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Prolyl-tRNA synthetase from *Thermus thermophilus* is eukaryotic-like but aminoacylates prokaryotic tRNA^{Pro}

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Aim. Cloning, sequencing and expression of the gene encoding prolyl-tRNA synthetase, a class IIa enzyme, from the extreme thermophile *T. thermophilus* HB8 (ProRSTT). **Methods.** Search for the ProRSTT gene was performed by Southern blot hybridization with chromosomal DNA, the digoxigenin-labeled PCR fragments of DNA being used as a probe. **Results.** The gene of *T. thermophilus* HB8 ProRS has been cloned and sequenced. The predicted 477-amino acid protein is significantly more similar in sequence to eukaryotic and archaeal than to eubacterial ProRS. Sequence analysis shows two distinct structural groups of ProRS which most likely had diverged early in evolution: (1) eukaryotic/archaeal group characterized by the absence of an insertion domain between motifs 2 and 3 and by the presence of an extra C-terminal domain beyond the normal class IIa anticodon binding domain; and (2) prokaryotic with a very large insertion between motif 2 and 3 and no extra C-terminal domain. **Conclusions.** *T. thermophilus* proS gene was overexpressed in *Escherichia coli* and overproduced ProRSTT was purified to high homogeneity. In spite of its eukaryotic-like features, *T. thermophilus* ProRS exhibited highly specific cross-species aminoacylation. The charging ability of the ProRSTT is restricted to prokaryotic tRNA^{Pro}.

Keywords: prolyl-tRNA synthetase from *Thermus thermophilus*, proS gene, cloning, sequencing, expression.

Introduction. Aminoacyl-tRNA synthetases (aaRSs) play a crucial role in the translation of the genetic code in all living organisms. The fidelity of protein synthesis depends to a large extent on the high specificity with which aaRSs charge their cognate tRNAs with the correct amino acid. In spite of the universality of the genetic code, there are barriers to aminoacylation across taxonomic domains that need the tRNA-synthetase coadaptation during the evolution. The adaptations made by aaRSs are sometime correlated with changes in the architecture and/or sequence, which are specific for a certain group of organisms [1, 2].

The aaRSs are divided into two quite distinct classes (class I and class II) on the basis of primary structure and the fold of catalytic domain [3, 4]. Class IIa prolyl-tRNA synthetase exists in several distinct archi-

tectures in different organisms. There are basically two distinct types of ProRS, each with two variants. The first is the eukaryotic-like type, characterized by a unique C-terminal domain. The second is the prokaryotic-like enzyme, lacking this domain [5, 6]. Each of these two types is recognizable by specific sequence characteristics which even extend to the residues in the catalytic domain. Both types share a common anticodon binding domain characteristic of class IIa synthetases. At another level of organization, a key difference is the existence and position of a unique editing domain (of about 180 residues) that has been shown to be responsible for hydrolyzing mischarged alanine [7, 8]. In higher eukaryotes and some bacteria there is no editing domain, although the genes encoding a separate editing-like domain, denoted ProX, have been identified (e. g. in mouse, human, *Clostridium sticklandii*). In lower eukaryotes (e. g. malaria, trypanosome, yeast), the editing do-

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main is found as an N-terminal extension. In the prokaryote-like systems, there is generally an editing domain inserted into the catalytic domain (most bacteria, e. g. *Escherichia coli*, *Mycobacterium tuberculosis*, *Enterococcus faecium*), but some bacteria (e. g. *Rhodospseudomonas palustris*, *Wolbachia*) and putative mitochondrial ProRS (e. g. human) have prokaryotic-like ProRS with the lack of insertion domain. In some systems a separate gene encoding an editing-like domain has been identified (ProX or YbaK) and their capability to edit the mischarged alanyl-tRNA^{Pro} in trans has been shown [8]. The investigation of tRNA^{Pro} recognition by the *E. coli* and human enzymes has also provided evidences of the divergent adaptation between tRNA and synthetase [9, 10]. Prokaryotic-like ProRS generally recognize A73 and G72 of acceptor stem of tRNA^{Pro}. On the other hand, in eukarya all cytoplasmic tRNA^{Pro} sequences have C73 and C72 and other determinants for recognition, for example by the human enzyme, which are located only in the anticodon. To understand the structural basis for tRNA^{Pro} recognition and amino acid specificity of the different ProRS types, intensive structural work was done. *T. thermophilus* ProRS has been crystallized [11] and its structure has been determined at 2.43 Å resolution, the first reported structure of ProRS [5, 6]. We have also crystallized the ProRS-tRNA^{Pro} complex [12] and have solved its structure [5, 6, 13]. Recently we have solved the structure of prokaryotic ProRS from *E. faecalis* [14]. The active site of prokaryotic-like ProRS of the pathogenic bacteria has an open conformation, while the active site of ProRS from *T. thermophilus* is more compact. Thus, the essential differences in the structures of these enzymes can be considered as start point for successful development of drugs that would selectively inhibit pathogenic bacteria, without side effects for the host human organism. Therefore, we cloned the *proS* gene of *T. thermophilus* as a part of structural and functional study on ProRSs. Here we report the cloning, sequencing and sequence analysis and aminoacylation activity of the recombinant *T. thermophilus* prolyl-tRNA synthetase using tRNA^{Pro} from different sources.

Materials and methods. *Purification of prolyl-tRNA synthetase from T. thermophilus and Activity Assays.* *T. thermophilus* HB8 was grown in 300-liters fermenter at 76 °C as described by Oshima and Imahori

[15]. The cells were harvested by centrifugation and broken with a French press. The enzyme was purified from the cell extract by successive chromatography on DEAE-Sephadex, Toyoperl HW-65, hydroxyapatite, and heparin-Sephadex column [11]. Approximately 30 mg of prolyl-tRNA synthetase from 1 kg of *T. thermophilus* cells were obtained. As judged by sodium dodecyl sulfate (SDS)-PAGE analysis, the purified enzyme was at least 95 % homogeneous.

The aminoacylation reaction proceeded at 37 or 60 °C, respectively, in 50 µl reaction mixture containing 100 mM Tris-HCl (pH 7.9), 10 mM magnesium chloride, 2.5 mM ATP, 2 mg/ml unfractionated *E. coli* tRNA («Boehringer Mannheim», Germany), 20 µM L-[¹⁴C]proline (330 mCi/mmol, «Amersham», USA) and a suitable amount of enzyme.

N-terminal and internal amino acid sequence analysis. The amino acid sequences of the N-terminal peptide and five internal peptides of purified *T. thermophilus* prolyl-tRNA synthetase were determined by the Protein and Peptide Group at EMBL, Heidelberg, by microsequencing.

Two peptides: KETGVQNAYFP and RAPIQVVI, were used to design oligonucleotides which were purchased from Genosys.

Recombinant DNA techniques, Southern hybridization, construction and screening of the genomic library. General recombinant DNA methods were performed according to Sambrook et al. [16]. Genomic DNA from *T. thermophilus* HB8 was prepared by the method of Marmur [17]. Degenerate oligonucleotides oligo 1 and oligo 2, were designed on the basis of amino acid sequence of two internal peptides, according to the preferential codon usage of *T. thermophilus*. Oligo 1: 5'-ACSGGSGTSCAGAACGCSTACTT-3'; oligo 2: 5'-GATSACSACCTGRATSGGSGC-3' (S = C or G; R = A or G). These two oligos were used as primers in PCR experiment with 1 µg of genomic DNA in 100 µl mixture containing 50 mM Tris-HCl buffer (pH 9.0), 20 mM (NH₄)₂SO₄, 0.2 mM dNTP and 2.5 U Tub DNA polymerase («Amersham»). The amplification was performed under the following conditions: 4 min at 95 °C; 35 cycles of 1 min at 95 °C, 2 min at 52 °C and 2 min at 72 °C. The product was analyzed on 1 % agarose gel and subsequently cloned into the PCR II vector («Invitrogen», USA). This PCR product was labelled using the digoxi-

genin DNA labelling kit («Boehringer Mannheim») and used as probe for Southern blotting and colony hybridization. The genomic minilibrary was constructed by ligation of a *Bam*HI digest of chromosomal DNA in *pUC19* after linearization by *Bam*HI digestion and dephosphorylation. DH5 was used as *E. coli* host for screening of the *T. thermophilus proS* gene.

DNA sequencing. PCR fragments and the chromosomal DNA subcloned fragments were sequenced by the dideoxynucleotide chain-termination method [18] using [³⁵S]-dATP or [³⁵S]-dCTP (ICN), the Sequenase version 2.0 DNA sequencing kit (USB) and Thermo Sequenase cycle sequencing kit («Amersham»).

Overexpression of *T. thermophilus* prolyl-tRNA synthetase in *E. coli* and purification of recombinant enzyme. For overexpression of the *T. thermophilus* prolyl-tRNA synthetase in *E. coli* the *pET17b* vector was used. The coding region of the *proS* gene was amplified from the genomic clone in 30 cycle PCR using Tub polymerase at the annealing temperature of 58 °C with oligonucleotides 3 and 4, which contain the recognition sites for *Nde*I and *Bam*HI, respectively. Oligo 3: 5'-CATATGGC-GAAGGAGAGAAGGGCCTAACCC-3'; oligo 4: 5'-GGATCCTATTAGTA-CGCCTTGGCGAAGACCACCG-3'. The PCR product was firstly cloned into the PCR II vector and then subcloned into *pET17b*. Finally, the 1279 bp *Kpn*I-*Dra*III fragment of a resulting vector was replaced by chromosomal *Kpn*I-*Dra*III fragment of *T. thermophilus proS* gene.

E. coli BL21(DE3)pLysS cells carrying the recombinant plasmid were grown at 37 °C in 6 liters of LB medium containing 100 µg/ml ampicillin. When the A600 of the culture reached the value of 0.8, expression was induced by the addition of 0.5 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside). The cells were harvested 4 h after induction. The pellet of cells was resuspended in 420 ml of lysis buffer (50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 10 mM 2-mercaptoethanol and 4 % (v/v) glycerol) and cells were disrupted by freezing/defreezing method. Then 4.2 ml of 1 M MgCl₂ and 5000 U of DNaseI («Boehringer Mannheim») was added to the mixture and incubated 25 min at 37 °C. Cell debris was removed by centrifugation (20 min, 13,000 g). The supernatant was heated at 70 °C for 40 min and centrifuged at 13,000 g for 30 min. Then the supernatant was dialyzed overnight against buffer A (20 mM Tris-HCl, pH 7.9,

5 mM MgCl₂, 0.1 mM PMSF, 1 mM dithiothreitol and 0.1 mM NaN₃) before application on DEAE-Sepharose column (2.6 × 25 cm), equilibrated with the same buffer. Protein was eluted with 1 l of linear gradient from 0.03 to 0.3 M sodium chloride in buffer A. The active fractions were collected, dialyzed, and chromatographed on heparin-Sepharose column (1.2 × 37 cm). The column was developed with a linear gradient of 0–0.25 M KCl in 0.5 l buffer A.

Results and discussion. *Cloning and sequencing of proS gene.* For further study on the structure-function relationships of ProRSTT we cloned the corresponding structural gene. The purified enzyme from *T. thermophilus* cells provided several short peptide sequences: N-terminal sequence of 20 residues and 5 internal tryptic peptides. Using sequence information from two peptides, the oligonucleotide primers were designed which allowed PCR amplification of a partial gene fragment (700 bp) of ProRSTT. The correspondence of this fragment to a putative *proS* gene was verified by DNA sequencing. The PCR fragment was labelled with digoxigenin and then used as a probe for Southern blot hybridization to *T. thermophilus* chromosomal DNA digested with several restriction enzymes (Fig. 1, A). The probe was hybridized to the *Bam*HI fragment of about 2.75 kb (Fig. 1, B). This 2.75 kb *Bam*HI was cloned into the *pUC19* plasmid. The positive clones were identified by colony hybridization and sequenced.

Analysis of the nucleotide sequence of proS gene and amino acid sequence of its product. The nucleotide sequence of 1900 bp DNA fragment containing the *T. thermophilus proS* gene was determined on the both strands. The open reading frame of this gene contains 1431 bp, from which the 477 amino acid residues protein was deduced (Fig. 2). The calculated relative molecular mass of this protein is 54453 Da. This is close to the value of 54 kDa estimated by SDS/PAGE and in agreement with the value of 110 kDa estimated by gradient PAGE under non-denaturing condition for the purified enzyme from *T. thermophilus* cells, taking into account a dimeric structure of ProRS. The aminoterminal sequence and five peptides were identical to those determined by amino acid sequencing, showing unambiguously that the correct gene has been identified (Fig. 2).

Comparison of the amino acid composition of the thermophilic and mesophilic, *E. coli* ProRS shows a

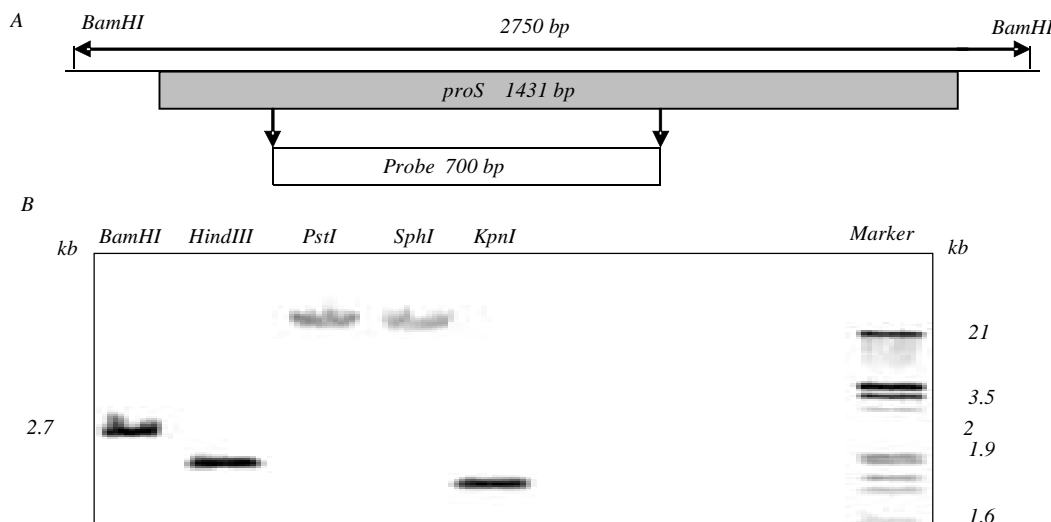


Fig. 1. Cloning of *T. thermophilus* *proS* gene: A – schematic presentation of the *Bam*HI fragment of *T. thermophilus* chromosomal DNA which covers the entire gene sequence of ProRS and the probe, which was used to search for this gene; B – Southern blot hybridization of the *T. thermophilus* HB8 chromosomal DNA with 700-bp PCR fragment as a probe

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MAKEKGLTPQSQDFSEWYLEVIQKAELADYGPVVRGTIVVRPYGYAIWENI  50
QQVLDRMFKETGHQNAYFPLFIPMSFLRKEAEHVEGFSPELAVVTHAGGE  100
ELEEPLAVRPTSETVIGYMWSKWIRSWRDLPQLLNQWGNVVRWEMRTRPF  150
LRTSEFLWQEGHTAHATREEAEEVRRMLSIYARLAREYAAIPVIEGLKT  200
EKEKFAGAVYTTTTIEALMKDGKALQAGTSHYLGENFARAFDIKFQDRDLQ  250
VKYVHTTSWGLSWRFIGAIIMTHGDDRGLVLPPRLAPIQVVIVPIYKDES  300
RERVLEAAQGLRQALLAQGLRVHLDDRDQHTPGYKFHEWELKGVPFRVEL  350
GPKDLEGGQAVLASRLGGKETLPLAALPEALPGKLDAFHEELYRRALAFR  400
EDHTRKVDTYEAFKEAVQEGFALAFHCGDKACERLIQEETTATTRCVPFE  450
AEPEEGFCVRCGRPSAYGKRVVFAKAY

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Fig. 2. The amino acid sequence of ProRSTT deduced from the nucleotide sequence of the *proS* gene. The amino acids underlined correspond to the peptide sequences determined by protein sequencing

decrease in asparagine, serine, methionine and isoleucine, and an increase in cysteine, tryptophan and histidine in content of the thermophilic protein [19]. Concerning cysteine, it appears 3 times more frequently in the *E. coli* proteins than in *Thermus* proteins [19]. But in the case of ProRS only one cysteine is present in the *E. coli* enzyme whereas 5 cysteines were found in *T. thermophilus* enzyme. All these cysteines are located in the C-terminal domain of *T. thermophilus* ProRS and as can be seen from the crystal structure of ProRSTT, four of them form a tetrahedral binding site for zinc ion [6]. The analysis of the frequency of the codons shows a pronounced preference for codons possessing G or C at the third position. It is as high as 94 %, which is similar to that in other *Thermus* genes [20].

The amino acid sequence of *T. thermophilus* *prolyl-tRNA synthetase* appears eukaryote-like. Comparison of the amino acid sequence of ProRSTT with all other known ProRSs surprisingly shows significantly more similarity to the enzymes from the cytoplasm of eukaryotes (e. g. 35 % homology to the yeast ProRS and 37 % to the *Drosophila melanogaster* and human

ProRS) than to eubacterial ProRSs (18 % homology to the *E. coli* ProRS and 14 % to the *Chlamydia trachomatis* ProRS). Phylogenetic analysis confirms the fact that there are two distinct structural groups of ProRS (Fig. 3). The first group is eukaryotic/archeal-like including ProRS from cytoplasm of eukaryotes, *Mycoplasma genitalium*, *Metanococcus jannaschii* (archae) and *T. thermophilus*, characterised by the absence of an insertion domain between motifs 2 and 3 and by the presence of an extra C-terminal domain beyond the normal class IIa anticodon binding domain. The second group is prokaryotic-like including ProRS from mitochondria of eukaryotes, *E. coli* and *C. trachomatis*, which have a very large insertion between motifs 2 and 3 and no extra C-terminal domain. These two forms had diverged early from a primitive ancestor of ProRS. ProRS represents the third *T. thermophilus* synthetase (in addition to GlyRS and IleRS) [1, 21] which has eukaryotic/archeal-like characteristics rather than prokaryotic-like, suggesting some possible horizontal gene transfer event.

T. thermophilus *prolyl-tRNA synthetase* aminoacylates tRNA from *E. coli* but not from yeast and bovine

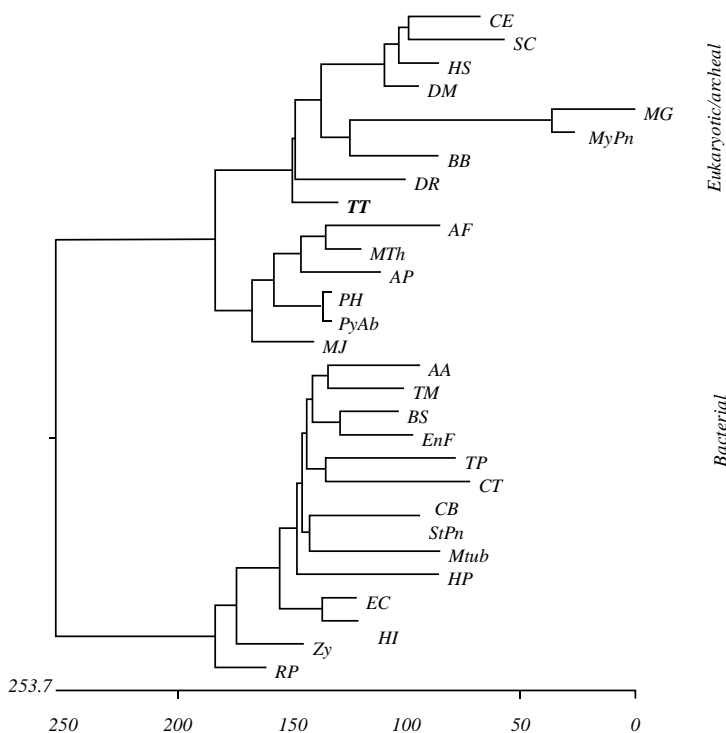


Fig. 3. Phylogenetic tree of ProRS sequences. The tree was rooted between the *Bacteria* and the *Archaea* plus *Eukarya*. Abbreviations: AA – *Aquifex aeolicus*; AP – *Aeropyrum pernix*; AF – *Archaeoglobus fulgidus*; BB – *Borrelia burgdorferi*; BS – *Bacillus subtilis*; CB – *Clostridium botulinum*; CE – *Caenorhabditis elegans*; CT – *Chlamydia trachomatis*; DM – *Drosophila melanogaster*; DR – *Deinococcus radiodurans*; EC – *Escherichia coli*; EnF – *Enterococcus faecalis*; HI – *Haemophilus influenzae*; HP – *Helicobacter pylori*; HS – *Homo sapiens*; MJ – *Methanocaldococcus jannaschii*; MyPn – *Mycoplasma pneumoniae*; MTh – *Mycobacterium tuberculosis*; MG – *Mycoplasma genitalium*; PH – *Pyrococcus horikoshii*; PyAb – *Pyrococcus alba*; RP – *Rickettsia prowazekii*; SC – *Saccharomyces cerevisiae*; StPn – *Streptococcus pneumoniae*; TM – *Thermotoga maritima*; TP – *Treponema pallidum*; TT – *Thermus thermophilus*; Zy – *Zymomonas mobilis*. The tree was generated using MegAlign with version 5.1 of DNA STAR package programs. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events

liver. To overexpress the ProRS from *T. thermophilus* in *E. coli* cells, the *proS* gene was cloned between *NdeI* and *BamHI* sites of the *pET17b* vector (see the Materials and methods). The overexpressed ProRSTT was purified by heat treatment of the crude cellular extract followed by ion-exchange chromatographies on DEAE-Sephrose and heparin-sepharose. SDS/PAGE analysis of the purified recombinant ProRSTT revealed a band (more than 98 % of purity) with molecular weight of 54 kDa, which is consistent with molecular weight expected from the deduced amino acid ProRSTT sequence and exactly corresponds to molecular weight of the enzyme purified from *T. thermophilus* cells.

Due to the fact that *T. thermophilus* ProRS resembles eukaryotic synthetases, one can expect that it might charge eukaryotic tRNA. We tested this suggestion by *in vitro* aminoacylation of total tRNA from *T. thermophilus*, *E. coli*, yeast and bovine liver by ProRSTT. Fig. 4 shows that *E. coli* and *T. thermophilus* tRNAs are aminoacylated by ProRSTT with the same efficiency. At the same time eukaryotic yeast and bovine tRNAs are very poor substrates for the *T. thermophilus* enzyme. Eubacterial (*E. coli*) ProRS recognizes the identity determinants of tRNA^{Pro} at the acceptor stem and anticodon loop [9]. The primary structures of two isoacceptor

forms of tRNA^{Pro} from *T. thermophilus*, determined in our laboratory, have revealed the same determinants in the acceptor stem as in case of *E. coli* tRNA^{Pro} [22]. They have A73 and G72 instead of C73 and C72 present in the structure of the acceptor stem of eukaryotic tRNA^{Pro}. When a certain aaRS crosses from one taxonomic domain to another it has to adapt to a new tRNA sequence. ProRSTT provides interesting example of adaptation which differs from known in the literature. For example, it has been reported that the eukaryotic-like bacterial *T. thermophilus* glycyl-tRNA synthetase is able to charge U73-containing bacterial tRNA^{Gly}, but the enzyme could also efficiently aminoacylate yeast tRNA^{Gly} containing A73 [1]. Thus, this eukaryotic-like bacterial enzyme adapted to prokaryotic tRNA without losing capacity to charge the eukaryotic one (as result of lacking sensitivity to N73). The case of co-adaptation of ProRSTT is more complicated and probably affected other parts of tRNA in addition to the acceptor stem. Although the structure of ProRSTT complexed with tRNA^{Pro} was solved by us, the tRNA acceptor stem appeared not to be ordered [5, 6], and therefore, the structural basis for the acceptor stem recognition by ProRS is still unknown. Thus, for further studies it is necessary to obtain new crystals of the ProRSTT-tRNA^{Pro}

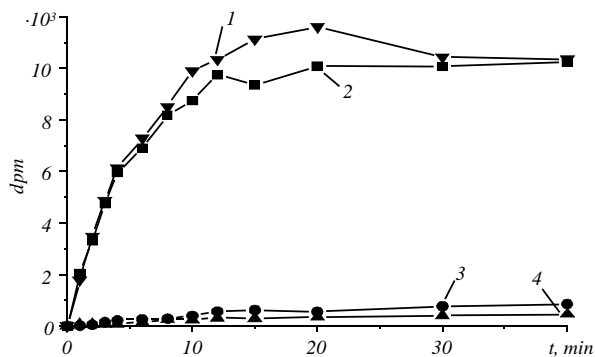
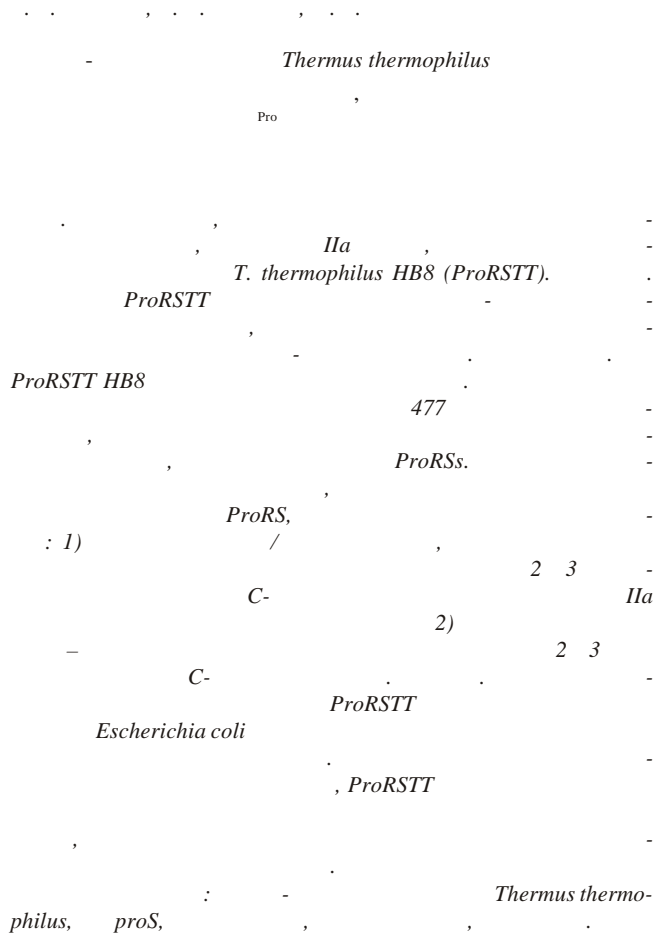


Fig. 4. Time-cours of aminoacylation of tRNA^{Pro} from different organisms by ProRSTT: 1 – *E. coli*; 2 – *T. thermophilus*; 3 – bovine; 4 – yeast

complex in which the tRNA acceptor stem is bound to the catalytic site of the enzyme. The differences between two groups of ProRSs are of great interest from both the evolutionary and drug design points of view. Thus, the described here genetic construct for overexpression of *T. thermophilus* ProRS is important to develop our structural and functional studies in both directions.

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