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In vivo assembly of plasmid-expressed ribosomal protein S7 of *Thermus thermophilus* into *Escherichia coli* ribosomes and conditions of its overexpression

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Abstract Researchers still have great difficulty in isolating individual ribosomal proteins from the ribosome in quantities high enough for structural research. To this end, when studying protein S7, we created an *E. coli* overproducer of the recombinant protein S7 of *Thermus thermophilus*. The vector for expression was pQE-32 having a strong promoter of *E. coli* phage T5 and six triplets of His at the 5′-end. This N-terminal six His tag of the fusion protein is responsible for binding to Ni-NTA-resin and allows purifying the protein in one step. The yield of the recombinant protein was 20% and more of the total cellular proteins. In addition we have shown that the recombinant thermophilic protein is incorporated in vivo into the ribosome of *E. coli* despite the fact that these proteins (thermophilic and mesophilic) have a rather low homology, only 52%. This fact provides a base for the system to study functions of individual proteins.

Key words: Overexpression; Ribosomal protein S7; Assembly (in vivo); *Thermus thermophilus*; *Escherichia coli*

1. Introduction

Protein S7, a small protein of the bacterial ribosome, playing an essential role in the assembly of the 30S small ribosomal subunit, binds to the 3′-domain of the 16S rRNA, and stimulates binding of some other proteins to form a well-defined ribonucleoprotein neighborhood [1–3]. Protein S7 regulates a balance of expression of the str-operon by the feed-back translation mechanism [4,5]. Protein S7 binds with tetracycline, an antibiotic, which inhibits tRNA binding with the A-site of the ribosome [6]. Cross-links of protein S7 have been located with the 16S rRNA [7,8], with the −3,−4 region of mRNA [9], with the anticodon loop of tRNA both in the A- and P-sites [10]. In addition protein S7 cross-links with initiation factor IF-3 [11].

Gaining an understanding of all these processes will require high-resolution structural information. Such information can be derived both from X-ray analysis data and from detailed knowledge of RNA–protein interactions. That is why we created an overproducer of protein S7 of *T. thermophilus*. We further discovered that the plasmid born S7 protein of *T. thermophilus* can replace the S7 protein of *E. coli* during the assembly of the ribosome in vivo.

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2. Materials and methods

2.1. Construction of the overproducer

The vector for expression, pQE-32 (QIAGEN Inc.), was kindly provided by the Department of Virology, A.N. Belozersky Institute of Physico-Chemical Biology. It has the *E. coli* phage T5 strong promoter with two lac operator sequences, a synthetic ribosomal binding site, six codons for His which binds to the Ni-NTA (nitrilo-tri-acetic acid) resin with high affinity, a polylinker, and transcriptional terminator 'to' from phage lambda [12].

The *rpsG* gene of *T. thermophilus* was from the Z14.2 plasmid, kindly provided by Dr. N.I.Matvienko (Institute of Protein Research, Pushchino, Russia) [13]. The *rpsG* gene was amplified from the plasmid by PCR with Pfu-polymerase (USB, USA) and two primers. The right primer was 17-mer, CTTGTGGATCCGGCCCG, and fully complementary to the original *Bam*HI site some 100 nucleotides downstream of the terminator codon of the S7 protein. The left primer was designed to have the *Bam*HI site in frame with the vector, and had two extra codons for Asn and Gly. These two amino acids may be used if it is needed to cleave out all but extra N-terminal amino acids:

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GCGCGGATCCCC AAC GGA CGG AGAAGGAGAGCAGAGGTC
     BamHI   Asn Gly Arg3 protein S7
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After purification of the PCR product in a low melting agarose gel and cutting it with *Bam*HI it was cloned into pQE-32 into the *Bam*HI site. The ligation mixture was transformed into *E. coli* JM109 by the DMSO method [14] with some modifications. The desired clone (pQS12.212) was subjected to a restriction analysis and sequencing by the dideoxy method. We found only one replacement in its primary structure: T216→C which fortunately does not change the primary structure of the protein.

2.2. Overexpression of protein S7

Overexpression was performed by overnight induction with IPTG of *E. coli* cells JM109/pQS12.212 at the stationary stage in the LB media. The cell pellets obtained were resuspended in Laemmli-SDS sample buffer and boiled for 3 min. Electrophoresis of the total proteins was carried out according to Laemmli in 15% PAG and the proteins were stained with Coomassie G-250 [15]. The level of expression of recombinant protein S7 was measured by scanning to be 20% and more of the total cellular proteins.

2.3. Isolation of the thermophilic S7 protein

Recombinant protein S7 of *T. thermophilus* was isolated from the *E. coli* cells either under denaturing or nondenaturing conditions as recommended by manufacturer [12]. In the first case cells were resuspended in a solution of 6 M guanidine chloride, and the solution should become translucent. Then it was directly loaded on the Ni-NTA-column, all proteins were washed out by a solution of 8 M urea, and the recombinant S7 protein was removed by 100 mM EDTA solution.

In the second case cells were disrupted by lisozyme, frozen and thawed in liquid nitrogen, the supernatant applied on the column, all proteins were washed with 0.3 M NaCl and recombinant protein S7 desorbed with the same EDTA solution.

2.4. Incorporation of the recombinant S7 protein of T. thermophilus into the E. coli ribosome in vivo

To study the incorporation of the recombinant thermophilic protein

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into *E. coli* ribosomes in vivo, *E. coli* cells JM109/pQS12.212 were grown up to the middle logarithmic phase (0.5 at 600 nm). All further operations were performed at +4°C. Cells were collected, resuspended in 200 μl of 10/100 buffer (10 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 100 mM NH_4Cl, 1 mM 2-mercaptoethanol), supplemented with 20 μl of lysozyme (10 mg/ml) and incubated for 30 min on ice. The tube was frozen and thawed three times in liquid nitrogen. The solution was clarified by centrifugation at 14,000 rpm for 20 min. The supernatant was spun in a SW 50.1 rotor at 45,000 rpm for 90 min. The ribosomal pellet was resuspended in 0.5 ml of 0.5/100 buffer and the subunits were separated by 10–30% sucrose gradient ultracentrifugation in a SW 41 rotor at 24,000 rpm for 16 h. Fractions of the 30S subunit were collected and analyzed by the same PAGE.

3. Results and discussion

As the first step in an attempt to study the structure and function of ribosomal protein S7 we decided to clone the *rpsG* gene of *T. thermophilus* into the expression plasmid. We selected a relatively new expression system, the pQE series. The vector is described in section 2. Using PCR and primers we cloned a coding region of the protein from the third codon for Arg till the end, i.e. till the original termination codon of the protein S7, all in all 156 codons. Adding 5 amino acids, coded by the primer, and 6 His we obtained the following protein with 171 amino acids altogether: just before the sequence for the protease cleavage (Asn-Gly) we introduced the codon for Pro, hoping that it will keep the 6 His tag away. This resulted in the following N-terminal structure of the fusion protein:

```
ATG AGA GGA TCT ----- GGG ATC CCC AAC GGA CGG ---------
Met Arg Gly Ser (His)6 Gly Ile Pro Asn Gly Arg3 --S7
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Upon induction with IPTG of *E. coli* cells JM109/pQS12.212 at the stationary stage, the yield of the overproduction of recombinant protein S7 of *T. thermophilus* was 20% and more of the total cellular proteins as evidenced by PAGE (Fig. 1, line 4). Its migration was notably faster than that of the host *E. coli* ribosomal S7 protein which has 179 amino acids.

The recombinant S7 protein can be isolated with the Ni-NTA-support either under denaturing or nondenaturing conditions with the yield of 30 μg per 1 ml of the cell culture at the stationary phase.

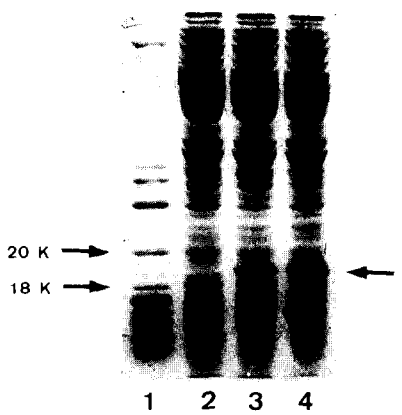

Fig. 1. PAGE separation of the total proteins of *E. coli* cells JM109/pQS12.212. Line 1, marker proteins of the 30S subunit from *E. coli* MRE600; line 2, total proteins from the stationary stage cells (for a day) which were induced with IPTG early in the growth (D_{600} ~0.5–0.6); line 3, total proteins from cells growing without IPTG for a day; line 4, total proteins from cells induced with IPTG at the stationary stage for 12 hrs. The right arrow points to protein S7 of *T. thermophilus*, and the left arrows point to the markers: 18 and 20 kDa (the latter is *E. coli* protein S7).

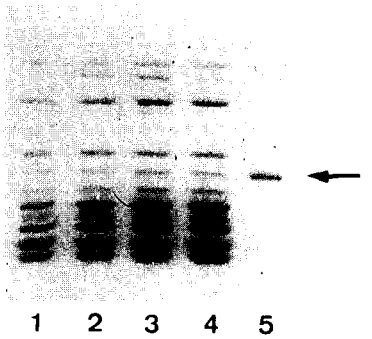

Fig. 2. PAGE separation of the 30S subunit proteins isolated from the 70S ribosomes treated with increasing concentrations of the salt. Line 1, 20/1000, (10 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 1000 mM NH_4Cl); line 2, 10/500; line 3, 10/350; line 4, 10/100; line 5, isolated recombinant protein S7 from *T. thermophilus*. The arrow shows the position of the S7 protein of *T. thermophilus*.

Interestingly when inducing the same strain with IPTG early in the growth (D_{600} ~0.5–0.6) no visible synthesis of the recombinant S7 protein of *T. thermophilus* occurred (Fig. 1, line 2). We have no reasonable explanation for this phenomenon. It is appropriate to suggest that it is the host ribosomes that affect its expression. There is indirect evidence for this supposition.

Even with two sites of repression the T5 promoter for *E. coli* JM109 was not completely repressed and was fairly leaky allowing the expression of the protein from the plasmid at any stage of the growth. Thus the overnight incubation of the cells led to the accumulation of a large amount of the recombinant protein S7 of *T. thermophilus*, though less than when inducing the stationary phase with IPTG (Fig. 1, lines 3,4).

An interesting question is whether it would be possible for thermophilic proteins to replace a host one in the course of ribosome assembly in vivo.

To test the possibility of heterological incorporation of recombinant protein S7 of *T. thermophilus* into *E. coli* ribosomes in vivo, we took advantage of the accumulation of this protein without IPTG and worked with the cells at the middle-logarithmic phase. We isolated ribosomes and washed them by ultracentrifugation in solutions with increasing concentration of NH_4Cl up to 1 M. Then ribosomal subunits were separated by sucrose gradient ultracentrifugation and the proteins of the 30S subunits were analyzed by PAGE. Surprisingly, recombinant protein S7 of *T. thermophilus* was found to remain within the 30S subunits of *E. coli* up to 1 M NH_4Cl, in amounts slightly less then the host *E. coli* S7 protein (Fig. 2). A surprising thing is that this happened to one of the key proteins of the assembly and functioning despite the fact that the homology of the primary structures of proteins S7 of *T. thermophilus* and *E. coli* was not high, just about 50%. This fact suggests that should the tertiary structure of the proteins be similar the distribution of conservative regions in the structure must be similar too thus allowing the interchangeability of the proteins when reconstituting the ribosome. Garnier analysis [16] using 'Genebee' program of the secondary structure of these proteins reveals some similarity in the distribution of their α-helical regions.

The possibility to make in vitro a complex between thermo-

philic protein S7 and the fragment of *E. coli* 16S rRNA binding site [1], is in vein with our in vivo data (to be published).

It has earlier been shown that heterological incorporation may happened to some of chloroplast ribosomal proteins [17,18]

Heterologous recombination in vitro of thermophilic ribosomal proteins [15] was also observed with 5S rRNA *E. coli* [19].

Our results have shown that such heterologous interactions are possible even for key protein of the assembly and functioning of ribosomes. The obtained data show great promise for studies of ribosomal proteins and their functions.

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