Published in final edited form as:

FEBS Lett. 2007 July 10; 581(17): 3197–3203.

The amino-terminal domain of pyrrolysyl-tRNA synthetase is dispensable *in vitro* but required for *in vivo* activity

Stephanie Herring^{2,*}, Alexandre Ambrogelly^{2,*}, Sarath Gundllapalli², Patrick O'Donoghue², Carla R. Polycarpo^{2,*}, and Dieter Söll^{1,2}

¹Department of Chemistry, Yale University, New Haven, CT 06520-8114, USA

²Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520-8114, USA

Abstract

Pyrrolysine (Pyl) is co-translationally inserted into a subset of proteins in the *Methanosarcinaceae* and in *Desulfitobacterium hafniense* programmed by an in-frame UAG stop codon. Suppression of this UAG codon is mediated by the Pyl amber suppressor tRNA, tRNA^{Pyl}, which is aminoacylated with Pyl by pyrrolysyl-tRNA synthetase (PylRS). We compared the behavior of several archaeal and bacterial PylRS enzymes towards tRNA^{Pyl}. Equilibrium binding analysis revealed that archaeal PylRS proteins bind tRNA^{Pyl} with higher affinity ($K_D=0.1-1.0\ \mu\text{M}$) than *D. hafniense* PylRS ($K_D=5.3-6.9\ \mu\text{M}$). In aminoacylation the archaeal PylRS enzymes did not distinguish between archaeal and bacterial tRNA^{Pyl} species, while the bacterial PylRS displays a clear preference for the homologous cognate tRNA. We also show that the amino-terminal extension present in archaeal PylRSs is dispensable for *in vitro* activity, but required for PylRS function *in vivo*.

Keywords

Pyrrolysine; pyrrolysyl-tRNA synthetase; tRNA; aminoacyl-tRNA synthetase

1. Introduction

Pyrrolysyl-tRNA synthetase is unique among the aminoacyl-tRNA synthetases (aaRSs), as it mediates the co-translational insertion of the unusual amino acid pyrrolysine into proteins [1, 2]. While the 20 canonical amino acids used in protein synthesis are genetically encoded by 61 sense codons, Pyl has been shown to be inserted into the methylamine methyltransferases of *Methanosarcina barkeri* and *Methanosarcina acetivorans* in response to an in-frame UAG stop codon embedded in the corresponding mRNAs [3,4]. The decoding of these particular UAG codons as Pyl is achieved by the presence of *pylT* that encodes the pyrrolysine-specific amber suppressor tRNA^{Pyl} [5]. Comparative genomic analyses have shown that closely related orthologs of *pylT* and *pylS*, the gene encoding PylRS, are present in the genomes of the other members of the *Methanosarcinaceae* family (*Methanosarcina mazei* and *Methanococcoides burtonii*). While sequence similarity implies these orthologs have similar function, no

Correspondence to: Dr. Dieter Söll, Department of Molecular Biophysics and Biochemistry, Yale University, P.O. Box 208114, 266 Whitney Avenue, New Haven, CT 06520-8114, USA, Tel: (203) 432-6200, Fax: (203) 432-6202, E-mail: dieter.soll@yale.edu

*S.H., A.A. and C.R.P. contributed equally to this work

^Φcurrent address: National Institutes of Health, Rockville, MD, USA.

[§]current address: Schering-Plough Research Institute, Union, NJ, USA.

[#]current address: Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil.

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biochemical data exist establishing their enzymatic activity. In addition to the *Methanosarcinaceae*, *pylT* and *pylS* are present in the strictly anaerobic bacterium *D. hafniense* [5]. PylRS from this organism has been shown to aminoacylate tRNA^{Pyl} *in vitro* [6].

Here we report the amino acid sequence and enzymatic activity of PylRS from an additional member of the *Methanosarcinaceae*, *Methanosarcina thermophila*, and compare the affinities and aminoacylation preference of three of the *Methanosarcinaceae* PylRSs and of the *D. hafniense* PylRS toward the known tRNA^{Pyl} species. Finally, we investigate the potential role of the archaeal PylRS amino-terminal domain..

2. Materials and methods

2.1 General

Oligonucleotide 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 [α -³²P]ATP (10 mCi/ml) was from Amersham Biosciences. N- ϵ -cyclopentylloxycarbonyl-L-lysine (Cyc) was from Sigma.

2.2 Cloning and purification of recombinant PylRS enzymes

As the *M. thermophila* genome sequence is not available, *pylS* and *pylT* genes were amplified from genomic DNA by PCR assuming reasonable levels of sequence identity of upstream and downstream regions with other sequenced *Methanosarcinaceae*. The amplified DNA was cloned into the pCR2.1–TOPO vector (Invitrogen) and sequenced multiple times. *M. thermophila pylS* nucleotide sequence was deposited in GenBank (accession number DQ017250). *M. thermophila pylS* was subcloned into pET15b vector (Novagen) using *NdeI* and *BamHI* as restriction sites. The *M. acetivorans pylS* gene was amplified by PCR based on the available genomic sequence, cloned into the pCR2.1–TOPO vector (Invitrogen), sequenced, and subcloned into pET15b vector (Novagen) using *NdeI* and *BamHI* as restriction sites. The *M. barkeri pylS* (Fusaro and MS strains) and *D. hafniense pylS* expression constructs were as described [2,6]. All clones were transformed into *Escherichia coli* BL21-(DE3)-RIL (Stratagene). The overexpression and purification of the recombinant His₆-PylRS enzymes was performed as described [2,6].

2.3 Cloning of *M. barkeri* Fusaro and *D. hafniense* *pylS* and *pylT* genes for in vivo activity assay

The *D. hafniense pylS* gene was amplified by PCR, cloned into the pCR2.1–TOPO vector (Invitrogen), sequenced, and subcloned into pCBS (Amp^r), using *NdeI* and *KpnI* as restriction sites. Full length and amino-terminal truncations of *M. barkeri* Fusaro *pylS* were generated by PCR with primers corresponding to the desired length. The PCR products were cloned into the pCR2.1–TOPO vector (Invitrogen), sequenced, and subcloned into pCBS vector using *NdeI* and *XhoI* as restriction sites. The truncation sites are: Δ_{1-16} (nucleotide 49, protein starts at M17), Δ_{1-30} (nucleotide 91, protein starts at M31), Δ_{1-73} (nucleotide 220, protein starts at M74), Δ_{1-106} (nucleotide 319, protein starts at M107). Point mutations in the *pylS* gene were inserted by PCR with the Quick Change Mutagenesis kit (Qiagen) using the wild type *M. barkeri* Fusaro *pylS*, cloned into pCBS as a template. The constructs were entirely sequenced in order to verify that the desired mutation had been obtained and that no other mutations had been inserted during PCR. The *D. hafniense* and *M. barkeri* Fusaro *pylT* genes were cloned into the pTECH vector as described [12].

2.4 Cloning and in vitro transcription of the tRNA^{Pyl} substrates

Transcripts of *M. barkeri* (strains Fusaro and MS), *M. burtonii* and *D. hafniense* tRNA^{Pyl} were prepared and 3' end-labeled as reported [6]. *In vitro* T7 RNA polymerase run off transcription was conducted according to standard procedures [7].

2.5 Filter binding assay

The ability of the PylRSs to bind [α -³²P]ATP 3'-labeled tRNA^{Pyl} transcript across species was determined using a standard filter binding assay [8]. 3'-labeled tRNA bound to PylRS (tRNA_{bound}) was captured on a nitrocellulose membrane (Millipore); unbound 3'-labeled tRNA (tRNA_{free}) was captured on a nylon Hybond N⁺ membrane (Amersham). The binding of tRNA_{bound} and tRNA_{free} were quantified and used to calculate the binding curve for K_D determination. The K_D of the PylRSs for the various tRNA^{Pyl} were determined by fitting the data to a simple binding isotherm: $\theta = [\text{enzyme}] / ([\text{enzyme}] + K_D)$ [9].

2.6 ATP-[³²P]PP_i exchange

The assay was used to measure PylRS activation of amino acid substrates, and performed as described [10]. Reactions (200 μ l final volume) were carried out at 37 °C (50 °C for *M. thermophila* PylRS) 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 [³²P]PP_i (1.6 cpm/pmol), 0.5 μ M PylRS enzyme and either, 10 mM Cys, 1 mM lysine or no amino acid. Adsorption of formed [³²P]ATP on acid-washed Norite, filtration, wash and quantification were performed as described [10].

2.7 PylRS aminoacylation of tRNA^{Pyl} with Cys

Aminoacylation assays were adapted from a recently described procedure [11]. Aminoacylation reactions (10 μ l) were carried out at 37 °C (50 °C for *M. thermophila* PylRS) in 100 mM Na-Hepes, pH 7.2, 25 mM MgCl₂, 60 mM NaCl, 5 mM ATP, 1 mM DTT, 10 mM Cys, 5 nM PylRS and 1 μ M 3'-[α -³²P]ATP labeled tRNA^{Pyl}. Nuclease P1 digest, thin layer chromatography, and quantification were performed as described [6].

2.8 Suppression of E. coli XAC/A24 lacI-lacZ nonsense mutation

In vivo suppression experiments were carried out using *E. coli* strain XAC/A24 as described [12]. *E. coli* strain XAC/A24 cells were co-transformed with plasmids carrying the *M. barkeri* Fusaro *pylT* (pTECH) and *M. barkeri* Fusaro wild type *pylS* or any of its variants (pCBS). *E. coli* strain XAC/A24 cells were also co-transformed with *D. hafniense* *pylT* (pTECH) and *D. hafniense* *pylS* (pCBS). The UAG suppression level was determined by quantitative analysis of the β -galactosidase activity, which was performed according to the standard procedure [13]. Values are the average of triplicate measurements and are reported as the percentage of mutant enzyme activity relative to that of the wild type enzyme produced by the *E. coli* I-Z40 strain, which carries the *lacI-lacZ* fusion with a wild type tryptophan codon in place of the UAG triplet.

2.9 In vivo binding affinity of tRNA^{Pyl} for PylRS using the yeast three hybrid system

The yeast three hybrid experiments were performed as described [21]. Effect of mutations in PylRS on tRNA^{Pyl} binding was measured using the *in vivo* yeast three hybrid method as reported [14].

2.10 Acid urea gel electrophoresis of aminoacyl-tRNA and Northern hybridization

Acid urea gel electrophoresis and Northern blot were performed as described [2]. The aminoacylation levels of tRNA^{Pyl} isolated from the various *E. coli* XAC/A24 strains

transformed with *D. hafniense* *pylS*, *M. barkeri* Fusaro *pylS* wild type or its variants, grown in the presence of 10 mM Cys was determined.

3. Results

3.1 Sequence variations in PylRS orthologs

The *pylS* and *pylT* sequences from the methanogenic archaea *M. barkeri*, *M. acetivorans*, *M. mazei* and *M. burtonii* as well as from the bacterium *D. hafniense* are available in the public sequence databases. Recently, a metagenomic analysis revealed an additional bacterial *pylS* homologue that is present in a δ -proteobacterial member of the marine worm *Olavius algarvensis* endosymbiotic community [15]. *M. thermophila*, another organism from the *Methanosarcinaceae* group, has been the object of many biochemical studies [16]; its genome sequence, however, has not yet been reported. In order to gain more insight on PylRS sequence variations, we cloned and determined the nucleotide sequence of the *M. thermophila* *pylS* gene (GenBank accession number Q1L6A3) (Fig. S1).

PylRS sequences can be subdivided into three regions: the highly conserved class II aaRS signature core domain at the carboxy-terminal, the unique amino-terminal domain, and a highly variable region linking these two domains. The linking region in archaeal PylRS differs substantially in sequence and in length. This region is relatively short in *M. barkeri* (16 aa) and *M. burtonii* (14 aa) PylRSs compared to the much longer linkers present in *M. thermophila* (74 aa), *M. mazei* (50 aa), and *M. acetivorans* (42 aa) PylRS proteins. In *M. thermophila*, the region is composed of five degenerate repeats of the PAPASTTA sequence (Fig. S1). The PylRSs of the *Methanosarcinaceae* range from 52%–75% sequence identity, and from 56%–82% when the linking region is excluded. Taken together, the other aaRSs from this taxonomic group show a slightly wider range of variability (57%–94% sequence identity).

The bacterial PylRSs from *D. hafniense* and the *O. algarvensis* endosymbiotic δ -proteobacteria differs markedly from the archaeal PylRSs, most notably through the absence of approximately 100 amino acids from their amino-terminal ends. Overall, the bacterial PylRSs share 23%–30% sequence identity with their archaeal counterparts. BLAST searches in the two bacterial genomes identified a distinct short open reading frame (*pylSn*) homologous to the archaeal amino-terminal domain downstream of *pylS* [5]. The bacterial *PylSn* sequences share 59% identity to each other, and ~33–39% identity to the amino-terminus of the archaeal PylRS consensus sequence (Fig. S1).

The five *Methanosarcinaceae* and *D. hafniense* have the same genomic organization of their *pyl* genes: *pylT* is directly upstream of *pylS* which is followed by three genes called *pylB*, *pylC* and *pylD*, whose products are involved in Pyl biosynthesis [17]. In *D. hafniense*, *pylSn* is found downstream of *pylD* [5]. In the worm endosymbiotic δ -proteobacterium, *pylSn* is found immediately downstream of *pylS* while *pylB*, *pylC* and *pylD* are located in a separate cluster elsewhere in the genome.

The archaeal tRNA^{Pyl} species are more conserved than their corresponding PylRSs. All *Methanosarcinaceae* tRNA^{Pyl} have identical or nearly identical nucleotide sequences (Fig. 1 and Fig. S1). In contrast, the *D. hafniense* tRNA^{Pyl} contains 23 nucleotide changes when compared to the archaeal consensus sequence (Fig. S1,[6]). Searches in genomes of the worm endosymbiotic bacterial community either by BLAST or using tRNA Scan-SE with various settings revealed the presence of a possible amber tRNA suppressor. However, this putative tRNA lacks the distinctive secondary structure of *pylT* and includes a number of mismatches in the acceptor stem (Fig. S1).

3.2 Archaeal PylRS has a higher affinity for tRNA^{Pyl} than *D. hafniense* PylRS

An equilibrium binding analysis was carried out to determine the relative PylRS affinities for the various tRNA^{Pyl} species. All archaeal PylRS enzymes bound both the archaeal and *D. hafniense* tRNA^{Pyl} transcripts with a K_D ranging from 0.15–1 μ M (Table 1). While *M. acetivorans* PylRS did not show any preference towards either the *D. hafniense* or archaeal tRNA^{Pyl}, *M. thermophila* and *M. barkeri* Fusaro PylRS bound the archaeal tRNA^{Pyl} slightly better than the *D. hafniense* tRNA^{Pyl} (Table 1). Overall, the nucleotide differences between the archaeal and *D. hafniense* tRNA^{Pyl} did not have a major effect on binding by the archaeal synthetases. The *D. hafniense* PylRS bound tRNA^{Pyl} with a lower affinity relative to the archaeal enzymes with K_D ranging from 5.3–6.9 μ M (Table 1). The overall lower affinity of the *D. hafniense* PylRS toward tRNA^{Pyl} could be due to the absence of the amino-terminal domain. All tested PylRSs were able to bind tRNA^{Pyl} specifically irrespective of the presence or absence of total yeast RNA (0.5 μ g/ μ l) used as competitor in the reaction mixture.

3.3 All PylRSs activate the Pyl analogue Cyl

Pyrrolysine is not a commercially available substrate, and its synthesis has proven both difficult and expensive [2,12,18]. The pyrrolysine analog, N- ϵ -cyclopentylloxycarbonyl-L-lysine (Cyl), is a reasonable substrate for *M. barkeri* Fusaro PylRS *in vitro* and *in vivo*, and for *D. hafniense* PylRS *in vitro* [6,12]. Here, the ability of *M. barkeri* (MS and Fusaro strains), *M. acetivorans* and *M. thermophila* PylRS enzymes to activate Cyl and promote the ATP-[³²P] PP_i exchange reaction was assayed. All tested PylRSs were able to activate Cyl while rejecting lysine (Figure 2A).

3.4 All PylRSs aminoacylate tRNA^{Pyl} with Cyl

Cyl was used to assay the ability of PylRS to aminoacylate tRNA^{Pyl} transcripts *in vitro* (Figure 2B-F) using the established Wolfson/Uhlenbeck assay [6,11]. Each of the different PylRS enzymes was shown to aminoacylate the entire set of tRNA^{Pyl} species *in vitro*. However, there were variations in aminoacylation rates depending on the enzyme:tRNA pair (Figure 2B-F). The archaeal enzymes showed no preference toward any of the various archaeal tRNA^{Pyl} species. Despite the sequence differences, the archaeal enzymes charged *D. hafniense* tRNA^{Pyl} with only a small decrease in the aminoacylation rate relative to the archaeal tRNA^{Pyl} species.

In contrast, the *D. hafniense* PylRS showed a clear preference toward the homologous tRNA^{Pyl} which it charged at a rate 10 times greater than the archaeal tRNAs (Figure 2F).

3.5 The archaeal PylRS amino-terminal domain enables activity *in vivo*

The robust enzymatic activity of *D. hafniense* PylRS demonstrates that the amino-terminal domain is dispensable for bacterial PylRS activity *in vitro* (this work and [6]). Nevertheless, its presence in archaeal PylRSs may be responsible for the significant improvement in archaeal PylRS affinity toward tRNA^{Pyl}, suggesting a positive contribution to tRNA binding from this part of the PylRS molecule. In order to assess whether this region is important for aminoacylation *in vivo*, we truncated the first 106 amino acids of the *M. barkeri* Fusaro PylRS. This more stringent test of aminoacylation takes into account the competition between non-cognate tRNAs in the cellular context. The functionality of the resulting truncated enzyme PylRS Δ_{1-106} was compared to those of *D. hafniense* and wild type *M. barkeri* Fusaro PylRSs as measured by a previously described *in vivo* suppression assay [12]. In this experiment, PylRS activity is correlated to the level of β -galactosidase generated by read-through of an in-frame amber codon located within the coding sequence of a chromosomally encoded *lacI-lacZ* fusion reporter system. The *E. coli* strain XAC/A24 which bears this reporter system, was transformed with plasmid borne copies of *D. hafniense* and *M. barkeri* Fusaro

(wild type or truncated) *pylS* genes in conjunction with the plasmid borne *M. barkeri* Fusaro or *D. hafniense pylT* genes. The transformants were subsequently grown in LB medium containing 10 mM Cyc.

The *E. coli* strain bearing the wild type *M. barkeri* Fusaro *pylS* generated robust β -galactosidase activity when co-transformed with either *M. barkeri* Fusaro or *D. hafniense pylT* (Table 2). These results confirmed our *in vitro* observation that *D. hafniense* and *M. barkeri* Fusaro tRNA^{Pyl} are equally good substrates for the wild type *M. barkeri* Fusaro PylRS. In contrast, neither the naturally truncated *D. hafniense* PylRS nor the *M. barkeri* Fusaro PylRS Δ_{1-106} in which the entire amino-terminal domain was deleted, promoted production of detectable β -galactosidase activity (Table 2). The *in vivo* charging level of tRNA^{Pyl} in these cells was assessed by acid urea gel electrophoresis [22] (Fig. 3). No tRNA^{Pyl} aminoacylation was observed in strains expressing *D. hafniense* PylRS or the amino-terminal truncated *M. barkeri* Fusaro PylRS (Fig. 3, Table 2). In contrast, good Cyc-tRNA^{Pyl} formation was observed in cells carrying the wild-type *M. barkeri* Fusaro PylRS. This suggests that the truncated enzyme did not productively interact with tRNA^{Pyl} in the cellular context when competing noncognate tRNAs are present. This was confirmed by *in vivo* binding experiments using the yeast three hybrid technique which showed that while the wild type *M. barkeri* Fusaro PylRS could bind tRNA^{Pyl}, such binding was completely abolished upon truncation of the *M. barkeri* Fusaro amino-terminal domain (data not shown). Taken together our results are consistent with a role for the PylRS amino-terminal domain in tRNA^{Pyl} binding.

In order to determine which region of the amino-terminus is important in promoting *in vivo* activity, we sequentially truncated the amino-terminal domain of the *M. barkeri* Fusaro PylRS. The position at which the truncations were made was selected based on secondary structure prediction; we respected the integrity of the proposed α -helices and β -sheets. Three amino-terminal truncations of 16, 30 and 73 amino acids (PylRS mutants Δ_{1-16} , Δ_{1-30} and Δ_{1-73}) were made in *M. barkeri* Fusaro *pylS* gene and the resulting enzyme functionality was assayed using the *E. coli in vivo* suppression method. While the PylRS truncated at positions 30 and 73 were unable to promote UAG suppression, some activity—though modest when compared to the wild type—could be detected when the cells were transformed with the shortest 16 amino acid truncation (Table 2). The *in vivo* ability of the wild type and truncated PylRSs to bind tRNA^{Pyl} was assayed using the yeast three hybrid method. While PylRSs truncated at positions 30 and 73 were unable to form a productive complex with their cognate tRNA, some binding could be recovered with the shortest truncation, although, as in the suppression experiment, to an extent much less than with the wild type PylRS (data not shown).

3.6 Residues from the archaeal PylRS amino-terminal domain affecting activity in vivo

Since the deletion of the first thirty residues significantly reduced *in vivo* suppression levels, we selected 12 positions in this region for mutagenesis (Table 2). Six of these residues are completely conserved in the PylRS sequences, while the remaining residues were selected based on their ability to engage in potential hydrogen bond interactions with tRNA^{Pyl}. We tested the ability of the corresponding mutant proteins to promote *in vivo* suppression. The K3A and I26G mutations resulted in a moderate 5-fold reduction in PylRS/tRNA^{Pyl} mediated UAG suppression. The most significant effects reduced suppression efficiency to 5% or less of the wild type levels, and these included the D2A, K4A, S11A, T13A, and H24A mutants as well as the D2A/K4A and S11A/T13A double mutants. The level of *in vivo* tRNA^{Pyl} aminoacylation was visualized by acid urea gel electrophoresis, and for the mutants assayed the charging levels agreed with the suppression results; Cyc-tRNA^{Pyl} was only observed in cells carrying PylRS mutants K3A, D7A and W16A (Fig. 3).

4. Discussion

Compared to the longer *Methanosarcinaceae* PylRSs the *D. hafniense* enzyme appears to be at a disadvantage. Since we do not know whether in *D. hafniense* PylSn and PylRS are both expressed as a non-covalently associated holoenzyme, we attempted to reconstitute this system in *E. coli*. Expression of PylSn in *E. coli* leads to a barely soluble protein, the addition of which to purified PylRS did not change the enzyme's behavior. Co-expression of *D. hafniense* PylSn and PylRS in *E. coli* did not yield the desired holoenzyme. While our archaeal PylRS mutant that is most similar to the *D. hafniense* PylRS (*M. barkeri* Δ_{1-106}) is active in forming Cys-tRNA^{Pyl} *in vitro*, it is only 4.3% as efficient as the wild type enzyme.

The *D. hafniense* enzyme, at least in the absence of PylSn, is only able to efficiently aminoacylate its homologous tRNA^{Pyl} *in vitro*, but not *in vivo*. These data when taken together with our experiments on PylRS-tRNA^{Pyl} binding in addition to our *in vitro* and *in vivo* aminoacylation assays of tRNA^{Pyl} by amino terminally truncated PylRSs lead to the conclusion that the amino terminal domain is required in the competitive cellular context and that the role of this domain is in tRNA binding and recognition. Consistent with this observation is the fact that the *M. barkeri* Fusaro PylRS makes contacts with nucleotides in the tRNA anticodon loop [14], while this is not the case for the *D. hafniense* catalytic core domain that recognizes the discriminator base G73 and the first base pair (G1:C72) of tRNA^{Pyl} [6].

We further probed the role of the amino-terminal domain with a series of PylRS mutants. Deletion mutants seem to indicate that the first 30 amino acids of this domain are essential for charging of tRNA^{Pyl} *in vivo*. Specific site directed mutations in this portion of the molecule revealed that D2, K4, S11, T13 and H24 play an important role in the PylRS tRNA^{Pyl} interaction. While the yeast three-hybrid data supported the notion that these mutations resulted in lower affinity of PylRS for tRNA^{Pyl}, the observed decrease in suppression efficiency could also result from perturbation of the tertiary structure of the protein.

For several reasons (see review [19]), PylRS is a unique enzyme among the tRNA synthetases. Not only does its tRNA recognition domain show no sequence similarity to any other proteins, this domain is present as a split gene in the bacterial examples [5,15]. While other aaRSs exist as split genes, such as the *Aquifex aeolicus* LeuRS, in which the two halves of the catalytic core domain are separated [23], or the free-standing editing domain in some crenarchaeal ThrRSs [24,25], the bacterial PylRSs are the first example of an aaRS that is split between the tRNA recognition domain and the catalytic core.

While we found that the catalytic core domain of the *D. hafniense* PylRS is not functional in *E. coli*, the situation could be markedly different in *D. hafniense* itself. Other aaRS split genes, including the *A. aeolicus* LeuRS [23] and AlaRS of *Nanoarchaeum equitans* [26], are expressed to form non-covalently bound, functional holoenzymes. Although the situation may be similar for the *D. hafniense* PylRS, why any of these genes exist in split forms is unknown. Indeed, the biochemical mystery of how the bacterial Pyl-encoding system functions, namely how or even if the PylRS amino-terminal and catalytic core domains associate in these bacterial examples is a fascinating and still open question. Since our results show that the amino-terminal domain is required for Pyl-tRNA^{Pyl} formation *in vivo*, we find it likely that PylSn has been selectively conserved in *D. hafniense* and the *Olavius* endosymbiont during the course of evolution. Future biochemical and genetic studies will be needed to resolve this question.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Carina Frauer, Darrick Li, Benfang Ruan and Kelly Sheppard for their assistance; Richard Villemur, William Metcalf and Kevin Hill for the gift of *D. hafniense*, *M. acetivorans* and *M. thermophila* genomic DNAs. We thank Nikos Kyrpides for the access to the *Olavius algarvensis* community meta-genomic sequences. This work was supported by grants from the National Institute of General Medical Sciences, the Department of Energy, and the National Science Foundation.

Abbreviations

aaRS, aminoacyl-tRNA synthetase; Pyl, pyrrolysine; PylRS, pyrrolysyl-tRNA synthetase..

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Fig 1.

Clover leaf structures of tRNA^{Pyl} from *M. barkeri* Fusaro, *M. burtonii*, and *D. hafniense*. Arrows indicate nucleotide variations in *M. barkeri* MS tRNA^{Pyl}. Circles represent absent nucleotides according to standard tRNA numbering [23].

Fig 2.

Activation and charging properties of various PylRSs. (A) Time course for the activation of Cyc by PylRS from *M. barkeri* Fusaro (◆), *M. barkeri* MS (▲), *M. thermophila* (●), *M. acetivorans* (□), and *D. hafniense* (○). All PylRS proteins were assayed in reactions with lysine (×) and no amino acid (▷), and no activation was observed. *In vitro* aminoacylation of tRNA^{Pyl} transcript with Cyc by PylRS from: (B) *M. barkeri* Fusaro, (C) *M. barkeri* MS, (D) *M. acetivorans*, (E) *M. thermophila*, (F) *D. hafniense*. tRNA^{Pyl} was from *M. barkeri* MS (■), *M. barkeri* Fusaro (●), *M. burtonii* (▲), and *D. hafniense* (○). Reaction conditions are described in Materials and Methods.

Fig 3.

In vivo aminoacylation of tRNA^{Pyl} as visualized by northern hybridization. Total tRNA was extracted from *E. coli* XAC/A24 strain transformed with plasmids bearing *M. barkeri* Fusaro *pylT* and either *M. barkeri* Fusaro PylRS mutants D2A (Lane 1), K3A (Lane 2), K4A (Lane 3), D7A (Lane 4), S11A (Lane 5), T13A (Lane 6), W16A (Lane 7), D2A/K4A (Lane 8), S11A/T13A (Lane 9), wild type *M. barkeri* Fusaro PylRS (Wt), wild type *D. hafniense* PylRS (Lane 11) and *M. barkeri* Fusaro PylRS truncated from its first 106 amino acids (Lane 12). All transformants were grown in the presence of 10mM Cyc. The resulting tRNA products were loaded onto an acid gel. After transfer to a nitrocellulose membrane, the samples were hybridized with a tRNA^{Pyl} specific oligonucleotide probe. The positions of tRNA^{Pyl} and Cyc-tRNA^{Pyl} are indicated.

Table 1

Dissociation constants (K_D , μM) of archaeal and bacterial PylRSs for all known tRNA^{Pyl} species as determined by nitrocellulose filter binding assay.

Enzymes	<i>D. hafniense</i> tRNA ^{Pyl}	<i>M. acetivorans</i> tRNA ^{Pyl} *	<i>M. barkeri</i> MS tRNA ^{Pyl}	<i>M. burtonii</i> tRNA ^{Pyl}
<i>M. barkeri</i> Fusaro PylRS	0.98 ± 0.07	0.32 ± 0.02	0.15 ± 0.02	0.43 ± 0.03
<i>M. thermophila</i> PylRS	1.00 ± 0.05	0.71 ± 0.06	0.16 ± 0.01	0.19 ± 0.02
<i>M. acetivorans</i> PylRS	0.50 ± 0.08	0.62 ± 0.02	0.25 ± 0.01	0.44 ± 0.03
<i>D. hafniense</i> PylRS	6.90 ± 0.41	6.50 ± 0.26	5.40 ± 0.18	5.30 ± 0.37

* The sequences of tRNA^{Pyl} from *M. barkeri* Fusaro, *M. thermophila*, and *M. acetivorans* are identical.

Table 2**Suppression efficiency of *M. barkeri* Fusaro PylRS variants**

Suppression efficiency was measured by β -galactosidase activity in *E. coli* strain XAC/A24. 100% corresponds to 3800 Miller units [13]. In the absence of Cyc, background suppression in all cases was less than 1%. *M. barkeri* Δ_{1-NN} refers to PylRS N-terminal truncations: Δ_{1-16} (protein start at M17), Δ_{1-30} (protein start at M31), Δ_{1-73} (protein start at M74), Δ_{1-106} (protein start at M107).

PylRS	tRNA ^{Pyl}	Suppression efficiency (%)
<i>M. barkeri</i> wild type	<i>M. barkeri</i>	100
	<i>D. hafniense</i>	98
<i>D. hafniense</i> wild type	<i>M. barkeri</i>	<1
	<i>D. hafniense</i>	<1
<i>M. barkeri</i> Δ_{1-16}	<i>M. barkeri</i>	10
<i>M. barkeri</i> Δ_{1-30}	<i>M. barkeri</i>	<1
<i>M. barkeri</i> Δ_{1-73}	<i>M. barkeri</i>	<1
<i>M. barkeri</i> Δ_{1-106}	<i>M. barkeri</i>	<1
<i>M. barkeri</i> D2A	<i>M. barkeri</i>	5
<i>M. barkeri</i> K3A	<i>M. barkeri</i>	20
<i>M. barkeri</i> K4A	<i>M. barkeri</i>	5
<i>M. barkeri</i> D7A	<i>M. barkeri</i>	95
<i>M. barkeri</i> S11A	<i>M. barkeri</i>	5
<i>M. barkeri</i> T13A	<i>M. barkeri</i>	5
<i>M. barkeri</i> W16A	<i>M. barkeri</i>	65
<i>M. barkeri</i> S18A	<i>M. barkeri</i>	95
<i>M. barkeri</i> R19A	<i>M. barkeri</i>	95
<i>M. barkeri</i> G21L	<i>M. barkeri</i>	95
<i>M. barkeri</i> H24A	<i>M. barkeri</i>	5
<i>M. barkeri</i> I26G	<i>M. barkeri</i>	20
<i>M. barkeri</i> D2A/K4A	<i>M. barkeri</i>	<1
<i>M. barkeri</i> S11A/T13A	<i>M. barkeri</i>	<1