G6, U7, U68, C69) (Figure 3). Moderate protection was also observed at residue G8, the single base separating the acceptor stem and the D-stem. Other than protection at the first base pair C27\(\text{G}44\), the anticodon stem digestion pattern is the same regardless of the presence or absence of PylRS. As with the RNase probes, no protection is observed in the anticodon loop. Since enhancement of cleavage was weak, significant opening of the tRNA structure to solvent is not occurring upon PylRS binding (Figure 3B).

In tRNAs, the DT-region is where the T-loop and D-loop make contacts with each other that maintain the L-shape structure. The conserved D- to T-loop contacts are between bases, and the phosphate backbone is left exposed in some areas. The backbone of the tRNA\text{P}y\text{I} T-Loop is occluded from solvent when bound to the synthetase, as expected if the synthetase uses the DT-region in the outer corner of the L-shape structure for recognition.

**Aminoacylation efficiency of D. hafniense tRNA\text{P}y\text{I} variants**

Based on the contacts observed in the footprinting experiments, a series of D. hafniense tRNA\text{P}y\text{I} variants were made and assayed for their ability to be aminoacylated with Cyc by PylRS.

Within the tertiary structure of a tRNA molecule the D-arm is stacked on the anticodon stem and is favorably positioned to play a role in interacting with the cognate synthetase. Moderate losses in aminoacylation efficiency resulting from the changes G10A\(\text{C}25\) (\(L = 38.2\)) and A11G\(\text{U}24\) (\(L = 26.8\)) in the D-arm suggest that this area, which is conserved throughout the tRNA\text{P}y\text{I} species, is important for forming a productive RNA-protein interface.
In the canonical tRNA structure, there are two residues (U8 and N9) separating the acceptor stem and D-arm. The highly conserved U8 interacts with A14 in the D-loop. While this A–U pair is preserved in the archaeal tRNA_Pyl, in *D. hafniense* positions 8 and 14 are both G. The changes G8C ($) L_8 = 8.0 $, and G8A ($ L_8 = 12.8 $) had only small affects on aminoacylation. The impact of G8A was dominated by an increase in $ K_m $. Interestingly, G8U ($ L_8 = 4.6 $) increased $ K_m $, but also increased $ k_{cat} $ slightly, resulting in a very slight overall loss in aminoacylation efficiency. While the base at position 8 appears not to have a large impact on aminoacylation efficiency, the 2'-OH of the ribose sugar of residue 8 may be involved in stabilizing the overall tRNA secondary structure, as it has been shown in tRNA_pro

where the 2'-OH interacts with G46 in the variable loop (21). Thus, it is possible the base changes are not important because the phosphate backbone is engaged in relevant contacts which are not affected by the mutations.

In the T-loop of tRNA_Pyl, protection was observed at A57 and A58. Changes of A58 to U, C or G were all deleterious to the aminoacylation efficiency of tRNA_Pyl ($ L_8 > 1000 $). A58 is almost universally conserved in tRNAs due to the interaction it makes with U54, both of which are conserved in all tRNA_Pyl isoacceptors. Thus, the U54-A58 contact is likely to be present in tRNA_Pyl, and essential for maintaining the tRNA tertiary structure. The formation of this bond would expose

![Diagram of tRNA_Pyl](image)
the phosphate backbone to solvent, which the footprinting results indicate is protected by the PylRS during binding.

At A57, changes to U and C had moderate effects on aminoacylation efficiency of \( L = 43 \) and 31, respectively, while the purine-to-purine change of A57G had only \( L = 5.6 \). Position 57 is generally conserved as a purine in canonical tRNAs because it is involved in a stacking interaction important for stabilization of the T-loop structure. This explains the larger loss in aminoacylation of the U and C mutations relative to G, and the conservative change G57 in the archaeal tRNAs.

A unique feature of the archaeal and bacterial tRNA\(^{Pyl}\) is the absence of the almost universally conserved sequences of T\(_{54}\)G\(_{55}\)C\(_{56}\) in the T-loop, and G\(_{16}\)G\(_{19}\) in the D-loop. When canonical tRNA molecules adopt their characteristic L-shape tertiary structure these two motifs are brought into contact and interactions between them help stabilize the overall tertiary structure. Specifically, the interactions occur between G18-Ψ55 and G19- C56. This also leaves other nucleotides exposed to solvent and available for interactions with a synthetase. As both the T\(_{54}\)G\(_{55}\)C\(_{56}\) and G\(_{16}\)G\(_{19}\) sequences are absent in tRNA\(^{Pyl}\), understanding how the D- to T-loop contacts are established is of interest.

We found that changing A56 in \( D. \) hafniense tRNA\(^{Pyl}\) to any of the other three nucleotides resulted in only small changes in aminoacylation relative to wild-type (\( L < 10 \)). There is evidence that the highly conserved G18-Ψ55 and G19-C56 sequences are not essential in certain tRNAs, such as \( E. \) coli tRNA\(^{\text{Ala}}\)\(_{CUA}\) (22). These contacts in tRNA\(^{Pyl}\) may be compensated for by creating other stabilizing interactions.

**DISCUSSION**

Recognition of a tRNA by its cognate aminoacyl-tRNA synthetase is dependent on the interaction of several factors. These include specific nucleotide–protein contacts, the overall tRNA structure, and the ability of the tRNA to undergo conformational changes upon binding by the cognate synthetase. The interplay of these factors ensures correct and productive interactions between a synthetase and tRNA by creating an exclusive RNA–protein interface upon which binding occurs. In the case of \( D. \) hafniense tRNA\(^{Pyl}\), the discriminator base and the first base pair were identified as important identity elements for PylRS. The discriminator base G73 could not be changed to any of the other three nucleotides without significantly reducing aminoacylation efficiency. Although changing the first base pair of the acceptor stem G1-C72 to A1-U72 decreases aminoacylation efficiency (\( L = 62.7 \)), A2-U71 decreases aminoacylation levels only in the presence of the A2-U72 change (\( L = 106.4 \)), and not by itself. The increased loss in aminoacylation efficiency upon changing both the first and second base pairs in the acceptor stem suggests that the A2-U71 does not make direct contact with the PylRS. The discriminator base, G1-C72 and G2-C71 are conserved throughout the archaeal tRNA\(^{Pyl}\) as well, suggesting these residues may play an important role in tRNA\(^{Pyl}\) aminoacylation across species.

The anticodon was shown not to be recognized by the \( D. \) hafniense PylRS. Most AARSs recognize the tRNA anticodon through a specialized RNA-binding domain appended to the catalytic core at the amino or carboxyl terminus of the synthetase. For example, the amino terminal of the class II synthetases AspRS and LysRS function as anticodon-binding domains (23,24). PylRS from \( D. \) hafniense has a natural 100 amino acid amino-terminal truncation when compared to the archaeal counterparts. Thus, it is still possible that the PylRS in \( Methanosarcinaceae \) uses this amino-terminal domain for anticodon recognition. Consequently, these results may differ in the \( Methanosarcinaceae \) context.

Although the anticodon stem and loop are relatively conserved between the archaeal and bacterial tRNA\(^{Pyl}\) isoacceptors, no contact was observed between PylRS and tRNA\(^{Pyl}\) in this region other than the first base pair of the anticodon stem. Instead, contacts were observed along the T\(_{9}\)C and D-arms and acceptor stem, which is also where the majority of the nucleotide changes between the bacterial and archaeal tRNA\(^{Pyl}\) are found. Of the 23 nucleotides unique to \( D. \) hafniense tRNA\(^{Pyl}\), 5 are in the anticodon stem, while 18 are in the D- or T-arms, or acceptor stem. This leaves open the possibility that the archaeal PylRS core relies on contacts different from those found here in the bacterial context.

In addition, the mutations in tRNA\(^{Pyl}\) may be in part rooted in the higher GC content of the \( D. \) hafniense genome (47%) relative to the archaeal genomes (39–42%). Of the 23 residue changes in the \( D. \) hafniense tRNA\(^{Pyl}\), 15 are changes to G or C, and only 8 are to A or T. Similarly, the \( D. \) hafniense pylS has 51.3% GC content, while the \( M. \) barkeri pylS has 42.7% GC.

Identity elements in tRNAs can confer global specificity through different mechanisms. One perspective is that each nucleotide makes essential contributions, and changes at these positions each result in large losses in aminoacylation efficiency (generally over 100-fold). This is the case for tRNAs such as yeast tRNA\(^{Phe}\), where the anticodon, and the bases A73 and G20 all act independently (25) and were shown to be sufficient in a transplantation experiment (26). Alternatively, the additive effect of many moderate identity elements can be the dominant mode of recognition. The functional relationship between identity elements of tRNA\(^{\text{Asp}}\) were investigated and shown that combined effects of multiple identity elements could be additive (27). For \( D. \) hafniense tRNA\(^{Pyl}\) only moderate identity elements were found; the D-stem base pairs G10-C25 and A11-U24, the base G8, the first base pair G1-C72 and discriminator base G73. These may have a combined effect that would be important for PylRS recognition. While we were unable to find discrete residues with larger (over 100-fold) contributions to aminoacylation efficiency, this may still be sufficient to ensure specific aminoacylation of tRNA\(^{Pyl}\) by PylRS as the major identity elements G73 and G1-C72 are only found together in two other tRNAs in \( D. \) hafniense, tRNA\(^{\text{Asp}}\) and tRNA\(^{\text{Ser}}\). In the in vivo competitive environment, the mischarging of these tRNAs...
by PylRS is unlikely as they are lacking both the tRNA\textsubscript{Pyl} secondary identity elements and unique secondary structure. The synthetases responsible for tRNA\textsubscript{Asp} and tRNA\textsubscript{Ser} aminoacylation depend on additional identity elements absent in tRNA\textsubscript{Pyl} (19) which presumably prevent misacylation of tRNA\textsubscript{Pyl} with aspartate or serine. Therefore, specific aminoacylation of tRNA\textsubscript{Pyl} by PylRS is likely to result from a combination of direct interactions with the identity set revealed here, and recognition of the overall non-canonical tertiary structure of tRNA\textsubscript{Pyl}. Future studies will determine whether the same is true for the archaeal PylRS and tRNA\textsubscript{Pyl}.

Interestingly, tRNA\textsubscript{Pyl} shares many unique structural features with the Bos taurus mitochondrial tRNA\textsubscript{Ser} with the UGA anticodon sequence (tRNA\textsubscript{Ser\_UGA}) including the elongated 6 base pair anticodon stem, a short 5 nucleotide D-loop, the presence of a single residue separating the acceptor and D-stems, and a 3 residue variable loop (20). Although non-canonical structural similarities are present, there is little sequence homology between tRNA\textsubscript{Ser\_UGA} and tRNA\textsubscript{Pyl}. A solution NMR study indicated that the packing of the of tRNA\textsubscript{Ser\_UGA} core is relatively compact due to the missing nucleotides (28). As the compact core was determined to be a result of the structural feature mentioned above, and not specific residues, it is likely that these conclusions hold true for tRNA\textsubscript{Pyl}. The presence of a compact tRNA core has precedent as being a stabilizing factor in the tRNA structure and subsequently leads to enhanced protein synthesis with these structural studies, suggest a global identity for D. hafniense tRNA\textsubscript{Pyl} that is likely dependent on the cumulative effect of several minor identity elements, along with the overall compact core structure.

This report also establishes the functionality of D. hafniense PylRS, despite the absence of approximately 100 amino acids from the amino-terminus of the bacterial enzyme relative to those from archaea. Further downstream of the pyl genes, the open reading frame pylSn encodes a putative protein with ~36% sequence identity to the archaeal PylRS amino-terminus, which initially raised the possibility of a split PylRS in bacteria. However, the PylSn protein is not required for D. hafniense PylRS activity in vitro. The high level of conservation between PylSn and the archaebal PylRS amino-terminus, however, suggests that PylSn may have another role in vivo. Whether the amino-terminus from the Methanosarcinaeae PylRS enzymes is important for enzyme activity or amino acid incorporation during translation in vivo will be determined in future studies.

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