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

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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 μM) than *D. hafniense* PylRS (K_D = 5.3–6.9 μM). 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.**
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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 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 [^{32}P]pyrophosphate [1–60 Ci/mmol (1 Ci = 37 GBq)], and [α-^{32}P] ATP (10 mCi/ml) was from Amersham Biosciences. *N*-ε-cyclopentyloxycarbonyl-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 *Nde*I and *Bam*HI 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 *Nde*I and *Bam*HI 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 His$_6$-PylRS enzymes was performed as described [2,6].

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Abbreviations: aaRS, aminoacyl-tRNA synthetase; Pyl, pyrrolysine; PylRS, pyrrolysyl-tRNA synthetase

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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 *Nde*I and *Kpn*I 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 *Nde*I and *Xho*I 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-^{32}P] 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: θ = [enzyme]/([enzyme]+K_D) [9].

2.6. ATP-[^{32}P]PP$_i$ exchange

The assay was used to measure PylRS activation of amino acid substrates, and performed as described [10]. Reactions (200 μl final volume) were carried out at 37 °C (50 °C for *M. thermophila* PylRS) in 100 mM Na-HEPES, pH 7.2, 10 mM $MgCl_2$, 50 mM KCl, 5 mM DTT, 2 mM KF, 2 mM ATP, 2 mM [^{32}P]PP$_i$ (1.6 cpm/pmol), 0.5 μM PylRS enzyme and either, 10 mM Cyc, 1 mM lysine or no amino acid. Adsorption of formed [^{32}P]ATP on acid-washed Norite, filtration, wash and quantification were performed as described [10].

2.7. PylRS aminoacylation of tRNA^{Pyl} with Cyc

Aminoacylation assays were adapted from a recently described procedure [11]. Aminoacylation reactions (10 μl) were carried out at 37 °C (50 °C for *M. thermophila* PylRS) in 100 mM Na-HEPES, pH 7.2, 25 mM $MgCl_2$, 60 mM NaCl, 5 mM ATP, 1 mM DTT, 10 mM Cyc, 5 nM PylRS and 1 μM 3′-[α-^{32}P] 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 Cyc 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 catalytic 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% to 75% sequence identity, and from 56% to 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 differ 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

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