

Ribosomal protein L22 from *Thermus thermophilus*: sequencing, overexpression and crystallisation

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Abstract The gene for the ribosomal protein L22 from *Thermus thermophilus* has been sequenced and overexpressed in *Escherichia coli*. A multiple sequence alignment was carried out for all proteins of the L22 family reported so far. The recombinant protein was purified and crystallized. The crystals belong to the space group $P2_12_12_1$, with cell parameters of $a = 32.6$ Å, $b = 66.0$ Å, $c = 67.8$ Å.

Key words: Ribosomal protein; Overexpression; Purification; Crystallisation; *Thermus thermophilus*

1. Introduction

L22 is a small core protein of the 50S ribosomal subunit. Its function in the ribosome is not clear. However, some data suggest that together with several other ribosomal proteins it may affect the folding of the ribosomal 23S RNA [1]. The main interest in L22 has been based on its involvement in the interactions of the antibiotic erythromycin with ribosomes. Erythromycin is known as an inhibitor of the elongation of the nascent peptide chain [2]. It was demonstrated that mutations in *Escherichia coli* L22 produced cells resistant to erythromycin [3]. Affinity labelling studies with erythromycin derivatives showed that the antibiotic binds to L22 as well as to the ribosomal proteins L2 and L4, which probably also interact with 23S RNA [1,4]. Erythromycin-resistant mutants of *E. coli* L22 and L4 have recently been characterised [5]. In L4 the resistance is caused by a single substitution of a conserved lysine residue by a glutamate, while in L22 by a deletion of a triplet Met-Lys-Arg (residues 82–84), conserved in homologous ribosomal proteins from several other organisms.

An understanding of the function of L22 in ribosomes, identification of a potential erythromycin and probably RNA binding sites will undoubtedly be facilitated by a knowledge of its three-dimensional structure. The structures of several ribosomal proteins from various sources have been determined by X-ray crystallography and nuclear magnetic resonance methods: the C-terminal fragment of L7/L12 [6], L30 [7], S5 [8],

L6 [9], L9 [10], S6 [11], S17 [12] and L1 (S. Nikonov et al., manuscript in preparation). None of these proteins possesses notable sequence homology to L22, although most of them include a folding unit believed to be involved in RNA binding.

Our experience has shown that proteins from thermophilic organisms are relatively easier to crystallise than their counterparts from mesophiles [13]. In order to obtain an amount of L22 sufficient for crystallisation and structure investigation, the gene (rpl22) from the extreme thermophile *Thermus thermophilus* was cloned, sequenced and overexpressed, and a purification procedure was developed. The complete sequence has been deposited in the EMBL Nucleotide Sequence Database (accession number X84708). The recombinant protein was crystallised by the hanging drop technique.

2. Experimental

2.1. Bacterial strains

T. thermophilus was grown as described by Zheltonosova et al. [14]. *E. coli* strains TG1, CJ236 and BL21(DE3) were grown on SOB medium and made competent by the method of Hanahan [15]. The transformed cells were grown on 2 × LB [16].

2.2. Recombinant DNA techniques

Chromosomal DNA was isolated from *T. thermophilus* strain VK1 using standard methods [17]. The expression plasmid pACA [18] contained the T7 RNA polymerase promoter for the efficient expression of cloned genes, and the fl origin of replication to enable production of single-stranded DNA. Uracil-containing single-stranded form of the vector was prepared from *E. coli* strain CJ236 (*dur*⁺, *ung*⁺) by the method of Kunkel et al. [19].

The coding region of the gene rpl22 was isolated by PCR with the primers: *L22*_{start} (5' phosphorylated) 5'-GTTTAACTTTAAGAAG-GAGATATACCATGGAAGCGAAAGCGATCGCC-3'; *L22*_{stop} 5'-CAGTGAAAGTGAAACTGAATCAATCTTATTTCCTGCTT-CTCCCC-3'. The PCR primers were constructed so that the produced PCR-fragment contained extensions in the 5' and 3' ends that were complementary to two regions of the expression vector. One strand of the PCR product was directly annealed to the uracil-containing single-stranded DNA of the vector using the 3' and 5' extensions. The annealing mixture consisted of purified PCR product in previously optimised concentration, 100 ng single-stranded vector, 0.5 mM each of dNTPs, 1 × PCR buffer and 1.5 U Tth polymerase. This mixture was incubated at 95°C for 3 min, 67°C for 0.5 min and 37°C for 1 min in a heating block. Immediately after this step the extension-ligation mixture (40 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, 30 mM NaCl, 15 mM DTT, 0.5 mM ATP, 4 U T4 DNA ligase, 1 U T7 DNA polymerase) was added. After 20 min of incubation at 23°C the reaction mixture was used for transformation of *E. coli* strain TG1.

DNA sequencing was performed by the M13-dideoxymethod [20] with Sequenase Version 2.0 Kit (US Biochemicals). Sequence compressions were solved with the help of Taq Track Sequence Deaza System

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(Promega). Universal pUC/M13 Forward Primer was used for M13 sequencing. The protein coding region after cloning into the expression vector was checked by direct sequencing from the plasmid DNA with Sequenase Version 2.0. Primers complementary to the expression vector were used for control sequencing of the cloned gene.

2.3. Cloning and sequence analysis

Plasmid pBR322-tthS8 [21] was used in sequencing studies. This plasmid contained the *spe* and *S10* operons of *T. thermophilus* (V. Vysotskaya, unpublished data). The gene *rpl22* was localised in a *HincII*–*XbaI* digestion fragment of pBR322-tthS8 with the size of approximately 1.5 kb and cloned in the M13mp19 vector after linearisation by *SmaI* and *XbaI*. Both strands of this fragment were sequenced. The gene *rpl22* encodes a polypeptide chain of 113 amino acids (*M_r* 12765).

The sequences for several proteins homologous to L22 were obtained from EMBL and Swiss-Prot databases. WISCONSIN software package Version 8.0.1-UNIX 1994 was used for DNA sequences analysis. Amino acid sequences were aligned by PileUp program of GCG package with a gap penalty of 3.0.

The ribosomal protein L22 was cloned into pACA vector using the 'sticky feet'-directed mutagenesis protocol [22]. The search for positive colonies which have the insertion of *rpl22* gene was done by screening of 120 colonies using PCR and the original 'sticky feet' primers. One plasmid, called pTthL22, isolated from the clone with correct gene sequence was transformed into *E. coli* strain BL21 (DE3). Protein production was induced by adding IPTG to the final concentration of 0.5 mM.

2.4. Purification procedure

The recombinant protein accumulated to a level of 15% of the total cellular protein (Fig. 1, lane 2). It was purified according to the following procedure: 3 g of wet cells were disintegrated by grinding with alumina and resuspended in 30 ml of 50 mM Na-acetate, pH 5.5, 150 mM MgCl₂, 800 mM NaCl. The suspension was centrifuged for 20 min at 15,000 × *g* to remove cellular debris and heated at 55°C for 10 min. After pelleting of precipitated *E. coli* proteins, the supernatant was diluted with 120 ml of 50 mM Na-acetate, pH 5.5, 50 mM NaCl and loaded on a CM-Sepharose column. After washing, the protein was eluted with a linear gradient from 0.15 to 1 M NaCl in 50 mM Na-acetate, pH 5.5. The fractions that were homogeneous on Coomassie blue stained SDS-PAGE (Fig. 1, lane 5) were pooled and concentrated to 27 mg/ml using a Centriprep concentrator (Amicon) with a molecular weight cutoff of 10,000 Da.

2.5. Crystallisation and X-ray data collection

The crystallisation experiments were performed at 6°C by the hanging drop vapour diffusion method [23]. The initial crystallisation conditions were found using Crystal Screen I (Hampton Research). Drops containing 3 ml of protein solution mixed with an equal volume of precipitant buffer were equilibrated against 1 ml of the same buffer. The crystals of the protein L22 were obtained at 6°C in drops equilibrated against a reservoir containing solution No. 48 of Crystal Screen I (3.0 M ammonium dihydrogen phosphate and 0.1 M Tris-HCl, pH 4.5). Long rod-like crystals appeared after 24 h and grew to a maximum size of 0.8 mm × 0.2 mm × 0.2 mm within three days. Crystals were mounted into sealed glass capillaries for X-ray studies.

X-ray diffraction data were collected with a Siemens area detector equipped with a 3-circle goniostat mounted on a Rigaku RU200BEH rotating anode. Data reduction was done by the XDS program suite [24,25].

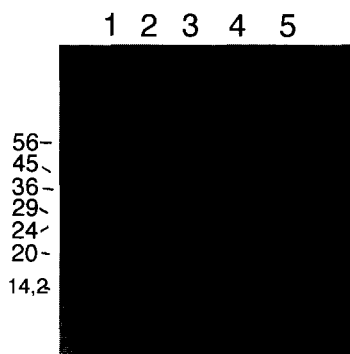


Fig. 1. Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue: (1) molecular mass standards (kDa); (2) total cell protein at 3 h after induction with IPTG; (3) soluble cell proteins; (4) soluble proteins after heating at 55°C; (5) protein L22 after CM-Sepharose chromatography.

3. Results and discussion

The deduced amino acid sequence of the ribosomal protein L22 from *T. thermophilus* was aligned with 5 eubacterial, 7 chloroplast and 2 archaeobacterial sequences of proteins homologous to L22 (Fig. 2). The alignment shows that eubacterial and chloroplast sequences are very similar and differ from the archaeobacterial protein. The main differences between the eubacterial and chloroplast sequences are found at the N- and C-terminal parts, whereas the core of the protein is well conserved. An interesting feature is the conservation of many basic residues, which seem to be clustered into two groups close to the N- and C-termini. Using *T. thermophilus* L22 numbering the first group consists of Arg¹¹, Lys¹⁶, Arg¹⁸, Arg²⁵, Lys²⁷, Lys⁴¹ and Lys⁴⁹, and the second of Lys⁸³, Arg⁸⁴, Arg⁸⁸, Arg⁹⁰, Arg⁹², Lys⁹⁸ and Arg⁹⁹. Arg⁹⁰ is substituted in some proteins by lysine, and in the case of *Bacillus stearothermophilus* by a methionine. As conserved arginine and lysine residues are often vital in interactions with nucleic acids [26], one may expect that these regions in L22 are involved in interactions with the ribosomal 23S RNA. Along with conservation of the basic amino acids there are several conserved glycine, alanine and proline residues, which are usually found in structurally restricted regions of proteins. These features might indicate that the secondary and tertiary structure of L22 in the core region is similar among different organism.

The main difference between the archaeobacterial, eubacterial and chloroplast sequences is the 30 residues insertion in archaeobacterial proteins after residue 39 (*T. thermophilus* numbering). There are only few amino acid residues which are conserved throughout all the organisms. However, it is interesting to note that the glycine and proline residues conserved

Fig. 2. Multiple alignment of fifteen amino acid sequences of the ribosomal protein L22. The first two sequences are archaeobacterial, the next six eubacterial and the final seven from plant chloroplast genome. Asterisks (*) indicate identities between all groups, plus signs (+) indicate identities between eubacterial and chloroplast groups, colons (:) indicate well-conserved amino acids (bold letters). Abbreviations: accession numbers and references are: Hma, *Halobacterium marismortui* – J05222, P10970 [28]; Hha, *Halobacterium halobium* [29]; Ala, *Acoelasma laidlawii* – M74771, P29222 [30]; Myc, *Mycoplasma-like organism* – M74770 [31]; Bst, *Bacillus stearothermophilus* [32]; Tma, *Thermotoga maritima* – Z21677 [33]; Eco, *Escherichia coli* [34]; Tth, *Thermus thermophilus* (present work); Sol, *Spinacia oleracea* – X13336, P09594 [35]; Nta, *Nicotiana tabacum* [36]; Msa, *Medicago sativa* – L00667; Mpo, *Marchantia polymorpha* – X04465, P06388 [37]; Gte, *Gracillaria tenuistipitata* – M32638, P16634; Egr, *Euglena gracilis* – M37463, P19166 [38]; Zma, *Zea mays* – Y00329, P06589 [39].

• HmaGI	SYSVEADPDT	TAKAMLRERQ	MSFKHSAITA	32			
• HhaMGI	SYSVDVDSEA	SAKAMLRERS	ISLKHSAITA	33			
• AlaM	EAKAIGKTIR	IAPRKVRLVV		21			
• MycMETK	KPKAIARKVS	IAPRKARLVV		24			
• BstM	QAKAVARTVR	IAPRKARLVI		21			
• Tma	.MKLQVPRNG	LKRSLFHKKR	KELSSSLPKI	EARAVAKYIR	49			
• EcoM	ETIAKRRHAR	SSAQKVRLVA		21			
• TthM	EAKAIARYVR	ISPRKVRLVV		21			
• Sol	MGFFKKKEKK	AEFDVFRLYG	LHHPEPGKCD	EITTRGYSIS	MSVDKARRVI	50		
• Nta	..MLKKKK..T	EVYALGEHIS	MSADKARRVI		27		
• Msa	VTPNNNNNNP	FACHVSTSQF	GVQETNKSya	EAVAVGKHIR	MSADKARRVI	10		
• MpoMQTNTSNK	KIRAVAKHIH	MSPHKVRRVV		28		
• GteMTNKT.T	KIQATGKYVR	LSTAKTRRVL		26		
• EgrMEQKKPL	ESSASIKYVR	ISPFKVRRIL		27		
• Zma	LVKYTPRIKK	KKGLRKLARK	VPTDRLLKFE	RVFKAQKRIH	MSVFKAQRVL	55		
			:	:	:	+	:	:
• Hma	REIKGKTAGE	AVDYLEAVIE	GDQVPVFKQH	NSGVGHKSKV	DGWDAGRYPE	82		
• Hha	REISGETVAD	AKEYLQAVID	EERSVPFKQH	NSGVGHRNDI	DGWDAGRYPE	83		
• Ala	DLIRGKNVKE	AQAILMFT..	PR	41		
• Myc	DLIRGKNIAQ	AQAILTFT..	PK	44		
• Bst	DLIRGKEVGE	RFAILRHT..	PK	41		
• Tma	NTIRGKSVEE	AFQILAFS..	PK	69		
• Eco	DLIRGKKVSQ	ALDILTYT..	NK	41		
• Tth	DLIRGKSLEE	ARNILRYT..	NK	41		
• Sol	DQIRGRSYAE	TLMILELM..	PY	70		
• Nta	NQIRGRSYEE	TLMILELM..	PY	47		
• Msa	DTIRGRPYEE	TLMILELM..	PY	120		
• Mpo	SQIRGRSYEQ	ALMILEFM..	PY	48		
• Gte	NQIKGKKYQE	AILILEFM..	TY	46		
• Egr	NQIKGRSAKE	ALMILKFM..	PY	47		
• Zma	DEIRWRYEE	TVMILNLM..	PY	75		
	:	*:::	:	:	+	*	:	:
• Hma	KASKAFDLDL	ENAVGNADHQ	.GFDGEAMTI	KHVAAHKVGE	QOGRKPRAMG	131		
• Hha	KASKDFLKLL	SNVSNNADQQ	.GFDAVEMVI	EHVAPHKVGE	SQGRKPRAMG	132		
• Ala	GASPVIAKVL	DSAIANRTHN	LNLNLENLFV	KEVWANESIT	MKRMLPRAKG	91		
• Myc	VAAPVILKLL	NSAVSNAVNN	LKLNREQLYV	KEVFNNEGLR	LKRMFPRAKG	94		
• Bst	AASPIIEKVL	KSAVANAENH	YDMDVNNLVI	SQAYVDEGPT	LKRFRPRAMG	91		
• Tma	KAARIMEKVL	KSRVANAENN	FGLSVENLYV	SECYVNDGPR	MKRIWPRGRG	119		
• Eco	KA AVLVKVL	ESAIAANAENH	DGADIDDLKV	TKIFVDEGPS	MKRIMPRAKG	91		
• Tth	RGAYFVAKVL	ESAAANAVNN	HDMLEDRLYV	KAAYVDEGPA	LKRVLPRAAG	91		
• Sol	RACYPPIFKLI	YSAAANASHN	KQFNKANLII	SKAEVNKGIT	LKKVKPRARG	120		
• Nta	RACYPILKLI	YSAAANASYN	MGSSEANLVI	SKAEVNGGTT	VKKLKPRARG	97		
• Msa	RACETILKIV	FSAGANASNN	LGLSKSSLVI	SKAEVNEGRT	MKRTRPRAQG	170		
• Mpo	RACNPILQLL	SSAAANANHN	FGLSKTNLFI	SEIQVNKGTF	FKRFQPRAGQ	98		
• Gte	KPCKIIEKIL	ESAGNNA.LN	LKYEKQNLII	KQAFANDGPK	LKRFPRAAGQ	96		
• Egr	KPSTLIFKLL	KSAVSNSIKN	YDEDANVLRV	LEARADAGPI	LKRLOCPHAQG	97		
• Zma	RASYPILKLV	YSAAANATHY	RDFDKTNLFI	TKAEVSRSTI	MKKIQTSSSR	125		
	:	:	+++	++	++	++	++	++
• Hma	RASAWNSPQV	DVELILEEPE	VED.....	154		
• Hha	RATTWNATLC	DVEIVVTETE	EVTA.....	156		
• Ala	SGHLIRKRTS	HITVVVAERE	111		
• Myc	SGDMIKKRTS	HITLVITSST	NLQTSKEEEQ	SGSKN	129		
• Bst	RASAINKRTS	HITIVVSEKK	EG.....	113		
• Tma	RADIIQKRTS	HITVVVRDRS	REDEYRKALE	ELQKKISSEE	159		
• Eco	RADRILKRTS	HITVVVSDR.	110		
• Tth	RADIIKRTS	HITVILGEKH	GK.....	113		
• Sol	RSYMIKRPTC	HITIVLRDIT	HFDSYDKFLE	SLTPKKLIAL	LGLMSTGRRR	170		
• Nta	RSFPPIKRSTC	HITIVMKDIS	LDDEY.....	VEM	YSLKKTWKK	135	
• Msa	RANRILKRTC	HITITVKGLP	AESVVEASSS	200		
• Mpo	RGYPIHKPTC	HITIVLNILP	K.....	119		
• Gte	RAFRIQKPTC	HITINLSIN.	115		
• Egr	RGFPPIKRTC	HITIIIV	113		
• Zma	T	126		
	:	:	+	+++	+++	+++	+++	+++

among the majority of the eubacterial and chloroplast sequences are also conserved in archaeobacteria.

As mentioned earlier, one of the *E. coli* erythromycin resistant strains has a mutant L22 with a deletion of the amino acid triplet Met⁸²–Lys⁸³–Arg⁸⁴ [3]. It is seen from Fig. 2 that two of these residues (Lys–Arg) are strongly conserved among the known eubacterial and chloroplast L22 sequences, but not in archaeobacteria. However, there is an Arg–Lys sequence conserved in archaeobacteria and shifted by just two positions relative to the Lys–Arg sequence in eubacteria and chloroplasts. Taking into account the similarity of properties of Lys and Arg, the interchange of positions may not affect the possible function of these residues. However, no experimental data regarding the effect of erythromycin on archaeobacteria are available.

It was noted earlier in the case of the ribosomal protein S5 that mutations resistant to the antibiotics streptomycin and spectinomycin were probably located close to the regions of protein–RNA interactions [8]. With respect to this it will be interesting to know the effect of these mutations on the three-dimensional structure of the proteins. Thus, the three-dimensional structure of L22 and its mutant forms should help in a better understanding of the mechanisms of resistance to antibiotics.

L22 crystals grown with hanging drop technique, diffracted to at least 3 Å resolution. The space group is $P2_12_1$, with cell dimensions $a = 32.6$ Å, $b = 66.0$ Å, $c = 67.8$ Å and one molecule in the asymmetric unit. These values correspond to V_m (volume of asymmetric unit/molecular weight) of 2.86 Å³/Da, which is in the range usually found for protein crystals [27]. The work on improving the crystal quality and the search for heavy atom derivatives is in progress.

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