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Genetic organization of the *Meiothermus ruber* Hsp70/ClpB chaperone system

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Chaperones are conservative proteins present in three kingdoms of life: bacteria, archaea and eukaryotes, with the main responsibility for assisted protein folding. Thermophilic organisms were also found to provide a complex cellular machinery of molecular chaperones. The question how thermophiles adapt could be addressed to the organization of the chaperone system also because chaperones must meet the general requirements that affect every enzymatic function at an elevated temperature. Despite the sequence homology of chaperones from thermophilic and mesophilic organisms, the organization of the *hsp70* cluster differs. The *clpB* gene is not included into the *hsp70* gene cluster of the moderately thermophilic bacterium *Meiothermus ruber*, but it was detected far away from the *hsp70* operon. ClpB from *M. ruber* is the second chaperone of this family, identified in thermophilic organisms so far, and it shows a high sequence homology to the *T. thermophilus* protein. The internal translation site of *M. ruber* ClpB upon expression in *E. coli* cells results in full-length and truncated proteins.

Key words: *Meiothermus ruber*, chaperones, *hsp70* operone, *clpB* gene

INTRODUCTION

Molecular chaperons are involved in a variety of crucial cellular functions including protein folding and proteolysis, protein translation, membrane transport. The Hsp70 (70 kDa heat shock proteins) family of protein chaperones assists a wide range of folding processes: the folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, control of the activity of regulatory proteins. All of these activities seem to be based on the property of Hsp70 to recognize and bind to the hydrophobic peptide regions of proteins in an ATP-dependent manner [1, 2]. Members of the Hsp70 protein family exist in Eubacteria, Eukarya and in some of Archaea [2]. The growing number of sequenced genomes offers a possibility to analyse the genetic and structural organization of the chaperones.

Thermophilic organisms and their proteins have attracted particular attention in the last 2–3 decades in relation with the problem how protein folding and maintenance of well defined structures are achieved. Although the native fold of a protein is encoded by its amino acid sequence, proteins of thermophilic organisms need the assistance of molecular chaperones to reach and maintain their native state efficiently [3, 4]. The description of the genetic organization and functional properties of molecular chaperones may help to identify the molecular mechanisms of these particular proteins. Only a few Hsp70 chaperone systems have so far been cloned and characterized from thermophilic bacteria: *Thermus thermophilus* [5], *Bacillus stearothermophilus* [6], *Thermotoga maritima* [7] and *Bacillus thermoglucosidasius* [8]. It has been reported that *Thermus thermophilus* expresses a homolog of the fourth member of the Hsp70 chaperone family, a protein factor necessary for the binding of DnaK (Hsp70) to DnaJ (Hsp40) [5, 9]. The gene of this 8-kDa protein, DafA, is located immediately downstream of the gene cluster *hsp70-hsp22-hsp40* on the *T. thermophilus* chromosome. The *hsp70* gene cluster of *T. thermophilus* also contains *hsp100*, a homolog of *clpB* of *E. coli*.

This article aims describing the genetic organization of the Hsp70 system and ClpB chaperone of *Meiothermus ruber*, an organism isolated from hot springs of Kamchatka. Moderately thermophilic *M. ruber* is the gram-negative bacterium not forming spores and with the lower optimal growth temperature in the range of 60–65 °C (versus about 70–75 °C for *Thermus thermophilus*). The bacterium does not oxidize sulfur. It contains a red carotenoid pigment similar to neuro-sporaxanthine and retrodehydro-gamma-carotene [10]. In comparison with the genetic organization of the best characterized Hsp70 systems from *E. coli* and *T. thermophilus*, particular properties that are connected to the adjustment to higher temperatures may be identified.

MATERIALS AND METHODS

Cloning of *clpB* gene from *M. ruber*

The probe used for Southern hybridization were the 660 bp and 540 bp fragments labeled with [α-^{32}P]dATP. The fragments were obtained by PCR from DNA of *M. ruber* using oligonucleotide primers (MWG, Germany) 5'-CTGCTTCCCCACCCCCGT-GGGCCC-3' and 5'-CTTCCCGACAAGGCCATTGACCT-3' (the PCR product obtained is the fragment of 660 bp) and the degenerate primers 5'-CCCGGCGTSGGGAAGACGGC-3' and 5'-AG-GTCIATGGCCTTGTCGGGIAG-3' (the PCR product obtained is a fragment of 540 bp). S designates G/C, I designates inosine. The

primers were designed to be complementary to the conserved regions of nucleotide binding domains of *clpB* from *T. thermophilus*, *Helicobacter pylori*, *Corynebacterium glutamicum*, *Synecoccus* sp., *Campylobacter jejuni*. Southern blot hybridization to genomic DNA from *M. ruber* was carried out by the method described by Sambrook and Rusell [11]. Fractions including fragments of approximately 800 bp and 2.7 kb from SmaI digests and the 3.0 kb fragment from PstI digest of *M. ruber* genomic DNA were ligated (all DNA restriction-modification enzymes purchased from Fermentas, Lithuania) into SmaI and PstI sites of the plasmid pUC19, respectively. DNA was transformed into the *E. coli JM109* strain by electroporation. Recombinant clones were transferred onto a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) and hybridized by the standard procedure using a labeled 800 bp fragment for the selection of clones bearing SmaI fragments and a labeled 540 bp fragment for the selection of clones bearing PstI fragments. Plasmid DNA was isolated by a standard procedure [11]. Both strands of DNA insert were sequenced in an Amersham Pharmacia Biotech ALFwin sequence analyser 2.00.

The putative promoter sequences and 30 nucleotides downstream of the start codon of *clpB* were determined by the inverted PCR method [12]. The PCR product of 600 bp was obtained using primers 5'-TGATGCTGCGGGACGCTGC-3' and 5'-TGCGGCAGGTCGATCTTCG-3'. The nucleotide sequence of the PCR product was determined at the Sequencing Center of the Institute of Biotechnology.

Nucleotide sequence accession numbers

Meiothermus ruber clpB sequence has been deposited in GenBank nucleotide sequence databases under the accession number AY303998.

Construction of expression plasmid for Mru. ClpB

Fragments of 0.6, 0.8 and 2.7 kb fragments containing *clpB* gene fragments were cloned into pUC19 plasmid and ligated via the internal SmaI, PauI and PstI sites. NdeI and HindIII sites were introduced into 5'- and 3'-ends of the clpB gene by PCR, respectively. The *clpB* was then ligated into the NdeI and HindIII sites of pET21b(+) [Novagen].

Expression of Mru.ClpB protein

Mru. ClpB was expressed in *E. coli* BL21(DE3) strain. *E. coli* cells were grown in LB broth containing 0.1 mg/ml ampicillin at 37 °C to OD_{600nm}~0.6–0.8. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, cells were grown for 2.5 h and collected by centrifugation. The cell pellet was disrupted by sonication and centrifuged. The supernatant (soluble fraction) and pellet (insoluble fraction) were then analysed by 12% SDS–PAGE.

RESULTS AND DISCUSSION

The genomic organization of *hsp70* operon from thermophilic bacteria

The *M. ruber hsp70* gene cluster was cloned using the PCR fragment as a hybridization probe and the degenerated oligonucleotide primers designed to be complementary to the conserved N-terminal region of Hsp70 from various bacterial species including *E. coli* and several thermophilic bacteria.

The sequence cloned from *M. ruber* consists of three genes in the order of *hsp70–hsp22–hsp40* [13]. The intergenic sequences between *hsp70* and *hsp22*, and *hsp22* and *hsp40* do not contain promoters; thus, these genes are organized into an operon structure. Sequence alignment of the deduced amino acid sequences of Mru.Hsp70, Mru.Hsp40 and Mru.Hsp22 revealed a homology with proteins from several thermophilic bacteria, but the highest score was obtained for proteins from *T. thermophilus* [13]. Alignment of deduced amino acid sequences of *T. thermophilus, M. ruber and B. stearothermophilus* revealed a high conservation in the ATP-ase domain located in the N-terminal domain of Hsp70 protein (data not shown). There are few promoter-like sequences upstream of the *hsp70* gene (Fig. 1).

Despite the sequence homology of chaperones from different organisms, the organization of *hsp70* loci differs (Fig. 2). Generally, genes of two co-chaperones, *hsp40* and *hsp22*, are included in the *hsp70* operon (Fig. 2, *Bacillus* species). An exception is *E. coli* where *hsp22* (*grpE*) is not included. The *hsp70* operon of *T. thermophilus* contains also *dafA* and *clpB*. The bacterial *hsp70* clusters are divided into three groups in respect to genes' position in the hsp70 operon: i) the operon consists of the genes in the order of *hsp70-hsp22-hsp40* (*Thermus thermophilus, Meiothermus ruber, Streptomyces coelicor*); ii) the operon consists of the genes in the order *hsp22–hsp70–hsp40* (*Bacillus subtilis*); iii) *hsp22* is not included into the operon (*E. coli*). The *M. ruber hsp70* cluster belongs to the first group of *hsp70* clusters.

No ORF was found for a protein corresponding to DafA of *T. thermophilus* immediately downstream of the *hsp40* gene of *M. ruber*. The *clpB* gene is not in the *hsp70* gene cluster,

Start	End	Score	Promoter sequence
352	397	0.99	TGTTTTTTTAGCCTACACCGATTTACAGGTATAAAGGTAGTCAAAAGAAC
530	575	0.82	GGTTCAAACTTGATAATACTAGACTCAACTCTATTGACTATATCAATAAT
557	602	0.96	ACTCTATTGACTATATCAATAATAACTGGTATCATATGGCTCAGTGAGCC

σ [70] promoter consensus sequence:
TAtAaT (-10)
TTGACa (-35)

σ [32] Heat shock promoter consensus sequence:
TNtCNCcCTTGAA (-35)
CCCCATtTa (-10)

Fig. 1. Promoter-like sequences upstream *hsp70* gene were determined using Neural Network Promoter prediction Program (http://www.fruitfly.org/seq_tools/promoter.html). Bold T indicates transcription initiation

but it was detected away from the *hsp70* operon. The features of the *clpB* gene of *M. ruber* are discussed further below. It should be noted that the molecular size of *T. thermophilus* and *M. ruber* chaperones is in general smaller than of the *E. coli* homologs.

Alignment of Tth.Hsp40, *E. coli* DnaJ and Mru.Hsp40 sequences revealed (Fig. 3) that Mru.Hsp40 and its homolog from *T. thermophilus* both lack the whole cysteine-rich region (Zn-binding domain) which is found in Hsp40s from mesophilic bacteria and is considered responsible for substrate binding [2, 14].

Thus, Mru.Hsp40 belongs to the second class of J-domain proteins that lack the Zn-binding domain [2]. The most conservative is the J domain containing the HPD motive (Fig. 3) which is essential for interaction with the ATP-ase domain of Hsp70. The content of proline residues (7.2%) is lower than in Hsp40 of *T. thermophilus* (10%). It should be noted that both *T. thermophilus* and *M. ruber* Hsp40 do not contain any cysteine residues, meanwhile *E. coli* Hsp40 contains even ten cysteine residues (Table 1).

Thermophilic proteins have shown an increase in the content of proline, charged amino acid residues (especially Glu, Arg, Lys) and hydrophobic amino acid residues, namely Ala and Leu [15]. The differences in amino acid composition of thermophilic and mesophilic chaperones are summarized in Table 1.

Features of *clpB* gene of *M. ruber*

M. ruber clpB was cloned using a PCR fragment as a hybridization probe and degenerated oligonucleotide primers designed to be complementary to the conserved Walker motives of two nucleotide binding domains (NBD) from various bacterial species. The cloned 3100 bp fragment has shown an ORF-deduced amino acid which was 78% identical to the *T. thermophilus* ClpB chaperone.

Prokaryotic ClpB facilitates resolubilization of protein aggregates during heat shock and cooperates with Hsp70 to enable their renaturation. Clp proteins are classified according to the number of nucleotide binding domains which are 220–250 amino acids in length and show a high level of sequence homology [16, 17]. Class two proteins contain only one NBD, whereas the class one members ClpA and ClpB contain two NBDs. *M. ruber* ClpB protein contains two nucleotide binding domains, and the size of middle region is 204 nucleotides which coincide with these of *T. thermophilus* ClpB. The size of the middle region was originally used as a basis for categorizing ClpB proteins into different subfamilies [16]. The amino acid analysis using CLUSTALW program has revealed *M. ruber* ClpB (850 amino acids) to belong to class 1 subfamily B of Clp/Hsp100 proteins [16].

A functional relationship between Hsp70 and ClpB was found [17], therefore the clpB gene is often present in the *hsp70* operon (*T. thermophilus*). However, bacterial *clpB* was also detected far away from the *hsp70* gene cluster, and *clpB* activity, is regulated by its own promoter (*E. coli*, Fig. 2) or *clpB* is included in the operon of other genes (*Pasteurella multocida*). The *M. ruber clpB* gene is not included into the *hsp70* operon, but it seems to be regulated by its own promoter sequence. A few promoter-like sequences have been detected upstream of the *clpB* gene. It should be noted that besides *T. thermophilus*

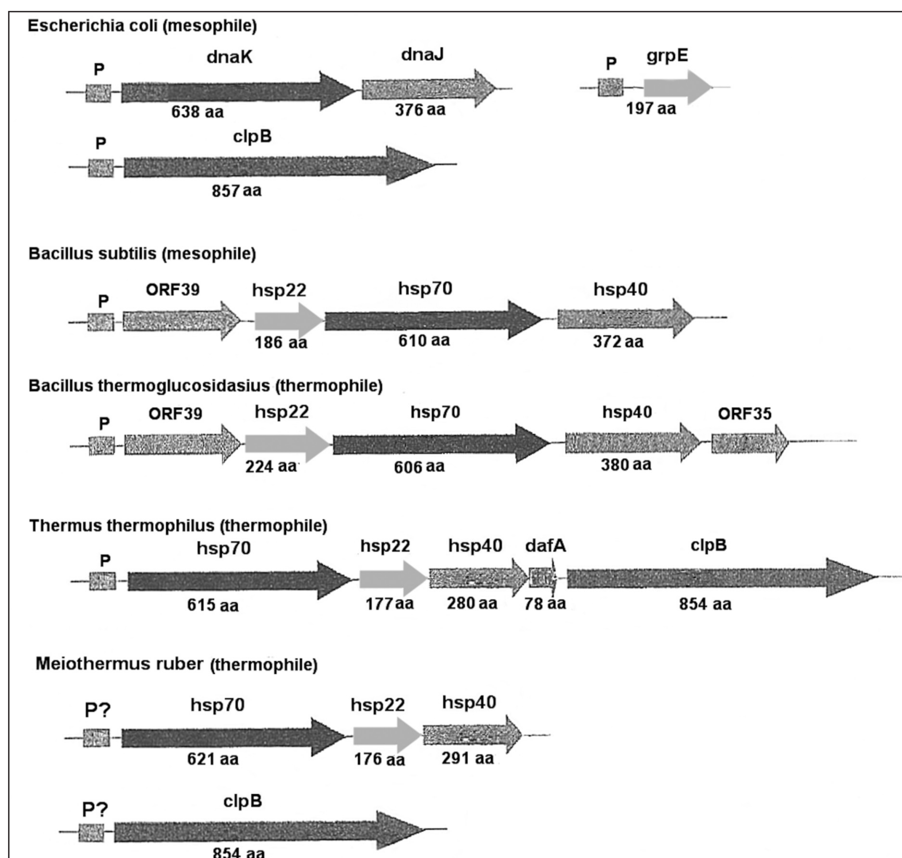

Fig. 2. Graphic representation of the *hsp70* (*dnaK*)-locus genes and their organization. The arrows represent the protein-coding regions of the genes. Figures below arrows are numbers of amino acids encoded. P indicates promoter. References used were: *E. coli, B. subtilis, B. thermoglucosidasius* [4]; *T. thermophilus* [5]

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                   10        20        30        40        50        60
                    |         |         |         |         |         |
MEIRU     -MAYKDYYKILGVPKNASEDEIKKAFKKLARKYHPDVNK-EPGAEEKFKEINEAYTVLSD
THETH     MAAKKDYYAILGVPRNATQEEIKRAYKRLARQYHPDVNK-SPEAEEKFKEINEAYAVLSD
ECOLI     -MAKQDYYEILGVSKTAEEREIRKAYKRLAMKYHPDRNQGDKEAEAKFKEIKEAYEVLTD

                   70        80        90       100       110       120
                    |         |         |         |         |         |
MEIRU     PEKRRYYDTYGAAAGSAGWQGPPPGPGGFGGFTGNVGDFSDFFQQLFGGRGGFGGLGDLF
THETH     PEKRRIYDTYGTTEAPP---PPPPGGYDFSGFD--VEDFSEFFQELFG-PGLFGGFG---
Ecoli     SQKRAAYDQYGHAAFEQ-------GGMGGGGFGG-GADFSDIFGDVFG--DIFGGGRG--

                  130       140       150       160       170       180
                    |         |         |         |         |         |
MEIRU     EQTPGRGARRVAGDLEAELPLSLEEAYRGGEKTISVG-----------------------
THETH     -----RRSRKGR-DLRAELPLTLEEAFHGGERVVEVA-----------------------
ECOLI     --R--QRAARGA-DLRYNMELTLEEAVRGVTKEIRIPTLEECDVCHGSGAKPGTQPQTCP

                  190       200       210       220       230       240
                    |         |         |         |         |         |
MEIRU     -----------------------------------------------AERLTVRIPPGVRE
THETH     -----------------------------------------------GRRVSVRIPPGVRE
ECOLI     TCHGSGQVQMRQGFFAVQQTCPHCQGRGTLIKDPCNKCHGHGRVERSKTLSVKIPAGVDT

                  250       260       270       280       290       300
                    |         |         |         |         |         |
MEIRU     GQKIRLAGKGRAG------GDLYLHVKLQSRPEMRLEGDDIYTVVEVPAPIAVVGGKVRV
THETH     GSVIRVPGMGGQGNP---PGDLLLVVRLLPHPVFRLEGQDLYATLDVPAPIAVVGGKVRA
ECOLI     GDRIRLAGEGEAGEHGAPAGDLYVQVQVKQHPIFEREGNNLYCEVPINFAMAALGGEIEV

                  310       320       330       340       350       360
                    |         |         |         |         |         |
MEIRU     QTLDGPVEITIPRRTQAGRKLRLAGKGWPRK-DKSRGDQYAEVRVTIPTNPSPEEERLYA
THETH     MTLEGPVEVAVPPRTQAGRKLRLKGKGFPG--PAGRGDLYLEVRITIPERLTPEEEALWK
Ecoli     PTLDGRVKLKVPGETQTGKLFRMRGKGVKSVRGGAQGDLLCRVVVETPVGLNERQKQLLQ

                  370       380       390
                    |         |         |
MEIRU     QLAELIKVQ-------------------------
THETH     KLAEAYYARA------------------------
ECOLI     ELQESFGGPTGEHNSPRSKSFFDGVKKFFDDLTR
```

Fig. 3. Alignment of Hsp40 from *M. ruber* (MEIRU), *T. thermophilus* (THETH) and *E. coli* (ECOLI). Identical amino acid residues are shown by letters on shaded background. Underlines indicate HPD motive. Boxed letters indicate cysteine-rich region (CXXCXGXGX)

Table 1. **Amino acid composition in chaperones of thermophiles and mesophiles**

Strain	Protein	Content of amino acids			
		Proline, %	Cysteine, %	Arginine, %	Alanine, %
T. thermophilus (extreme thermophile)	Hsp70	5.0	0.3	6.7	11.2
M. ruber (moderately thermophile)		4.3	0.2	5.5	11.6
E. coli (mesophile)		3.6	0.2	3.9	11.0
T. thermophilus	Hsp40	10.0	0.0	9.6	8.9
M. ruber		7.2	0.0	8.2	8.2
E. coli		4.3	2.7	7.2	7.2
T. thermophilus	Hsp22	4.0	0.0	10.7	11.9
M. ruber		5.1	0.0	6.2	10.2
E. coli		4.6	0.0	5.6	12.2

Table 2. **Comparison of ClpB from *M. ruber* with those of several bacterial species**

Strain	Sequence identity, %
	ClpB
Thermus thermophilus (thermophile)	78
Corynebacterium glutamicum (mesophile)	53
Heliobacter pylori (mesophile)	49
Campylobacter jejuni (mesophile)	48
Escherichia coli (mesophile)	57
Streptomyces coelicor (mesophile)	57

and *M. ruber*, the *clpB* gene has not been identified in any other thermophilic organism so far.

Sequence alignment (CLUSTALW program) of the deduced amino acid sequence of Mru.ClpB revealed a homology with proteins from various bacterial species (Table 2). The highest score was obtained for the protein from *T. thermophilus*.

ClpB is found in almost all organisms studied to date. Despite their functional similarity, bacterial and eukaryotic ClpB have one striking and highly conserved difference. Although, like in eukaryotes, two different-sized forms of ClpB occur (79 and 93 kDa) in eubacteria, both proteins originate from a single gene as a result of an internal translation initiation site within the *clpB* transcript. The specific function of the truncated form remains unknown, although it is thought to have a regulatory role in the activity of the full-length ClpB protein. *M. ruber* ClpB contains a conserved valine residue (Val-141) encoded by the GUG codon identical to the Val-141 of Tth.ClpB and Val-149 of *E. coli* ClpB, where it is used as an internal translation start (Fig. 4). Thus, as a result of an internal translation initiation site within the *clpB* transcript, two different-sized forms originate from a single gene: a full-length protein (95 kDa) and a shortened version (80 kDa) lacking the N-terminus [17].

The recombinant Mru.ClpB gene was expressed under the control of an inducible T7 promoter in *E. coli* (Fig. 5). The protein was recovered in both soluble and insoluble fractions from the total cell lysates. The solubility was increased by lowering

```
                          130       140       150       160       170       180
                            |         |         |         |         |         |
THETH      -------PGLPGLEALKGALKELRGGRTVQTEHAESTYNALEQYGIDLTRLAAEGKLDPV
MEIRU      -------YGGLQASAVRQALQEIRGGRTVNSEHAEGTYNALEQYGLDLTRQAEEGKLDPV
ECOLI      TLADILKAAGATTANITQAIEQMRGGESVNDQGAEDQRQALKKYTIDLTERAEQGKLDPV
```

Fig. 4. Alignment of ClpB N-terminus part from *T. thermophilus* (THETH), *M. ruber* (MEIRU) and *E. coli* (ECOLI). Identical amino acid residues are shown by letters on shaded background. Val is marked in box

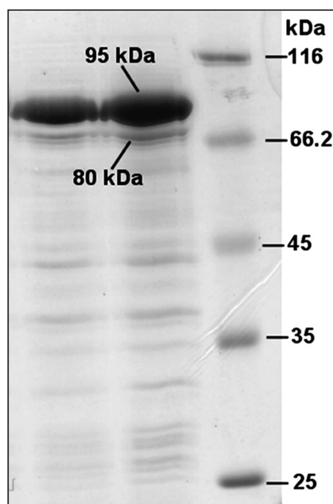

Fig. 5. Expression of Mru.ClpB in *E. coli* cells. Lines 1 and 2 indicate total cell lysate after induction of 1 mM of IPTG. Line 3 contains molecular weight standards

the temperature to 30 °C. In contrast to *E. coli* ClpB [18] upon expression in *E. coli* cells, the full-length Mru.ClpB (95 kDa) predominates and no truncated form (80 kDa) of Mru.ClpB was found in significant amounts.

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MEIOTHERMUS RUBER ŠAPERONŲ SISTEMOS HSP70/CLPB GENETINĖ ORGANIZACIJA

Santrauka

Dauguma šaperonų yra konservatyvūs ir gyvybiškai būtini ląstelės baltymai, aptinkami visose organizmuose: archėjose, bakterijose ir eukariotuose. Šaperonai yra atsakingi už aktyvų baltymų struktūrų formavimą. Domėjimąsi termofilinių mikroorganizmų šaperonais skatina baltymų susivyniojimo ir jų natyvios struktūros pasiekimo bei išlaikymo aukštoje temperatūroje problema. Nepaisant to, jog termofilinių mikroorganizmų baltymų pirminė aminorūgščių seka lemia baltymo termostabilumą, termofilai naudoja šaperonus natyviai baltymų būsenai pasiekti ir išlaikyti. Tik kelios termofilinių bakterijų Hsp70 šaperonų sistemos yra klonuotos. Šaperonų baltymų genetinės organizacijos nustatymas padėtų išaiškinti šių baltymų veikimo mechanizmus. Nors mezofilinių ir termofilinių organizmų šaperonų seka panaši, juos koduojančių genų organizacija skiriasi. ClpB šaperoną koduojančio geno nėra nuosaikiai termofilinės bakterijos *Meiothermus ruber*, išskirtos iš Kamčiatkos karštųjų versmių, *hsp70* geno klasteryje. *clpB* genas aptiktas nutolęs nuo *hsp70* operono. Prieš *clpB* nustatytos kelios potencialios promotorinės sekos. Reikia pažymėti, jog ClpB iki šiol nenustatytas jokiuose kituose, be *T. thermophilus*, termofiliniuose mikroorganizmuose (Swiss-Prot ir TrEMBL duomenų bazės), o jo amino rūgščių seka labiausiai panaši (78%) į *T. thermophilus* ClpB baltymo amino rūgščių seką. Prokariotuose aptinkami dviejų molekulinių masių (viso ilgio ir sutrumpintos formos) ClpB, nes *clpB* transkripte yra vidinis transliacijos iniciacijos signalas. *M. ruber* ClpB, kaip ir *E. coli* bei *T. thermophilus* ClpB, aptiktas vidinis transliacijos iniciacijos signalas – tripletas, koduojantis valiną, lokalizuotas netoli pirmojo ATP-azinio domeno. Skirtingai nuo rekombinantinio *E. coli* ClpB, ekspresavus Mru.ClpB *E. coli* ląstelėse, neaptikta didesnio sutrumpintos formos (80 kDa) šaperono kiekio, o vyravo viso ilgio (95 kDa) rekombinantinis ClpB baltymas.