

## Sequence Divergence of Seryl-tRNA Synthetases in Archaea

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The genomic sequences of *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum* contain a structurally uncommon seryl-tRNA synthetase (SerRS) sequence and lack an open reading frame (ORF) for the canonical cysteinyl-tRNA synthetase (CysRS). Therefore, it is not clear if Cys-tRNA<sup>Cys</sup> is formed by direct aminoacylation or by a transformation of serine misacylated to tRNA<sup>Cys</sup>. To address this question, we prepared SerRS from two methanogenic archaea and measured the enzymatic properties of these proteins. SerRS was purified from *M. thermoautotrophicum*; its N-terminal peptide sequence matched the sequence deduced from the relevant ORF in the genomic data of *M. thermoautotrophicum* and *M. jannaschii*. In addition, SerRS was expressed from a cloned *Methanococcus maripaludis* *serS* gene. The two enzymes charged serine to their homologous tRNAs and also accepted *Escherichia coli* tRNA as substrate for aminoacylation. Gel shift experiments showed that *M. thermoautotrophicum* SerRS did not mischarge tRNA<sup>Cys</sup> with serine. This indicates that Cys-tRNA<sup>Cys</sup> is formed by direct acylation in these organisms.

Aminoacyl-tRNA formation is a crucial process in the cell, ensuring translation of the genetic mRNA with the required exquisite accuracy. Aminoacyl-tRNA synthetases catalyze the esterification of an amino acid onto their cognate tRNA; this direct process is the major route of aminoacyl-tRNA formation. However, there is also an indirect route, relying on tRNA-dependent transformation of an amino acid incorrectly charged to tRNA (3, 7). Aminoacyl-tRNA synthetases are highly conserved in evolution; the amino acid sequences and structures of a synthetase specific for a certain amino acid normally show a high degree of conservation in different organisms (4). This aided the task of gene assignments in the analysis of genomic sequences. However, there are two exceptions. The known genomic sequences of two archaea, *Methanococcus jannaschii* (2) and *Methanobacterium thermoautotrophicum* (15), lack recognizable open reading frames (ORFs) for lysyl- and cysteinyl-tRNA synthetases. We now know that some archaea and some bacteria contain an unusual class I lysyl-tRNA synthetase structurally unrelated to the canonical class II lysyl-tRNA synthetase present in most bacteria and eukaryotes (8, 9). However, it is still unknown how Cys-tRNA<sup>Cys</sup> is formed. tRNA-dependent amino acid transformation (7) may be a route involving misacylation of tRNA<sup>Cys</sup> with serine by seryl-tRNA synthetase (SerRS) and subsequent thiolation of the tRNA-bound serine in a reaction similar to the formation of selenocysteinyl-tRNA<sup>Sec</sup> (1). Here we report experiments which show that *M. thermoautotrophicum* SerRS does not misacylate tRNA<sup>Cys</sup>. Thus, it is unlikely that amino acid transformation is the pathway for Cys-tRNA<sup>Cys</sup> formation in methanogenic archaea.

### MATERIALS AND METHODS

**General.** *M. thermoautotrophicum* Marburg (DSM 2133) was grown anaerobically at 65°C on 80% H<sub>2</sub>-20% CO<sub>2</sub>-0.1% H<sub>2</sub>S as previously described (14). The cells were harvested anaerobically and stored at -80°C until use. tRNA was purified from frozen *M. thermoautotrophicum* or *Methanococcus maripaludis* cells by standard procedures (19), except that DEAE-cellulose chromatography was included as a final step; 20 mg of tRNA was obtained from 50 g of cell mass. *Escherichia coli* tRNA was purchased from Sigma. Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed as described previously (6), and gels were stained with Coomassie brilliant blue (12). Protein concentrations were determined with the assay kit from Bio-Rad. Proteins were prepared for N-terminal sequencing (performed by the Keck Biotechnology Resource Laboratory, Yale University) by blotting from an SDS-gel onto an Immobilon-P membrane (Millipore) (17). Standard molecular biology methods were as described previously (13).

**SerRS assay.** Enzyme activity was measured as described previously for other SerRS enzymes (11). The reaction mixture contained 0.1 M HEPES (pH 8.0), 10 mM magnesium acetate, 10 mM KCl, 10 mM dithiothreitol, 1 mM ATP, 50 mM serine, 3.6 mM <sup>3</sup>[H]serine (specific activity, 19.7 Ci/mmol), and tRNA (1 mg/ml). Incubations were performed at 60°C for the *M. thermoautotrophicum* enzyme and at 37°C for the *M. maripaludis* or *E. coli* SerRS. One unit of enzyme activity is defined as 1 pmol of serine charged per min per mg of protein.

**Purification of SerRS from *M. thermoautotrophicum* cells.** All steps described were performed at 4°C. Frozen *M. thermoautotrophicum* cells (10 g) were resuspended in 30 ml of buffer A (50 mM Tris-HCl [pH 7.6], 10 mM magnesium chloride, and 2 mM dithiothreitol) plus 100 U of RNase-free DNase (Boehringer Mannheim), disrupted by passing the suspension twice through a French pressure cell at 1.5 MPa, and centrifuged at 100,000 × g for 60 min. The resulting S-100 extract was diluted with an equal volume of buffer A and applied to a Q-Sepharose FF HiLoad column. After the column was washed with 50 ml of buffer A the SerRS-containing protein fraction was eluted with 0.2 M NaCl in buffer A. The eluate was pooled, dialyzed against buffer A, and adjusted to 1.5 M potassium acetate before being separated by hydrophobic interaction chromatography on phenyl-Sepharose HP HiLoad 16/10, developed with a decreasing potassium acetate gradient (1.5 to 0 M), and washed with buffer A. SerRS eluted in the absence of potassium acetate and the protein fractions were pooled and applied to a MonoQ 5/5 column; SerRS eluted at 0.2 M NaCl. The proteins were eluted with a linear salt gradient from 0 to 0.4 M NaCl. Active fractions were pooled, concentrated, and further separated by gel filtration on Superose 12 in buffer A.

**Cloning of the *M. maripaludis* *serS* gene.** An *M. maripaludis* genomic λ Zap Express library was screened with <sup>32</sup>P-labeled oligonucleotides. The oligonucleotide sequences were from regions of the *M. jannaschii* and *M. thermoautotrophicum* *serS* gene with high conservation in known *serS* genes. A clone which contained the complete *serS* coding region was isolated and sequenced. The clone included 8 bp upstream of the *serS* ATG start codon at the 5' end and the gene for a 50S ribosomal protein downstream. A forward primer with an *Nde*I site at the ATG start codon and a backward primer a few base pairs downstream

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TABLE 1. Purification of *M. thermoautotrophicum* SerRS

Fraction	Total amt of protein (mg)	Sp act (pmol min <sup>-1</sup> mg <sup>-1</sup> )	Total activity (pmol)	Enrichment factor (fold)	Yield (%)
S-100	307.4	7.6	2,334		
Q-Sepharose	44.1	43.3	1,905	5.7	81.6
Phenyl-Sepharose	4.62	109	504	14.3	21.6
MonoQ 5/5	0.61	417	254	55	10.9
Superose 12	0.016	6,530	105	859	4.5

of the stop codon containing a *BspI* site were used in a PCR on DNA isolated from this clone. The PCR product was cut with restriction enzymes and directly cloned into expression vector pET11a (Invitrogen). This plasmid (pET11a-SerS) was sequenced and transformed into BL21(DE3) for expression of the protein.

**Expression and isolation of *M. maripaludis* SerRS.** *E. coli* BL21(DE3) cells containing pET11a-SerS were grown at 30°C in Luria-Bertani medium with 100 µg of ampicillin per ml. Expression of the protein was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 5 h, the cells were collected by centrifugation and resuspended in 25 mM HEPES (pH 7.2)–10 mM KCl–5 mM dithiothreitol–4 mM β-mercaptoethanol–10% glycerol. The cells were disrupted by sonication, and a cell-free S-100 fraction was obtained by centrifugation for 1 h at 100,000 × g. To separate the expressed archaeal protein from the *E. coli* SerRS, the S-100 fraction was applied to a MonoQ HR 10/10 column previously equilibrated with 50 mM HEPES (pH 7.2)–10 mM magnesium chloride–2 mM β-mercaptoethanol. The proteins were eluted from the column with a linear gradient from 0 to 0.5 M NaCl. Fractions containing *M. maripaludis* SerRS, eluting around 0.2 M NaCl, were pooled, adjusted to 1.5 M ammonium sulfate, and loaded onto phenyl-Sepharose HiLoad. The column was developed with a linear gradient from 750 to 0 mM ammonium sulfate. The *M. maripaludis* SerRS was eluted from the column at 0.66 M ammonium sulfate, while the *E. coli* SerRS was eluted at 0.20 M.

**tRNA separation on acid-urea gels and Northern blot hybridization.** Aminoacyl-tRNA for separation on acidic polyacrylamide-urea gels was recovered from charging-reaction mixtures by extraction with phenol (equilibrated with 10 mM sodium acetate [pH 5.0]–50 mM NaCl) followed by ethanol precipitation. Aliquots of charged and uncharged tRNA were resuspended in sample buffer (0.1 sodium acetate [pH 5.0], 8 M urea, 0.05% bromophenol blue, 0.05% xylene cyanol) and fractionated on a 6.5% polyacrylamide gel as described previously (18). tRNA was transferred onto a Nytran membrane (Schleicher & Schuell) and fixed to the membrane by baking at 70°C for 2 to 3 h. Northern hybridization was carried out for 12 h at 42°C, as described previously (18), with 5'-<sup>32</sup>P-labeled oligonucleotides complementary to positions 25 to 49 of *M. thermoautotrophicum* tRNA<sup>Cys</sup> or positions 25 to 46 of tRNA<sup>Ser</sup> (mixed probe of three tRNA<sup>Ser</sup> sequences).

**Phylogenetic tree analysis.** The phylogenetic tree was generated from complete SerRS sequences available in GenBank or Swissprot. The tree was based on maximum-likelihood analysis of quartets of aligned amino acid sequences by using the Puzzle program (16).

**Nucleotide sequence accession number.** The nucleotide sequence of the *M. maripaludis* *serS* gene has been deposited in GenBank under accession no. AF009822.

## RESULTS

**Purification of *M. thermoautotrophicum* SerRS.** Examination of the *serS* ORF in the *M. jannaschii* and *M. thermoautotrophicum* genomic sequence revealed a protein which showed the poorest alignment with all known SerRS enzymes. Therefore, we decided to purify SerRS from *M. thermoautotrophicum*, to correlate it to the ORF by microsequencing its amino-terminal region, and to study its biochemical properties. SerRS was purified from frozen *M. thermoautotrophicum* cells by standard column chromatographic techniques, using seryl-tRNA formation of unfractionated *M. thermoautotrophicum* tRNA as the assay (see Materials and Methods) (Table 1). After purification on four columns, the enzyme was about 860-fold purified. The protein fraction was judged by SDS-gel electrophoresis to be over 90% pure; the SerRS protein had a mass of 62 kDa. The SerRS preparation acylated unfractionated *M. thermoautotrophicum* tRNA to a level of 140 pmol of serine per  $A_{260}$  unit of tRNA. This level of tRNA<sup>Ser</sup> is similar to what is found in *E.*

TABLE 2. Heterologous charging by SerRS

SerRS source	Charging of:		
	<i>E. coli</i> tRNA	<i>M. maripaludis</i> tRNA	<i>M. thermoautotrophicum</i> tRNA
<i>E. coli</i>	++ <sup>a</sup>	–	–
<i>M. maripaludis</i>	++	++	+
<i>M. thermoautotrophicum</i>	+	++	++

<sup>a</sup> *E. coli* tRNA was charged to 160 pmol/ $A_{260}$  unit by *E. coli* SerRS.

*coli*. The amino-terminal sequence of the gel-purified SerRS was established to be MKFKLKGIIKLSK. This is identical to the first 13 amino acids of the deduced sequence of ORF Mt1076 (15). Thus, this ORF encodes SerRS.

**Cloning and expression of *serS* from *M. maripaludis*.** To provide another example of this uncommon SerRS we decided to clone *serS*, the gene encoding this enzyme, from the mesophilic archaeon *M. maripaludis*. A genomic clone was isolated by screening a genomic λ Zap Express library with degenerate oligonucleotides designed from the conserved regions of the *serS* gene from *M. thermoautotrophicum* and *M. jannaschii*. The nucleotide sequence was determined; the *serS* ORF also encoded a less common SerRS. The *serS* gene was subcloned into pET11a for expression of SerRS in *E. coli*. The expressed protein was mostly insoluble at 37°C, but soluble and active SerRS could be obtained by expression at a lower temperature (30°C). The expressed archaeal protein was separated from the *E. coli* SerRS by phenyl-Sepharose column chromatography. The partially purified SerRS charged *M. maripaludis* tRNA to a level of 120 pmol of serine/ $A_{260}$  unit of tRNA.

**Serylation of various tRNAs by the archaeal SerRS enzymes.** The ability of SerRS from both methanogens and from *E. coli* to charge homologous and heterologous unfractionated tRNA samples was tested (Table 2). *E. coli* SerRS charged neither *M. thermoautotrophicum* nor *M. maripaludis* tRNA. While the *M. jannaschii* and *M. thermoautotrophicum* tRNA<sup>Ser</sup> species contain the identity elements required for recognition by *E. coli* SerRS, the presence of archaea-specific base modifications (see, e.g., reference 10) could be the reason for the lack of charging of *M. thermoautotrophicum* and *M. maripaludis* tRNA by *E. coli* SerRS. However, both archaeal SerRS enzymes

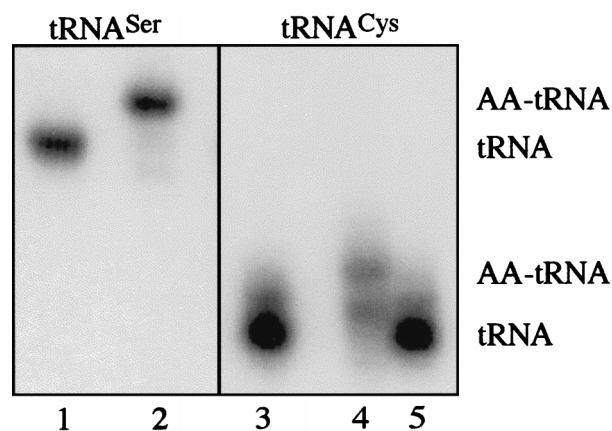


FIG. 1. Northern blot analysis of unfractionated *M. thermoautotrophicum* tRNA charged with purified *M. thermoautotrophicum* SerRS. Charged and uncharged tRNA was separated (18), and the blots were probed with a tRNA<sup>Ser</sup> and tRNA<sup>Cys</sup> probe. Lanes: 1 and 3, uncharged tRNA; 2 and 5, tRNA charged with serine by *M. thermoautotrophicum* SerRS; 4, tRNA charged with cysteine by *E. coli* CysRS. AA, amino acid.

	motif I	motif II	motif III	
Hsa	GYIPIYTFPF	FRQEVGSHGRDTRGIFRVHQFEKIE	ATMCATTRTICAI	Eukarya
Sce	GYIPLQAPVM	FRREAGSHGKDAWGVFRVHAFEKIE	STLAATQRALCCI	
Ath	GFTGLQPPFF	FRKEAGSHGRDTLGIQFRVHQFEKIE	STLTATERTICCI	
Dme	GFKLISVFDI	YRAETSGL.QEEKGIYRVHQFNKVE	GTATAIPRLIAL	
Eco	GYSENYVPYL	FRSEAGSYGRDTRGLIRMHQFDKVE	GSGLAVGRTLVAV	Bacteria
Bsu	NYTEVIPPYM	FRSEAGSAGRDRGLIRQHQFNKVE	GSGLAVGRTVAAI	
Taq	GFLPMTLPSY	FRSEAGSFGKDVRLMRVHQFHKVE	NTALATPRILAML	
Hma	EYVDVLPPI	FRREAGEHGTETRGYVRVHQFHKVE	GSGLAVPRVLVAI	Archaea
Afu	DFTIVSPPYM	FRKEAGAHGKDTKGIQFRVHQFNKVE	STAIATTRAITAI	
Pho	GFTVPVIPPYM	FRKEAGTAGKDTKGIQFRVHQFHKVE	STAIATSRIVVAI	
Mja	GFQECLFPKL	YRWEAGG...ARGLDRVNEFLRVE	CTGYGITRWLVGY	Methanogenic Archaea
Mth	GFVECLFPKL	YRWEAGG...SKGLDRVHEFQRVE	CTGIGLSRWIYGF	
Mma	GFDECLFPKL	YRWEAGG...AKGLDRVNEFLRGE	CTGYGLSRWLIGF	
	gφxxφxxPφφ	FRxE--(4-12)-->RxxxFxxxE	gφgφgφeRφφφφφ	

FIG. 2. Alignment of motifs 1, 2, and 3 (4) from a number of representative SerRSs. The sequences (accession numbers) are from *Homo sapiens* (X91257) (Hsa), *Saccharomyces cerevisiae* (X04884) (Sce), *Arabidopsis thaliana* (Z70313) (Ath), *Drosophila melanogaster* (Y14823) (Dme), *Escherichia coli* (X04017) (Eco), *Bacillus subtilis* (D26185) (Bsu), *Thermus aquaticus* (sp:P34945) (Taq), *Haloarcula marismortui* (X91007) (Hma), *Archaeoglobus fulgidus* (AE000962) (Afu), *Pyrococcus horikoshii* (AB009490) (Pho), *Methanococcus jannaschii* (U67550) (Mja), *Methanobacterium thermoautotrophicum* (AF009823) (Mth), and *Methanococcus maripaludis* (AF009822) (Mma). They were aligned with the Clustal program (16), and the motif regions are presented.

charged *E. coli* tRNA in addition to archaeal tRNA. The *M. thermoautotrophicum* SerRS charged *E. coli* tRNA to a lesser extent (72 pmol per  $A_{260}$  unit) than did the *E. coli* enzyme (160 pmol per  $A_{260}$  unit), whereas *M. maripaludis* SerRS recognized *E. coli* tRNA well. All archaeal tRNA<sup>Ser</sup> isoacceptors are equal in length and contain 16 bases in their variable loop; two *E. coli* serine isoacceptors have longer variable loops (18 and 21

bases). Possibly, the longer tRNA<sup>Ser</sup> species are not substrates for the *M. thermoautotrophicum* enzyme.

We then attempted to determine whether *M. thermoautotrophicum* SerRS is able to mischarge *M. thermoautotrophicum* tRNA<sup>Cys</sup> with serine. The *M. thermoautotrophicum* genomic sequence predicts the presence of three tRNA<sup>Ser</sup> and one tRNA<sup>Cys</sup> species. As in other organisms, the tRNA<sup>Ser</sup> species have a long variable loop whereas the tRNA<sup>Cys</sup> is 16 nucleotides shorter. Thus, these tRNAs should appear as separate bands on gel electrophoresis (18). We acylated unfractionated *M. thermoautotrophicum* tRNA with serine and separated charged and uncharged tRNA by acidic polyacrylamide-urea gel electrophoresis (Fig. 1). The positions of tRNA<sup>Cys</sup> and tRNA<sup>Ser</sup> in the charged and uncharged forms were identified by Northern hybridization with appropriate <sup>32</sup>P-labeled oligonucleotides. Half the gel was hybridized with the tRNA<sup>Ser</sup> probe (Fig. 1), and the other was hybridized with the tRNA<sup>Cys</sup> oligonucleotide (Fig. 1). A major portion of the tRNA<sup>Ser</sup> was acylated, as shown by the lower gel mobility (upper band in Fig. 1, lane 2). However, *M. thermoautotrophicum* tRNA<sup>Cys</sup> was not misacylated (Fig. 1, compare lanes 3 and 4) by partially purified homologous SerRS (leading to some degradation of tRNA). However, the *M. thermoautotrophicum* tRNA could be charged with cysteine by purified *E. coli* CysRS (Fig. 1, lane 4). Hence, the *M. thermoautotrophicum* SerRS does not mischarge tRNA<sup>Cys</sup> with serine.

## DISCUSSION

The *serS* genes in the three methanogenic archaea *M. thermoautotrophicum*, *M. maripaludis*, and *M. jannaschii* display only low similarity to *serS* genes present in bacteria, eucarya, and even some archaea (*Archaeoglobus fulgidus*, *Pyrococcus furiosus*, *Pyrococcus horikoshii*, *Pyrobaculum aerophilum*, and *Haloarcula marismortui*). The genomic annotated sequence alignment had raised some doubt about the correct identification of this gene in these organisms. As a class II aminoacyl-tRNA synthetase, SerRS is defined by the presence of three sequence motifs (4, 5). Figure 2 shows a sequence alignment of these motifs from a number of SerRS enzymes of eukaryotic,

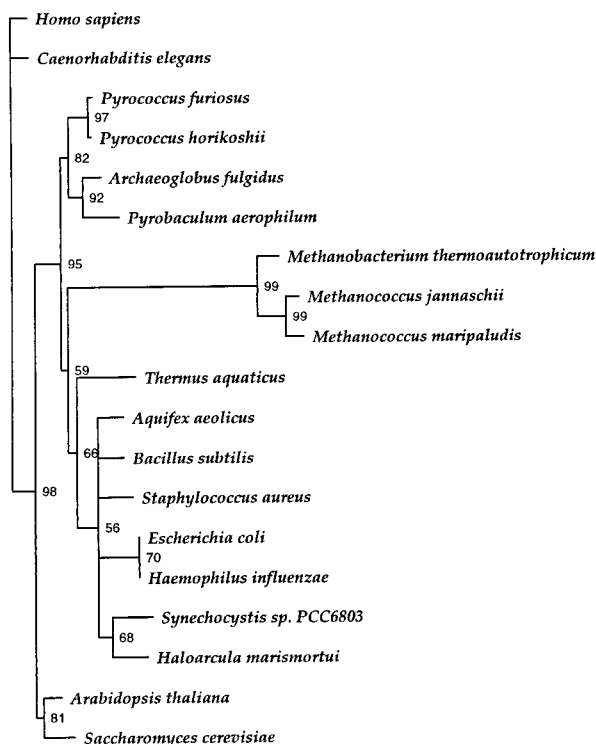


FIG. 3. Phylogenetic analysis of SerRS alignments by maximum likelihood analysis, using the Puzzle program (16).

bacterial, and archaeal origins. As can be seen, the overall similarity between the SerRS from the methanogenic archaea and SerRS proteins from other organisms is lower. In addition, motif II has a gap which is absent in other SerRS proteins, including those from the other archaea *Haloarcula marismortui*, *Pyrococcus horikoshii*, and *Archaeoglobus fulgidus*. The compilation also reveals some sequence insertions elsewhere in the sequence of the methanogenic archaeal enzymes (data not shown).

However, as demonstrated above, this enzyme is the active SerRS in these methanogenic archaea. An evolutionary tree (Fig. 3), based on maximum-likelihood methods, furthermore reveals that the SerRS of the methanogens cluster in a distinct clade separate from all other SerRSs including those of other archaea. It may also be pertinent that based on our current sequence knowledge, SerRS from the archaeon *Haloarcula marismortui* does not cluster with any of the archaea.

The absence of a recognizable cysteinyl-tRNA synthetase in the genomes of *M. thermoautotrophicum* and *M. jannaschii*, taken together with the pronounced difference of their SerRS sequence (see above), lent credence to the notion that in these organisms Cys-tRNA<sup>Cys</sup> might be produced by a tRNA-dependent thiolation of Ser-tRNA<sup>Cys</sup> resembling the synthesis of selenocysteinyl-tRNA<sup>Sec</sup> (1). Our in vitro experiments with purified SerRS do not support this idea. However, if serylation of tRNA<sup>Cys</sup> required proteins in addition to *M. thermoautotrophicum* SerRS, an unlikely scenario based on our current knowledge of aminoacyl-tRNA formation, we would not have detected mischarging.

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