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## Misacylation of pyrrolysine tRNA *in vitro* and *in vivo*

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### Abstract

*Methanosarcina barkeri* inserts pyrrolysine (Pyl) at an in-frame UAG codon in its monomethylamine methyltransferase gene. Pyrrolysyl-tRNA synthetase acylates Pyl onto tRNA<sup>Pyl</sup>, the amber suppressor pyrrolysine tRNA. Here we show that *M. barkeri* Fusaro tRNA<sup>Pyl</sup> can be misacylated with serine by the *M. barkeri* bacterial-type seryl-tRNA synthetase *in vitro* and *in vivo* in *Escherichia coli*. Compared to the *M. barkeri* Fusaro tRNA, the *M. barkeri* MS tRNA<sup>Pyl</sup> contains two base changes; a G3:U70 pair, the known identity element for *E. coli* alanyl-tRNA synthetase (AlaRS). While *M. barkeri* MS tRNA<sup>Pyl</sup> cannot be alanylated by *E. coli* AlaRS, mutation of the MS tRNA<sup>Pyl</sup> A4:U69 pair into C4:G69 allows aminoacylation by *E. coli* AlaRS both *in vitro* and *in vivo*.

### Keywords

tRNA<sup>Pyl</sup>; pyrrolysine; mischarging; *Methanosarcina barkeri*

## 1. Introduction

A number of deviations from the universality of the genetic code are known [1]. One of them is the recoding of in-frame UAG codons as pyrrolysine (Pyl) sense codons in the *Methanosarcinaceae*, a group of methanogenic archaea, and in two evolutionarily unrelated bacteria [2,3]. The co-translational insertion of Pyl is contingent on the presence of the special amber suppressor tRNA<sup>Pyl</sup> (encoded by *pylT*) [4], and an unusual aminoacyl-tRNA synthetase (aaRS), pyrrolysyl-tRNA synthetase (PylRS, encoded by *pylS*), specific only for this modified amino acid [4–6]. PylRS specifically recognizes Pyl and tRNA<sup>Pyl</sup> [5,6] and generates Pyl-tRNA<sup>Pyl</sup> both *in vitro* and *in vivo*. Transformation of *E. coli* with the archaeal *pylT* and *pylS* genes showed that both gene products are also functional in a bacterial setting [6–9]. Monitoring of UAG read-through efficiency by various reporter systems indicated that the stop codon is suppressed only when tRNA<sup>Pyl</sup> and PylRS are present and the *E. coli* strain is grown in a culture media containing an exogenous source of Pyl [N-ε-cyclohexylloxycarbonyl-L-lysine (Cyc), a structural analog of Pyl] [6–9].

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The molecular basis of PylRS specificity towards tRNA<sup>Pyl</sup> was attributed to the unusual secondary/tertiary structure of the tRNA [8,10] previously seen only in the bovine mitochondrial tRNA<sup>Ser</sup> [11,12]. A series of specific interactions mediated by nucleotides located in the acceptor stem and flanking the tRNA<sup>Pyl</sup><sub>CUA</sub> anticodon were also shown to contribute to the recognition of tRNA<sup>Pyl</sup> by PylRS [8,10]. In contrast to most aaRSs, PylRS does not recognize its tRNA substrate via interaction with nucleotides of the anticodon [8,10, 13]. This observation made possible the insertion of Cys at a UGA codon upon engineering of a complementary UCA anticodon in tRNA<sup>Pyl</sup> [10]. Crystallization of *Methanosarcina mazei* PylRS in a complex with Pyl and Cys allowed the visualization of the enzyme's active site and shed some light on Pyl recognition by archaeal [13,14] and bacterial PylRS [15].

In addition to Pyl and Cys recognition, recent work describes the engineering of the PylRS active site to accommodate the lysine analogs, N-benzyloxycarbonyl lysine and N-acetyl lysine, so as to allow their incorporation into proteins in human [16] and *E. coli* [17] cells.

The tRNA<sup>Pyl</sup> sequences of two *M. barkeri* strains are known; *M. barkeri* Fusaro and *M. barkeri* MS tRNA<sup>Pyl</sup> differ in two bases (see Fig. 1), but both share the unique tRNA structure with the one found in the *Bos taurus* mitochondrial tRNA<sup>Ser</sup> species [18]. In these tRNAs the junction between the acceptor stem and D stem is shortened by one nucleotide, the anticodon stem consists of six base pairs instead of the classical five pairs, and the D loop is significantly shorter compared to the canonical cloverleaf structures [5,18]. The *M. barkeri* MS tRNA<sup>Pyl</sup> has a G3:U70 base pair; this pair was shown to be the primary identity element for tRNA<sup>Ala</sup> recognition by *E. coli* AlaRS [19,20]. Since the PylRS and tRNA<sup>Pyl</sup> are assumed to be a functional orthogonal pair for genetic code expansion, we wanted to study this further by probing the aminoacylation of tRNA<sup>Pyl</sup> by non-cognate aaRSs.

## 2. Materials and Methods

### 2.1 General

Oligonucleotide synthesis, DNA sequencing and Edman degradation were performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University. [<sup>14</sup>C]Serine (155 mCi/mmol) and [<sup>14</sup>C]alanine (150 mCi/mmol) were from Amersham Biosciences (Uppsala, Sweden).

### 2.2 Construction of tRNA, minihelix, and enzyme constructs

*M. barkeri* strain Fusaro and strain MS tRNA<sup>Pyl</sup> were produced and purified as described in [5]. Minihelices were constructed based on the sequences of *M. barkeri* MS tRNA<sup>Ala</sup> (tRNA<sup>Ala</sup>), *M. barkeri* MS tRNA<sup>Pyl</sup> (tRNA<sup>Pyl</sup>), and an *M. barkeri* MS tRNA<sup>Pyl</sup> mutant (A4:U69→C4:G69) (tRNA<sup>Pyl</sup> C4:G69). The minihelices are shown in Fig. 4B with their designated names (indicated in parentheses in the previous sentence). The pGF-1b plasmid containing the *E. coli* suppressor tRNA<sup>Lys</sup> gene was obtained from W. McClain (University of Wisconsin, Madison). The pET-15b plasmids containing the *M. barkeri* Fusaro bacterial and methanogenic SerRS genes were as described [21]. The pET15 *E. coli* AlaRS construct was a gift from M. Frugier (IBMC, Strasbourg, France).

### 2.3 Aminoacylation Assays

Aminoacylation was performed at 37° C in 100 mM HEPES-KOH pH 7.2, 20 mM MgCl<sub>2</sub>, 5 mM KCl, 5 mM dithiothreitol, 5 mM ATP, 300 μM [<sup>14</sup>C]Ser or [<sup>14</sup>C]Ala, 2 μM tRNA transcript, and 1 μM enzyme. 20 μL aliquots were taken at 1, 5, 20, 40, and 60 min and spotted onto Whatman filters (3MM). The filters were washed twice with 10% TCA for 15 min each, and once with 5% TCA for 15 min. The filters were then rinsed with ethanol and allowed to dry. Radioactivity was measured with a Beckman Coulter LS 6500 scintillation counter.

## 2.4 TrpA mutant auxotrophy

To determine charging of a tRNA<sup>Pyl</sup> mutant with alanine *in vivo*, an *E. coli trpA94* strain (amber mutation in position 94 of *trpA*) was used. Active TrpA protein can only be made by the insertion of Ala or Gly into this position [22]. Cells were transformed with pGF-1b plasmid carrying the tRNA<sup>Pyl</sup> C4:G69 mutant and plated on M9 minimal medium supplemented with ampicillin, either with or without Trp and grown at 37°C.

## 2.5 Suppression efficiency

Read-through efficiency using the dual luciferase/ $\beta$ -galactosidase reporter system was performed as indicated earlier [9].

## 2.6 Suppression of a UAG codon in an *E. coli folA* reporter gene

A mutant *E. coli folA* gene (encoding dihydrofolate reductase, DHFR) containing a UAG codon in place of codon 3 was amplified from genomic DNA, cloned into the pCR 2.1-TOPO plasmid vector (Invitrogen, Carlsbad, CA), sequenced, and subcloned into pRSFDuet-1. *E. coli* BL21 (DE3) competent cells were cotransformed with wild-type *M. barkeri* Fusaro tRNA<sup>Pyl</sup> (cloned in pTECH), the *folA* reporter construct (cloned in pRSF) and the *M. barkeri* bacterial type SerRS in pET15b. The resulting strains were grown in Luria broth at 37°C. Production of the recombinant DHFR was induced with 1 mM isopropyl- $\beta$ -D-thiogalactoside when cells reached an A<sub>600</sub> of 0.6. Cells were harvested after 17 h. DHFR was purified using a Ni-NTA affinity column and then blotted onto a PVDF membrane. The first eight amino acids were identified by Edman degradation. Background was determined in the same conditions but in the absence of any overexpressed proteins.

BL21(DE3) competent cells were also cotransformed with wild-type *M. barkeri* Fusaro tRNA<sup>Pyl</sup> (cloned in pTECH), the *folA* reporter construct (cloned in pRSF) and *M. barkeri* PylRS in pET15b. Cells were grown in the presence of Cyc, recombinant DHFR was purified and sequenced by Edman degradation as indicated above.

## 3. Results and Discussion

### 3.1 Mischarging of *M. barkeri* Fusaro tRNA<sup>Pyl</sup> with serine *in vitro*

The unique secondary structure of *M. barkeri* Fusaro tRNA<sup>Pyl</sup> is only shared by mammalian mitochondrial tRNA<sup>Ser</sup><sub>UGA</sub> [23] (Fig. 1). Therefore, we investigated whether tRNA<sup>Pyl</sup> could be a substrate for the two *M. barkeri* SerRSs. *M. barkeri* contains two *serS* genes that encode two different types of SerRSs: a bacterial-type SerRS present in most organisms, and a highly divergent SerRS only found in some methanogenic archaea [24]. The methanogenic and bacterial-type SerRSs use overlapping identity elements to recognize the same set of tRNA<sup>Ser</sup> species [21]. *In vitro* aminoacylation reactions showed that the bacterial-type SerRS was able to acylate up to 15% of the available *M. barkeri* tRNA<sup>Pyl</sup> transcript with serine after 60 min (Fig. 2). In contrast, the methanogenic SerRS showed no detectable charging of tRNA<sup>Pyl</sup> (Fig. 2).

### 3.2 Mischarging of *M. barkeri* Fusaro tRNA<sup>Pyl</sup> with serine *in vivo*

We then determined whether the mischarging of tRNA<sup>Pyl</sup> with serine observed *in vitro* also occurs *in vivo*. To do so, we measured the level of UAG read-through in *E. coli* in the presence of the two *M. barkeri* SerRSs using a plasmid-based reporter system that contains in frame the genes encoding  $\beta$ -galactosidase (*lacZ*) and luciferase (*luc*). The two genes are transcribed as a single mRNA unit; an in frame UAG codon is located between the two open reading frames. In the absence of read-through only the  $\beta$ -galactosidase is produced. Upon read-through a  $\beta$ -galactosidase-luciferase fusion protein is synthesized. Luciferase enzymatic activity directly

correlates to the frequency of read-through, and when normalized to  $\beta$ -galactosidase activity allows sample-to-sample comparison. Read-through efficiency is expressed as the ratio of luciferase to  $\beta$ -galactosidase enzymatic activities observed with the in frame UAG-containing reporter plasmid over the same ratio obtained when the stop codon is a standard lysine codon. This reporter system, previously used to study internal ribosome reentry sites [25], permits to reliably measure read-through even when it occurs at very low level.

We quantified UAG suppression efficiency in the presence of tRNA<sup>Pyl</sup> and each one of the two *M. barkeri* SerRSs and compared these values to those obtained with the appropriate negative and positive controls. When tRNA<sup>Pyl</sup> was co-expressed with the bacterial type SerRS, read-through to up to 8.6% was detected and only to about 3.3% with the methanogenic SerRS (Fig. 3). In the absence of any overexpressed enzyme, background read-through was measured as 1.3% (Fig. 3). These results indicated that when expressed in *E. coli* the two *M. barkeri* SerRSs are able to misacylate *M. barkeri* tRNA<sup>Pyl</sup> with serine at a low but detectable level. To gauge the significance of such read-through we compared it with UAG suppression observed in the presence of PylRS, tRNA<sup>Pyl</sup> and Cys, a structural analog of Pyl (Fig. 3). Read-through in the presence of the bacterial-type SerRS accounted for only 11% of that observed with PylRS and Cys. The *in vivo* observations are in line with our above *in vitro* results that showed the ability of the *M. barkeri* bacterial-like SerRS to seryl-acylate tRNA<sup>Pyl</sup>. More surprising was the fact that we could detect read-through activity with the methanogenic enzyme while no aminoacylation could be detected *in vitro*. We attribute this apparent discrepancy to the higher sensitivity of the *in vivo* assay for very low mischarging activities. Neither the methanogenic-type *M. barkeri* SerRS nor *E. coli* SerRS formed significant amounts of Ser-tRNA<sup>Pyl</sup>, and suppression was entirely dependent on the presence of tRNA<sup>Pyl</sup> (Fig. 3).

### 3.3 Direct evidence for serine incorporation into protein

Next we sought to confirm the direct insertion of serine into a reporter protein in *E. coli*. We utilized an *E. coli folA* (encoding DHFR) reporter gene containing an in-frame UAG codon at position 3 and a six-histidine codon repeat at the 3' end. *E. coli* cells were co-transformed with tRNA<sup>Pyl</sup>, the *folA* gene reporter and either *M. barkeri* bacterial-type *serS*, or an empty plasmid for background determination. For each condition, a DHFR-His<sub>6</sub> was purified by Ni-NTA chromatography and SDS gel electrophoresis. N-terminal sequencing of the purified DHFR-His<sub>6</sub> from cells co-transformed with the bacterial-type *serS* revealed the presence of Ser at position 3 in the protein. Pro, Gln and Val were also detected at the third position albeit in much smaller amounts. Pro was found to be at position 3 of the protein sample extracted from the cells lacking tRNA<sup>Pyl</sup> (negative control).

To ensure that our reporter system was functioning properly we measured the incorporation of Cys at position 3 of the recombinant DHFR-His<sub>6</sub> in cells grown on Cys and containing the tRNA<sup>Pyl</sup>:PylRS pair. Expression of the DHFR-His<sub>6</sub> was significantly higher than in the previous experiments and allowed purification of the protein to homogeneity. Amino acid 3 of the purified DHFR-His<sub>6</sub> was identified as Cys, in line with earlier mass spectroscopy data [17]. No other amino acid was detected in position 3 (data not shown).

### 3.4 Misacylation of *M. barkeri* MS tRNA<sup>Pyl</sup> with alanine in vitro

*M. barkeri* MS tRNA<sup>Pyl</sup> differs from *M. barkeri* Fusaro tRNA<sup>Pyl</sup> at two positions: the first nucleotide of the variable loop is U44 instead of C44, and in the acceptor stem MS tRNA<sup>Pyl</sup> contains a G3 instead of A3 (Fig. 1). The G3A deviation results in the formation of the wobble base pair G3:U70. A G3:U70 pair is also the primary identity element for tRNA<sup>Ala</sup> recognition by AlaRS [19,20].

We then tested whether *M. barkeri* MS tRNA<sup>Pyl</sup> could be a substrate for *E. coli* AlaRS. As seen in Fig. 4A, the *M. barkeri* MS tRNA<sup>Pyl</sup> is not misacylated with alanine by *E. coli* AlaRS *in vitro*, possibly the result of a tRNA anti-determinant whose presence prevents AlaRS recognition. A good candidate for an anti-determinant in wild-type MS tRNA<sup>Pyl</sup> is the A4:U69 base pair, as insertion of A4:U69 into tRNA<sup>Ala</sup> disrupts the acceptor stem helix and reduces recognition by AlaRS [19]. We tested whether this particular base pair plays a role in precluding MS tRNA<sup>Pyl</sup> from being misacylated with alanine by *E. coli* AlaRS. Inspection of the *M. barkeri* MS tRNA<sup>Ala</sup> sequence revealed a C4:G69 base pair underneath the G3:U70 main identity element. We decided to replace *M. barkeri* MS tRNA<sup>Pyl</sup> A4:U69 with a C4:G69 pair. AlaRS is one of the few aaRSs shown to be able to charge stem-loop helices derived from its cognate tRNA<sup>Ala</sup> [26]. We then constructed three minihelices (Fig. 4B) based on (i) *M. barkeri* MS tRNA<sup>Ala</sup> (tRNA<sup>Ala</sup>), (ii) acceptor and T-stem sequences of *M. barkeri* MS tRNA<sup>Pyl</sup> (tRNA<sup>Pyl</sup>), and (iii) a C4:G69 *M. barkeri* MS tRNA<sup>Pyl</sup> mutant (tRNA<sup>Pyl</sup> C4:G69). The *M. barkeri* MS tRNA<sup>Pyl</sup> minihelix, like the full length tRNA<sup>Pyl</sup> is not misacylated by *E. coli* AlaRS. The control *M. barkeri* MS wild-type tRNA<sup>Ala</sup> stem-loop helix was charged close to 50%. The C4:G69 mutant minihelix was acylated with Ala but remained a poor substrate for AlaRS since it was charged to only 2% (Fig. 4C).

### 3.5 Misacylation of *M. barkeri* MS tRNA<sup>Pyl</sup> with alanine *in vivo*

To determine whether *M. barkeri* MS tRNA<sup>Pyl</sup> and its C4:G69 mutant can be misacylated with Ala *in vivo*, we made use of the *E. coli* *trpA94* amber mutant strain that will only grow in the absence of exogenously added Trp is the amber codon is suppressed by either Gly or Ala [22]. We transformed this strain with plasmids containing either the *M. barkeri* MS wild type tRNA<sup>Pyl</sup>, the C4:G69 tRNA<sup>Pyl</sup> mutant, an *E. coli* tRNA<sup>Ala</sup> amber suppressor, or an empty vector. The transformed strains were plated on M9 minimal medium with or without Trp (Fig. 5).

After incubation at 37 C for 72 h, the *E. coli* strain *trpA94* transformed with the tRNA<sup>Pyl</sup> C4:G69 mutant showed growth on plates without Trp, albeit at a slower rate (72 h) compared to the *E. coli* tRNA<sup>Ala</sup> amber suppressor (24–48 h) (Fig. 5). This result indicated the misacylation of the mutant tRNA<sup>Pyl</sup> with Ala, and the production of a sufficient amount of active TrpA enzyme, permitting cell survival. The cells transformed with *M. barkeri* MS wild-type tRNA<sup>Pyl</sup> or with the empty plasmid did not show any growth after 72 h (Fig. 5), confirming our *in vitro* results. Lastly, as expected, all transformants grew on minimal plates supplemented with Trp (Fig. 5).

## 4. Outlook

The current study demonstrates two instances of tRNA<sup>Pyl</sup> misacylation, the formation of Ser-tRNA<sup>Pyl</sup> and Ala-tRNA<sup>Pyl</sup>. *M. barkeri* bacterial-type SerRS recognizes its three tRNA<sup>Ser</sup> isoacceptors via interaction of amino acids in the long  $\alpha$ -helix domain with nucleotides of the extended variable loop of the tRNA<sup>Ser</sup>; this specificity mechanism is shared with *E. coli* SerRS [21]. Bovine mitochondrial tRNA<sup>Ser</sup> lacks such an extended variable loop and presents a non-canonical structure; a mitochondrial SerRS:tRNA<sup>Ser</sup> complex requires an additional N-terminal enzyme domain interacting with nucleotides of the D- and T-loops [23]. Future studies will establish details of the interaction between SerRS and tRNA<sup>Pyl</sup>, and should also provide more insight into possible uses of misacylated suppressor tRNA<sup>Pyl</sup> protein synthesis.

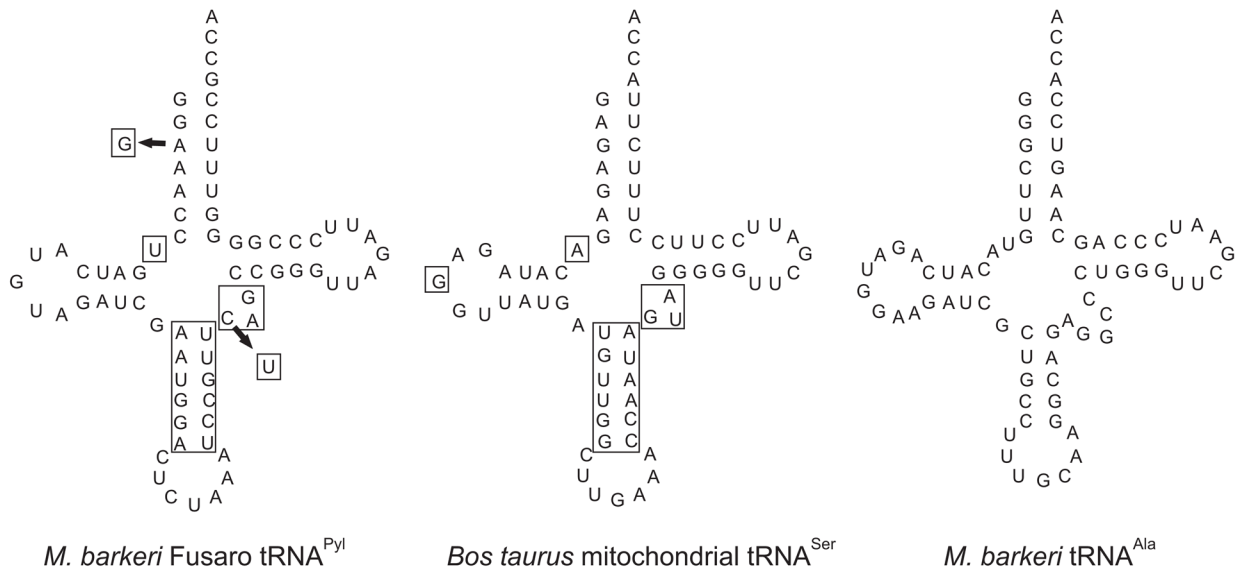
## Acknowledgements

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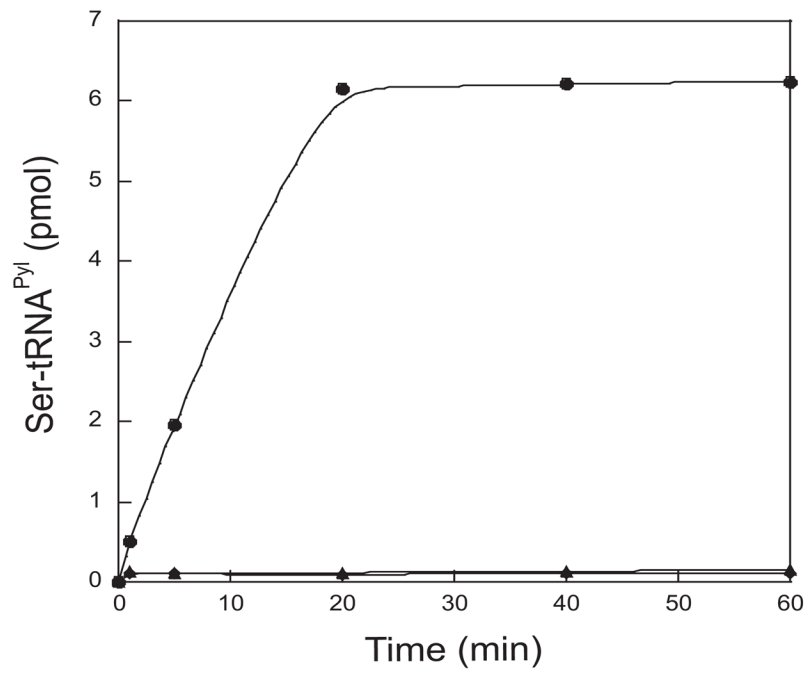
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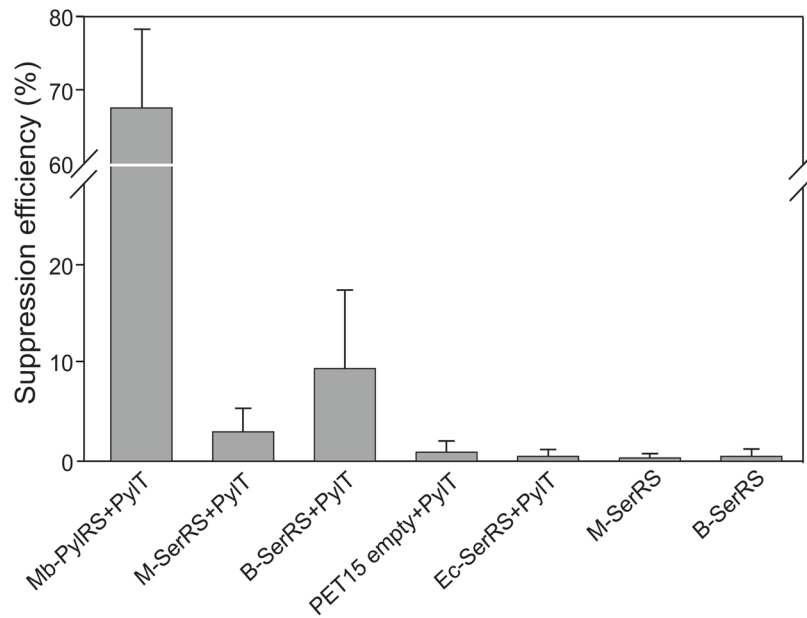
**Fig. 1.**

Cloverleaf representation of *M. barkeri* Fusaro tRNA<sup>Pyl</sup>, *Bos taurus* mitochondrial tRNA<sup>Ser</sup>, *M. barkeri* MS tRNA<sup>Pyl</sup> and *M. barkeri* tRNA<sup>Ala</sup><sub>UGC</sub>. Differences in *M. barkeri* MS tRNA<sup>Pyl</sup> are indicated by arrows. The boxes indicate the unusual structure shared by the tRNAs.

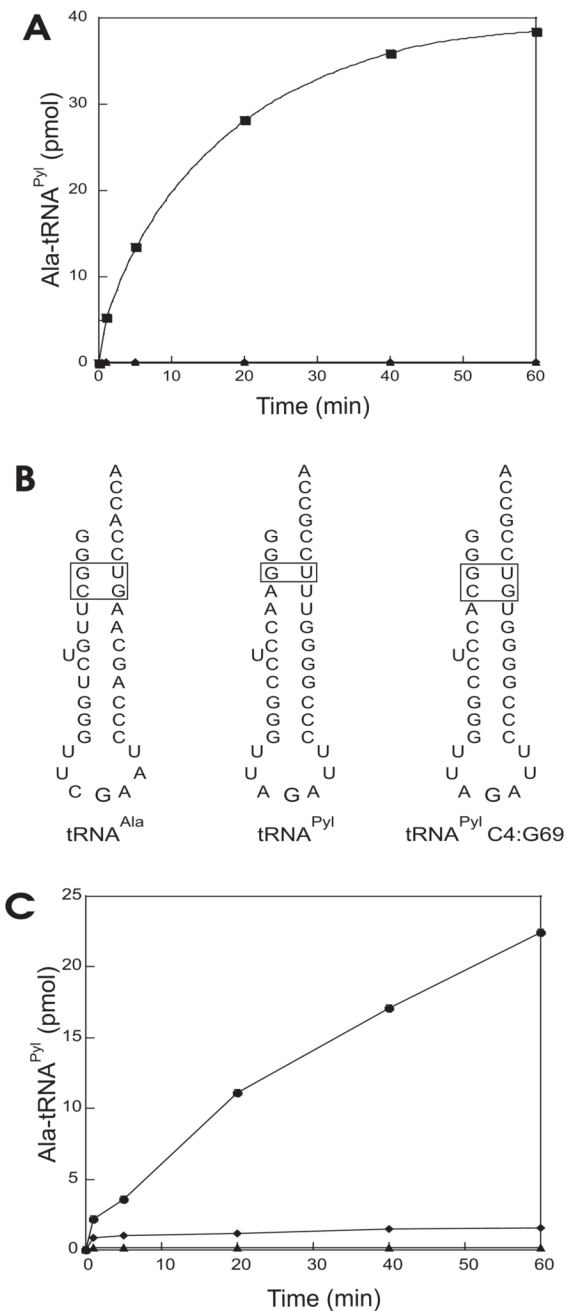




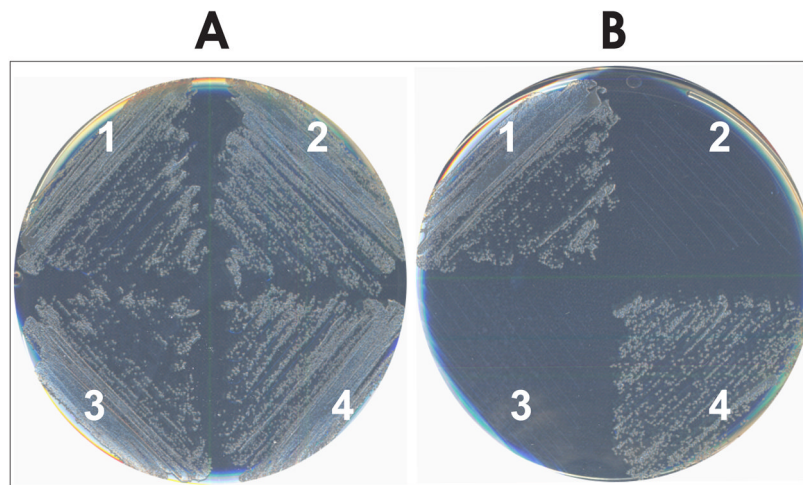
**Fig. 2.** Misacylation of *M. barkeri* Fusaro tRNA<sup>Pyl</sup> with serine *in vitro*. tRNA<sup>Pyl</sup> and bacterial-type SerRS (●), tRNA<sup>Pyl</sup> and methanogenic type SerRS (▲), no enzyme (◆).



**Fig. 3.** Incorporation of serine and Cys in a  $\beta$ -galactosidase/luciferase fusion reporter protein. Bars represent UAG read-through efficiency: *M. barkeri* tRNA<sup>Pyl</sup> and either *M. barkeri* bacterial like SerRS (B-SerRS), *M. barkeri* methanogenic SerRSs (M-SerRS), *E. coli* SerRS (Ec-SerRS) or an empty expression vector (pET15 empty). As positive control, *M. barkeri* tRNA<sup>Pyl</sup> and *M. barkeri* PylRS (Mb-PylRS) in the presence of Cys was measured. Negative controls include measurements with either M-SerRS or B-SerRS without PylT.



**Fig. 4.** Misacylation of *M. barkeri* MS tRNA<sup>Pyl</sup> with alanine *in vitro*. (A) *In vitro* aminoacylation of wild type *M. barkeri* MS tRNA<sup>Pyl</sup>. Total *E. coli* tRNA with *E. coli* AlaRS (■), *M. barkeri* MS tRNA<sup>Pyl</sup> with *E. coli* AlaRS (▲), no enzyme (◆). (B) Mini-helices based on the sequences of, from left to right, *M. barkeri* MS tRNA<sup>Ala</sup> (tRNA<sup>Ala</sup>), *M. barkeri* MS tRNA<sup>Pyl</sup> (tRNA<sup>Pyl</sup>), *M. barkeri* MS C4:G69 tRNA<sup>Pyl</sup> mutant (tRNA<sup>Pyl</sup> C4:G69). (C) *In vitro* aminoacylation of mini-helices. MS tRNA<sup>Ala</sup> mini-helix with *E. coli* AlaRS (●), MS tRNA<sup>Pyl</sup> mini-helix with the *E. coli* AlaRS (▲), MS tRNA<sup>Pyl</sup> C4:G69 mutant mini-helix with *E. coli* AlaRS (◆).



**Fig. 5.** Suppression of an *E. coli* strain *trpA94* with the *M. barkeri* MS tRNA<sup>Py1</sup> C4:G69 mutant. *E. coli* transformants were streaked on M9 medium (A) with Trp (B) without Trp. *E. coli* transformed with 1, C4:G69 *M. barkeri* MS tRNA<sup>Py1</sup> mutant; 2, *M. barkeri* MS tRNA<sup>Py1</sup> wild type; 3, empty plasmid; 4, tRNA<sup>Ala</sup> amber suppressor.