Analysis of the AAA+ chaperone *clpB* gene and stress-response expression in the halophilic methanogenic archaeon *Methanohalophilus portucalensis*

Chao-Jen Shih and Mei-Chin Lai

Department of Life Sciences, National Chung Hsing University, Taichung, Taiwan.

Correspondence

Mei-Chin Lai

mclai@dragon.nchu.edu.tw

ClpB is a member of the protein-disaggregating chaperone machinery belonging to the AAA+ superfamily. This paper describes a new *clpB* gene from the halophilic methanoarchaeon *Methanohalophilus portucalensis*, which has not been reported previously in Archaea. The partial sequence of *clpB* was identified from the investigation of the salt-stress response of *Meh. portucalensis* by differential-display RT-PCR (DDRT-PCR). Furthermore, the complete *clpB* sequence (2610 nt) and its upstream genes encoding the type I chaperonin GroEL/ES were obtained through inverse PCR, Southern hybridization and sequencing. The G+C ratio of *clpB* is 49.6 mol%. The predicted ClpB polypeptide contains 869 aa and possesses a long central domain and a predicted distinctly discontinuous coiled-coil motif separating two nucleotide-binding domains (NBD1 and NBD2). NBD1 has a single Walker A and two Walker B motifs and NBD2 has only one of each Walker motif, a characteristic of HSP100 proteins. Two repeated Clp amino-terminal domain motifs (ClpN) were identified in ClpB. The putative amino acid sequence shared 75.6% identity with the predicted *clpB* homologue annotated as ATPase AAA-2 of *Methanococcoides burtonii* DSM 6242. Preliminary phylogenetic analysis clustered *Meh. portucalensis* ClpB (*MpClpB*) with the low G+C Gram-positive bacteria. Stress response analysis of *clpB* by Northern blotting showed up to 1.5-fold increased transcription levels in response to both salt up-shock (from 2.1 to 3.1 M NaCl) and down-shock (from 2.1 to 0.9 M NaCl). Both *clpB* and *groEL/ES* transcript levels increased when the temperature was shifted from 37 °C to 55 °C. Under heat stress *clpB* transcription was repressed by the addition of the osmolyte betaine (1 mM). In conclusion, a novel AAA+ chaperone *clpB* gene from a halophilic methanogen that responded to the fluctuations in temperature, salt concentration and betaine has been identified and analysed for the first time.

Received 27 February 2007

Revised 7 April 2007

Accepted 13 April 2007

INTRODUCTION

Molecular chaperones are ubiquitous in bacteria and eukaryotes. They can help nascent polypeptides to fold correctly and reactivate aggregated proteins which were damaged by stress (Lee & Tsai, 2005). One would expect that molecular chaperones also function in archaea that can survive in extreme environments, and help in their survival and adaptation under stresses such as temperature, pH, salt and pressure.

Abbreviations: DDRT-PCR, differential-display RT-PCR; HSP, heat-shock protein; NBD, nucleotide-binding domain.

The GenBank/EMBL/DDBJ accession number for the *clpB*, *groEL* and *groES* sequences of *Meh. portucalensis* is DQ387867.

A supplementary figure showing the sequence alignment and secondary structure elements of ClpB/Hsp101/Hsp104 is available with the online version of this paper.

Molecular chaperones of all organisms can be classified into four types: prefoldins, the molecular chaperone machine (composed of DnaK, DnaJ and GrpE) and group I and II chaperonins (Conway de Macario *et al.*, 2003). Prefoldins are composed of an α -subunit and a β -subunit, and their major function is to work with chaperonins to facilitate the correct folding of cellular proteins (Martin *et al.*, 2004). Prefoldin genes can be found in all the archaeal genomes that have been sequenced (Macario *et al.*, 2004). It has been verified that the mRNA levels of the prefoldin α -subunit gene of *Methanocaldococcus jannaschii* is upregulated during heat-shock stress (Boonyaratankornkit *et al.*, 2005). The molecular chaperone machine is considered to be formed by Hsp70 (DnaK), Hsp40 (DnaJ) and a nucleotide-exchange factor (e.g. GrpE in prokaryotes and BAG-1 in eukaryotes); it catalyses the refolding of denatured proteins and assists the folding of

newly synthesized proteins, preventing aggregation. Heat-shock proteins (HSPs) have been identified and characterized from various bacteria and archaea. Among archaea, *Methanosarcina acetivorans* has all versions of chaperonins and HSPs (Galagan *et al.*, 2002; Conway de Macario *et al.*, 2003) and has features in common with bacterial and eukaryotic heat-shock systems. Surprisingly, the major chaperone classes Hsp100/Hsp83 are absent from the genomes of the hyperthermophilic archaea, although they are present in mesophilic archaea (Laksanalamai *et al.*, 2004). Group I chaperonins, such as GroEL and GroES, catalyse correct polypeptide folding. It has been verified that the group I chaperonins exist in archaea after whole-genome sequence analysis of *Msa. acetivorans* was performed (Galagan *et al.*, 2002) and it has been conjectured that the bacterial chaperonin genes were obtained by lateral gene transfer (Deppenmeier *et al.*, 2002; Klunker *et al.*, 2003), but this has been disputed (Macario *et al.*, 2006). Group II chaperonins, which mediate protein folding, such as thermosome subunits and Hsp60, exist exclusively in eukaryotes and archaea and so far have not been found in bacteria (Laksanalamai *et al.*, 2004). Among the extreme thermophiles, group II chaperonins have been investigated in *Thermoplasma* spp. (thermosomes), *Sulfolobus* spp. (archaeosomes) and *Pyrococcus* spp. (rosettasomes) (Kagawa *et al.*, 1995; Quate-Randall *et al.*, 1995; Waldmann *et al.*, 1995). Overall, all four types of molecular chaperone systems have been identified in mesophilic archaea, especially *Msa. acetivorans*, but not in all archaea (Laksanalamai *et al.*, 2004; Conway de Macario *et al.*, 2003).

The Clp/Hsp100 family, a class within the AAA+ (ATPases associated with various cellular activities) superfamily (Dougan *et al.*, 2002; Neuwald *et al.*, 1999; Schirmer *et al.*, 1996), is involved in a variety of functions, such as protein quality control (refolding, disaggregation and degradation), membrane fusion and vesicular transport, DNA replication and repair, and cytoskeleton regulation (Ogura & Wilkinson, 2001). Clp/Hsp100 proteins play an essential role as members of both the chaperone and the protease machinery (Schirmer *et al.*, 1996). The Clp/Hsp100 family includes eight subfamilies (ClpA, B, C, D, M, N, X and Y) (Schirmer *et al.*, 1996). ClpA and ClpX are associated with the ClpP protease (Singh *et al.*, 2000, 2001) and are involved in protein degradation and quality control. In contrast, ClpB/Hsp104 does not associate with a cellular protease (Woo *et al.*, 1992), but participates in the multi-chaperone system that efficiently inhibits and reverses protein aggregation (Ben-Zvi & Goloubinoff, 2001; Zolkiewski, 1999). Bacterial ClpB and its eukaryotic orthologues, plant Hsp101 and yeast Hsp104, are heat-shock-induced, and are essential proteins for the stress response (Lee *et al.*, 2004; Lee & Tsai, 2005; Queitsch *et al.*, 2000; Sanchez & Lindquist, 1990; Squires *et al.*, 1991). Among archaea, ClpA/B homologues are predicted to be encoded by only a few methanogen genomes to date, including *Methanospirillum hungatei* YP_502751 (optimum

growth temperature 37 °C), *Methanococcoides burtonii* YP_566814 (23.4 °C) and the distant ClpB-type homologue of *Methanothermobacter thermoautotrophicus* AAB84790 (65–70 °C) which could have been acquired by horizontal or lateral gene transfer from bacteria (Neuwald *et al.*, 1999; Laksanalamai *et al.*, 2004).

The strictly anaerobic *Methanohalophilus portucalensis* strain FDF1 can grow over a range of external NaCl concentrations from 1.2 to 2.9 M (Mathrani & Boone, 1985; Boone *et al.*, 1993; Lai *et al.*, 1991). To counter the changing osmotic stress, the cell could transport betaine or *de novo* synthesize betaine, β -glutamine and *N*^ε-acetyl- β -lysine as compatible solutes to respond to salt concentration of the external environment (Lai *et al.*, 1991, 1999, 2000, 2006). In addition to the accumulation of organic solutes, the internal K⁺ concentration also increased from 0.6 to 1.1 M (Lai *et al.*, 1991). In order to elucidate the molecular mechanisms of salt adaptation and stress response of the halophilic methanoarchaeon *Meh. portucalensis*, differential-display RT-PCR (DDRT-PCR) was used. The differential expression of genes of *Meh. portucalensis* in response to hyper-saline stress (3.1 M NaCl) and hypo-saline stress (0.9 M NaCl) was studied and a *clpB* gene was identified and characterized. In this report, the AAA+ chaperone *clpB* gene of *Meh. portucalensis* is described for the first time as is its stress-response expression.

METHODS

Organisms, vector and growth conditions. *Meh. portucalensis* strain FDF1 (=OCM59) was isolated from the solar saltern of Figueria da Foz, Portugal, by Mathrani & Boone (1985) and provided by R. Mah, School of Public Health, University of California, Los Angeles, USA (Boone *et al.*, 1993; Lai *et al.*, 1991). The cells were routinely incubated at 37 °C in defined medium that contained 2.1 M NaCl l⁻¹ and 40 mM trimethylamine as the sole carbon and energy source (Lai *et al.*, 1991). Sterile medium was prepared under a N₂/CO₂ atmosphere (4:1) by a modification of the Hungate technique (Balch *et al.*, 1979). The medium was anaerobically dispensed into serum bottles, which were then sealed with butyl rubber stoppers and aluminium crimp closures and autoclaved at 121 °C for 20 min. The methanogenic substrate and Na₂S₉H₂O were prepared under the N₂ atmosphere and were added to the sterile medium at 40 mM and 1 mM respectively just prior to cell inoculation. Sealed serum bottles were inoculated with a 5 % volume of late-exponential phase culture by using a N₂-flushed syringe. Cell growth rates were monitored by removing 1 ml of culture with a N₂-flushed syringe into a cuvette containing Na₂S₂O₃ and measuring the OD₅₄₀.

The pGEM-T easy vector (Promega) was used to clone the DDRT-PCR products. The yT&A cloning vector (Yeastern Biotech) was used to clone the *MpclpB* gene. The pUC18 cloning vector was used for cloning the 5' end and region upstream of *MpclpB*. *Escherichia coli* strains DH5 α and JM101 were used as the hosts. *E. coli* was grown in Luria-Bertani (LB) medium or on solid medium at 37 °C (Sambrook & Russell, 2001). Ampicillin was added to the medium at a concentration of 100 μ g ml⁻¹.

Chromosomal DNA and RNA extraction. Chromosomal DNA of *Meh. portucalensis* was isolated and purified by a modification of the methods of Jarrell *et al.* (1992) and Johnson (1985). Cells (500 ml)

were harvested at late-exponential phase by centrifugation and lysed with lysis buffer (10 mM Tris, 1 mM EDTA, 2% SDS and 100 µg protease K ml⁻¹). After phenol/chloroform extraction, nucleic acids were precipitated with 2-propanol and collected with a glass rod. The salt contained in the DNA was dissolved in 70% cold ethanol and nucleic acids were dissolved in 0.1 × standard saline citrate buffer (SSC). After treatment with RNase (50 µg ml⁻¹), the extraction and washes were repeated to purify the chromosomal DNA, which was dissolved in 0.1 × SSC.

To prepare RNA, mid-exponential-phase cells (50 ml, OD₅₄₀ ~0.6–0.8, ~10⁹ cells) were harvested and lysed with 1 ml of RareRNA (Genepure Technology) and incubated for 5 min at room temperature. After the addition of 0.3 ml chloroform, the mixture was shaken vigorously for 15 s, further incubated on ice for 5 min, and centrifuged at 12 000g and 4 °C for 10 min. The aqueous phase, containing RNAs, was transferred into a new tube and the nucleic acids were precipitated with 2-propanol. The RNA pellet was dried by Speed Vac (SC100, Savant) then dissolved in 20 µl DEPC-treated water. Total RNA concentration was determined from A₂₆₀.

Cloning the *clpB* gene of *Meh. portucalensis*. Total RNA (5 µg) from control (cells grown in medium containing 2.1 M NaCl) and experimental samples (cells grown in medium containing 0.9 or 3.1 M NaCl) was subjected to reverse transcription in a GeneAmp PCR System 2400 (Perkin Elmer). The cDNA synthesis reactions were carried out for 1 h at 42 °C by using ImProm-II reverse transcriptase (Promega). The reaction was stopped by incubation at 95 °C for 5 min. The cDNAs were further amplified by PCR with two synthesized arbitrary primers P1 (5'-GCCAAGCTCCAG-3') and P2 (5'-GGCGTCATCGAC-3') and *Taq* DNA polymerase (Takara). The resulting products were run in a 1.5% TAE/agarose gel, and the differentially expressed fragments were excised from the gel and purified using the GENECLEAN III (BIO 101 Systems, Q-BIOgene). These cDNAs were further ligated into the pGEM-T easy vector (Promega) as described by the manufacturer, and positive clones were sequenced by an automated sequencer (Applied Biosystems model 373A). Sequence comparisons were performed using BLAST from the NCBI web site (<http://www.ncbi.nlm.nih.gov/BLAST/>). A 1326 bp fragment (designated *clpB*-1.3) was obtained and sequenced to verify that this DNA fragment was a portion of the *clpB* gene.

In order to clone the full-length *clpB*, inverse PCR and Southern hybridization were used. Two oligonucleotide primers, forward primer InverseF (5'-ATGAACGAACACCAAACTT-3') and reverse primer InverseR (5'-GACGATTTCTCTCTCCTCGTAT-3'), were designed from the 3' and 5' regions of *clpB*-1.3, respectively, to amplify the 5' end and 3' ends of *clpB* from *Meh. portucalensis* by inverse PCR. Chromosomal DNA of *Meh. portucalensis* FDF1 (0.1 µg) was digested with *Eco*RI and religated with T4 DNA ligase (New England BioLabs) as the template and *Taq* DNA polymerase (Takara) was added for inverse PCR. The products of inverse PCR were subcloned into the pGEM-T easy vector (Promega) and sequenced. A 2042 bp segment of *clpB* was obtained (designated *clpB*-2.0).

In order to obtain the complete *clpB*, Southern hybridization was performed as follows. Chromosomal DNA of *Meh. portucalensis* FDF1 was digested with *Pst*II (New England BioLabs) and Southern blotting was performed based on the methods of Lai *et al.* (2004) and Sambrook & Russell (2001) with modifications, by using a Renaissance random primer fluorescein labelling kit (Perkin Elmer Life Sciences) and *clpB*-2.0 as a probe. Denatured DNA was blotted onto a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) and the labelled DNA was reassociated in a solution containing 50% formamide, 5 × Denhardt's solution and 0.5% (w/v) SDS in 5 × SSC buffer. After incubation overnight at 42 °C, blots were analysed with a Renaissance nucleic acid chemiluminescence reagent and an anti-fluorescein HRP conjugate detection system supplied by

NEN Life Science. Hybridization signals were detected by autoradiography. The positive DNA fragment was subcloned into pUC18 (Promega) and confirmed by DNA sequencing. Two specific primers were designed for amplification of the complete *clpB* gene from the *Meh. portucalensis* genome. The forward primer ClpBoeF (5'-CGA-CCTAATTAATGGACCTGAATAATTCA-3') was based on the Southern fragment and the reverse primer ClpBoeR (5'-ATTGCGGCCGCATGTATGTTCTTCT-3') was based on the inverse PCR fragment *clpB*-2.0. PCR was performed with ClpBoeF and ClpBoeR and Takara *Taq* DNA polymerase by using the *Meh. portucalensis* chromosomal DNA as template. The PCR product, designated *clpB*-2.6, was subcloned into *γ*T&A cloning vector and sequenced. All DNA sequences presented in this report were carefully checked and confirmed by at least two sequencing results.

Plasmids were extracted and purified as described in the Viogene Mini-M plasmid DNA extraction system user's guide. The method of DNA elution followed the protocol in the application manual for GENECLEAN III (BIO 101 Systems, Q-BIOgene).

Transcript analysis by Northern hybridization. Mid-exponential-phase cells (OD₅₄₀=0.6–0.8) of *Meh. portucalensis* FDF1 (50–60 ml) were harvested and total RNA was prepared according to the protocol for the RareRNA kit (Genepure Technology) described above. The amount of RNA was quantified by spectrophotometry (Ultraspex 1000, Pharmacia Biotech). The transcription level of 16S rRNA of *Meh. portucalensis* FDF1 was used as an internal control for Northern analysis and the 16S rDNA probe was generated by specific 16S rDNA primers (coccus1 primer, 5'-CGACTAAGCCATGCGAGTC-3', and reverse3 primer, 5'-GTGACGGGCGGTGTGTGCAAG-3') (Lai *et al.*, 2004). Both *clpB*-2.6 and 16S rDNA probes were prepared with the Renaissance random primer fluorescein labelling kit (Perkin Elmer Life Sciences). RNA samples (36 µg each) were loaded into a formaldehyde/agarose gel prepared as described by Sambrook & Russell (2001). RNA gel electrophoresis was performed at 20 V for 16 h then the RNA was transferred to Hybond-N+ nylon membrane (Amersham) with a vacuum transfer system (Hybaid). Further fixation, hybridization, washing and detection were performed as described previously (Lai *et al.*, 2002). TINA software (Version 2.09e; raytest Isotopenmeßgeräte) was used to analyse the hybridization signals. The relative transcript levels were calculated as the specific value for the *clpB* signal divided by that for the 16S rRNA signal.

To test the effect of the stress response on *clpB* transcription, 50 ml mid-exponential-phase culture grown in defined medium with 2.1 M NaCl at 37 °C was used as a control. For salt/osmotic up-shock, anaerobically prepared 3.85 M NaCl-containing defined medium (66 ml) was added to 50 ml culture to give a final NaCl concentration of 3.1 M. For the salt/osmotic down-shock, defined medium without NaCl (66 ml) was added to the residual 50 ml culture to give a final NaCl concentration of 0.9 M. Control tests were performed by adding the same amount of defined medium with 2.1 M NaCl. All tested cultures (0.9, 2.1 and 3.1 M NaCl) were further incubated at 37 °C for different time periods. To investigate the effect of temperature on *clpB* transcription, mid-exponential-phase cells incubated at 37 °C were shifted to 15, 20, 25, 30, 33, 40, 45, 50 and 55 °C. For a detailed time-course study measurements were obtained at 5, 10, 15, 30, 60, and 120 min. The effects of the osmolyte betaine on *clpB* transcription under temperature and salt stress were examined by using two parallel sets of mid-exponential-phase cultures (OD₅₄₀ 0.7) grown at 2.1 M NaCl, 37 °C. Cultures were tested with salt/osmotic stress and temperature stress as described above, with or without the addition of betaine (1 mM). More than two different culture batches were tested for each stress response.

To test the relationship of *groEL/ES* and *clpB* transcripts under heat shock, mid-exponential-phase cells incubated at 37 °C were shifted to 45 and 55 °C. The *groEL*-0.6 probe, generated with the specific primers

groEL-F: (5'-AGGTGGCGTGGCAGTAATCAAA-3') and groEL-R (5'-CTTTGGAGTGTCTTCTGA-3'), and the *clpB*-2.6 probe were labelled with the DIG DNA labelling kit (Roche Applied Science). The test was run in triplicate with the same batch of cultures.

DNA and amino acid sequence analyses. Sequence comparison of ClpB was performed by using information from the NCBI web site (<http://www.ncbi.nlm.nih.gov/BLAST/>). Conserved domain and motif analyses were carried out by using the SMART web site (<http://smart.embl-heidelberg.de>). The ClpB/Hsp100 amino acid sequences were obtained from the database of the NCBI web site (<http://www.ncbi.nlm.nih.gov>). Multiple sequence alignments were constructed by using the SDSC Biology Workbench website (<http://workbench.sdsc.edu>) with the CLUSTALW program (Thompson *et al.*, 1994). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004). The coiled-coil structures were analysed using the COILS program from ISREC, Switzerland (http://www.ch.embnet.org/software/COILS_form.html). Sequences were run with the weighted MTIDK matrix.

RESULTS

Meh. portucalensis clpB cloning and analyses

By DDRT-PCR of *Meh. portucalensis* mRNA from 0.9, 2.1 and 3.1 M NaCl cultures, fourteen differentially expressed cDNA fragments were obtained from the 0.9 or 3.1 M NaCl cultures (data not shown). Two of the differentially expressed genes from the 3.1 M NaCl culture shared high identity (70 % and 74 %) with the genes encoding transmembrane protein (MttP) and monomethylamine methyltransferase (MtmB), which participate in the unique energy-acquiring methanogenesis of methanogens. Three putative stringent-response-related genes were also identified by DDRT-PCR as the ribosomal protein L29, which was down-regulated in 0.9 M NaCl culture, and the translation elongation factor 1A and the lysyl tRNA synthetase, which were up-regulated in 0.9 M NaCl culture. The predicted genes for cobalamin biosynthesis protein (CbiD) (upregulated in 3.1 M NaCl culture), methenyl-H4MPT cyclohydrolase (upregulated in 0.9 M NaCl culture) and sulfite reductase (upregulated in 0.9 M NaCl culture) were also identified to be involved in the salt-stress response. Predictably, three known stress-response gene homologues were identified. One was the DNA mismatch repair protein Muts (upregulated in 3.1 M NaCl culture), the second was the universal stress protein USPA (upregulated in 0.9 M NaCl culture) and the third the differentially expressed cDNA fragment (1326 bp) that was obtained from the 3.1 M NaCl culture and had a deduced amino acid sequence that shared 55 % identity with the putative ClpB protein of *Thermosynechococcus elongatus* BP-1.

The 1326 bp cDNA fragment, designated *clpB*-1.3, possessed a nucleotide-binding domain (NBD), which was similar to NBD2, and also possessed the conserved Walker motifs (Walker A and Walker B) typical of a bacterial-type ClpB chaperone (Schirmer *et al.*, 1996). By using the *clpB*-1.3 as template, inverse PCR was performed and an extended 2042 bp partial *clpB* gene (*clpB*-2.0) with

the downstream sequence was obtained. There was a 652 bp overlap between *clpB*-1.3 and *clpB*-2.0 and all these sequences were 100 % identical. However, inverse PCR could not complete the 5' end of *clpB* so a labelled *clpB*-2.0 DNA probe was generated and the upstream and 5' end of *clpB* gene (total 1986 bp) was obtained through Southern hybridization and was further cloned and sequenced. There was a 1153 bp overlap between *clpB*-2.0 and this fragment and all sequences were 100 % identical. Combining the results of DDRT-PCR, inverse PCR and Southern blotting, the complete *clpB* gene (2610 bp) and its upstream and downstream adjacent genes were obtained. The full length *clpB* gene (2610 bp) was amplified from the chromosomal DNA of *Meh. portucalensis* for further overexpression and Northern investigation by using the specific primers ClpBoeF and ClpBoeR designed as described in Methods. The sequence of PCR-amplified full-length *clpB*-2.6 was 100 % identical with sequences obtained from the combination of DDRT-PCR, inverse PCR and Southern blotting.

The G+C content of *clpB* from *Meh. portucalensis* is 49.6 mol%, which is close to the G+C content of its genome (44 mol%). This is similar to the G+C contents of the *Mec. burtonii* ATPase AAA-2 gene (45.4 mol%), *Mes. hungatei* ATPase AAA-2 gene (47.5 mol%) and *Met. thermoautotrophicus* ClpA/B gene (47.4 mol%), which are close to the G+C contents of their genomes at 40, 45 and 49 mol%, respectively. Analysis of the promoter region of *Meh. portucalensis* *clpB* indicated that the predicted TATA box (5'-TATAAA-3') was located at the -37 to -42 position from the translation start codon and the BRE motif 5'-AAAA-3' preceded the TATA box. The Shine-Dalgarno sequence 5'-GGAGG-3' was located six nucleotides upstream from the start codon (Fig. 1). Sequence analysis indicated that *groEL* and *groES* were located upstream of *clpB* in the same direction and CDP-diacylglycerol-serine O-phosphatidyltransferase and phosphatidylserine decarboxylase genes were located downstream in the opposite direction (Fig. 1). The deduced amino acid sequence of the complete *groEL* (1611 bp designed as *MpgroEL*) and the complete *groES* (276 bp designed as *MpgroES*) that we obtained shared 74 % identity with the GroEL of *Methanosarcina barkeri* str. Fusaro (AAZ70491) and 71 % identity with the GroES of *Msa. barkeri* str. Fusaro (AAZ70490). The complete CDP-diacylglycerol-serine O-phosphatidyltransferase gene shared 56 % amino acid identity with the CDP-diacylglycerol-serine O-phosphatidyltransferase (YP_566813) of *Mec. burtonii* DSM 6242, and the partial phosphatidylserine decarboxylase-related gene shared 67 % amino acid identity with the phosphatidylserine decarboxylase related protein (YP_566812) of *Mec. burtonii* DSM 6242.

Sequence analyses and characteristics of AAA+ chaperone ClpB from *Meh. portucalensis*

The predicted polypeptide of ClpB from *Meh. portucalensis* (*MpClpB*) contains 869 aa with a calculated molecular

Fig. 1. Gene order in the *clpB* locus and *clpB* regulatory regions in *Methanohalophilus portucalensis*. DNA sequences of the promoter region and the region separating *MpgroEL* and *MpclpB* are also shown. Apparent transcription regulatory elements, as well as start and stop codons, are indicated with bars. Arrows indicate the major inverted repeat of the *groEL* gene. Solid lines indicate the completely sequenced genes. Dashed lines indicated incompletely sequenced genes. The lower two gene organizations show the homologous genes of *Methanococcoides burtonii* (*MbClpB*) and *Methanospirillum hungatei* (*MhclpB*, *MhgroEL* and *MhclpB*).

mass of 98 070.24 Da and a calculated isoelectric point of 5.09. Compared to the other 16 ClpB/Hsp100/Hsp101/Hsp104 proteins from bacteria and eukaryotes (Table 1), the molecular mass of *MpClpB* is smaller than that of proteins from plants and yeast but larger than that from bacteria and protozoa. Except for the *LdHsp100* from *Leishmania donovani* (pI 7.43), the calculated isoelectric points of all ClpBs were acidic. *MpClpB* contains 39.59 % hydrophobic, 16.80 % basic, 18.41 % acidic and 25.20 % neutral amino acids. The identity of the amino acid sequence between *MpClpB* and the *MbATPase*, a ClpB homologue we identified from the genome database of *Mec. burtonii* (Saunders *et al.*, 2003), is 75.6 % (Table 1). Results of a preliminary study of the phylogenetic relationships among ClpB/Hsp100 proteins within the three domains are shown in Fig. 2. The phylogenetic tree revealed that *MpClpB* from *Meh. portucalensis* and its archaeal homologue *Mec. burtonii* *MbATPase* as well as *Mes. hungatei* *MhATPase* are grouped with bacterial ClpBs and distant from the eukaryotic ones. Methanoarchaeal ClpBs (*MpClpB*, *MbATPase* and *MhATPase*) are more closely related to those from the low-G + C Gram-positive bacteria such as *Bacillus cereus* (*BcClpB*) than they are to

those from the high-G + C Gram-positive bacteria such as *Corynebacterium glutamicum* (*CgClpB*) (Fig. 2).

Analysis of the amino acid sequence of *MpClpB* revealed that there were two typical Clp signature motifs, i.e. the highly conserved NBD1 and NBD2; furthermore, NBD1 had a single Walker A (GXXGXGKT) and two Walker B (hhhhDE) motifs and NBD2 had only one of each Walker motif (Barnett *et al.*, 2000; Schirmer *et al.*, 1996; Squires & Squires, 1992) (Fig. 3a and Supplementary Figure S1). Two repeated Clp amino-terminal domain motifs (ClpN), which are typical of ClpA and ClpB and most ClpC proteins (Barnett *et al.*, 2000), were also identified in *MpClpB* (Fig. 3a and Supplementary Figure S1). A long middle region (M) separating the two ATP-binding sites, characteristic of ClpB proteins, was also detected in *MpClpB* and was 124 aa in length (~130 aa for other ClpBs). For all known ClpB proteins, this M region contains a predicted coiled-coil motif (Celerin *et al.*, 1998; Lee *et al.*, 2003) formed by a bundle of α -helices that are wound into a superhelix (Lupas, 1996; Martin *et al.*, 2004).

Using the COILS program, two major peaks with probability greater than 0.5 of forming a coiled-coil structure were

Table 1. Percentage identity of *Meh. portucalensis* ClpB with bacterial ClpB and eukaryotic Hsp100/Hsp101/Hsp104

Organism	Protein	Length (aa)	pI	Identity with <i>MpClpB</i> (%)	Accession no.
<i>Methanohalophilus portucalensis</i> FDF1	<i>MpClpB</i>	869	5.09	100	DQ387867
<i>Methanococcoides burtonii</i> DSM6242	<i>MbATPase</i>	869	4.97	75.6	YP_566814
<i>Methanospirillum hungatei</i> JF-1	<i>MhATPase</i>	865	5.47	51.0	YP_502751
<i>Methanothermobacter thermoautotrophicus</i> Delta H	<i>MtClpA/B</i>	616	4.91	30.0	AAB84790
<i>Bacillus cereus</i> ATCC 10989	<i>BcClpB</i>	866	5.19	57.8	Q73BY1
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	<i>LlClpB</i>	867	4.86	53.1	O68185
<i>Thermus thermophilus</i> HB8	<i>TtClpB</i>	854	5.53	56.4	BAA81745
<i>Corynebacterium glutamicum</i>	<i>CgClpB</i>	852	4.81	52.5	P53532
<i>Mycobacterium tuberculosis</i> H37Rv	<i>MtClpB</i>	848	5.04	53.4	P63288
<i>Haemophilus influenzae</i> Rd KW20	<i>HiClpB</i>	856	5.69	53.4	P44403
<i>Escherichia coli</i> K12	<i>EcClpB</i>	857	5.18	51.8	P63284
<i>Saccharomyces cerevisiae</i> X2180	<i>ScHsp104</i>	908	5.14	41.5	P31539
<i>Trypanosoma brucei</i> brucei	<i>TbHsp100</i>	868	6.16	46.4	P31543
<i>Leishmania donovani</i>	<i>LdHsp100</i>	867	7.43	47.6	CAB08073
<i>Zea mays</i>	<i>ZmHsp101</i>	912	5.76	49.5	AAD25223
<i>Glycine max</i>	<i>GmHsp101</i>	911	5.77	48.4	AAA66338
<i>Arabidopsis thaliana</i> Columbia	<i>AtHsp101</i>	911	5.73	48.6	P42730

predicted. The first peak falls within the N-terminal domain (amino acids 92–119, score 0.76), and the second peak coincides with the position of the middle region (amino acids 411–444, score 1.00; 465–492, score 0.61; 507–534, score 0.517) (Fig. 3b). The middle region in *MpClpB* contains three segments of periodic seven-residue repeats a-b-c-d-e-f-g, in which the first position (a) and the fourth (d) are occupied by predominantly hydrophobic

amino acids, whereas the neighbouring positions (g and e) are occupied by charged residues (Fig. 3c), which is similar to the middle region of *MbATPase* (data not shown). Analysis of the other 16 ClpB/Hsp100/Hsp101/Hsp104 proteins (Fig. 2) revealed that almost all of them could form a coiled-coil structure in the middle region, with one exception, *Met. thermoautotrophicus MtClpA/B*, which does not contain the middle region.

Fig. 2. Phylogenetic relationship of ClpB/Hsp100/Hsp101/Hsp104 proteins. Bootstrap values are shown at nodes (percentage of 1000 replicates). Bar, 0.1 aa substitutions per 100 aa.

In addition to the coiled-coil structure in the middle region, our analysis of *MpClpB* showed another coiled-coil structure located in front of the middle region (the first peak in Fig. 3b). This first peak was also detected in *MbATPase*, *BcClpB*, *LiClpB*, *AtHsp101*, *GmHsp101*, *ZmHsp101*, *TbClpB* and *CgClpB*. Moreover, a third peak behind the middle region was detected only in *LiClpB* (data not shown). Comparing the positions of the first peaks for coiled-coil structure of these Clp proteins, those from *MpClpB*, *MbATPase*, *BcClpB*, *AtHsp101* and *TbClpB* were located in the N-terminal region, while those from *LiClpB*, *GmHsp101*, *ZmHsp101* and *CgClpB* were located in NBD1. The distinctly discontinuous coiled coils are formed in the middle region of some ClpB proteins such as *MpClpB* (Fig. 3b), *MbATPase*, *BcClpB*, *TbClpB*, *CgClpB* and *ScHsp104*; however, only *MpClpB* and *MbATPase* generate three distinct segments. The Tyr653 residue of *E. coli* ClpB, which is potentially involved with the disaggregation process as a substrate-binding site or driving translocation, is conserved in *MpClpB* as Tyr657. A highly conserved serine residue, Ser409, within a putative phosphorylation domain adjacent to the predicted coiled-coil region (Celerin *et al.*, 1998), was also found in *MpClpB* (Fig. 3a and Supplementary Figure S1).

Transcriptional stimulation of *clpB* by salt stress

The effect of salt stress on transcription of *Meh. portucalensis clpB* was investigated by Northern hybridization using *clpB*-2.6 as probe. Signals for 16S rRNA of *Meh. portucalensis* were used as an internal control. The relative transcript levels were calculated from the specific value of the *clpB* signal divided by that of the 16S rRNA signal. In cells grown on defined medium with 2.1 M NaCl, the optimal concentration for growth, a single band was detected (Fig. 4a). This indicated that the *clpB* gene was transcribed constitutively without salt shock and we used this transcript level as the basal transcription level to compare the effect of hypersaline or hyposaline stress. With an extended time frame (from 1 to 2 h), the transcription level of *clpB* was slightly increased. This is due to the dramatic changes in culture density, with an over twofold dilution of mid-exponential-phase cultures with fresh medium. The dilution stress as well as oxygen introduced during the addition of medium may stimulate the transcription of *clpB*. Under salt up-shock from 2.1 M NaCl to 3.1 M NaCl, the *clpB* transcript level was increased 1.57-fold within 1 h and 1.52-fold after 2 h (Fig. 4). Similarly, under salt down-shock from 2.1 M NaCl to

Fig. 3. (a) Schematic representation of *MpClpB* structural regions. N, amino-terminal region; NBD1, nucleotide-binding domain 1; M, middle region; NBD2, nucleotide-binding domain 2; C, carboxyl-terminal region. Probability scores for coiled-coil formation were aligned with the different regions of ClpB. The two repeated Clp amino-terminal domain motifs (ClpN) are in diagonally striped boxes. Conserved Walker motifs (Walker A and Walker B) are shown as grey and white boxes, respectively. (b) Probability of forming coiled-coil supersecondary structures in *Meh. portucalensis MpClpB*. The y-axis indicates the probability of forming a coiled-coil with a maximum value of 1.0. The x-axis represents the length of amino acid sequence. The COILS program was run with the MTIDK matrix, with weights at positions a and d of 2.5 and at positions b, c, e, f and g of 1.0 and a window of 28 aa. (c) The middle region sequence of ClpB. The regions showing apparent coiled-coil heptad repeats (a through g) are indicated in italics.

Fig. 4. Transcription levels of *Meh. portucalensis clpB* during salt shock shown by Northern blotting. Total RNA (36 µg) was subjected to denaturing agarose gel electrophoresis. After transfer to nylon membranes, RNA was hybridized with (a) the *clpB*-2.6 probe and (b) the 16S rDNA probe. (c) The relative transcript levels were calculated with TINA software. White bars, relative transcript levels of *clpB* after 60 min incubation; grey bars, relative transcript levels of *clpB* after 120 min incubation.

0.9 M NaCl, the *clpB* transcript level was increased 1.03-fold with 1 h and 1.60-fold at 2 h (Fig. 4). The transcript level of *clpB* was increased at both salt up- and down-shock, indicating that the expression of the molecular chaperone *clpB* may be required for cell survival and adaptation while encountering salt stresses.

Transcriptional stimulation of *MpclpB* and *MpgroEL/ES* by temperature stress

Bacterial ClpB and its eukaryotic orthologues, plant Hsp101 and yeast Hsp104, are heat-shock induced and are essential proteins of the stress response (Queitsch *et al.*, 2000; Sanchez & Lindquist, 1990; Squires *et al.*, 1991; Lee *et al.*, 2004; Lee & Tsai, 2005). To investigate whether this archaeal ClpB gene from a halophilic methanogen also responds to heat shock as do those of bacterial and eukaryotic cells, mid-exponential-phase cultures of *Meh. portucalensis* grown optimally at 37 °C were incubated at 45 °C and Northern hybridization was performed with *clpB*-2.6 as the probe. As shown in Fig. 5, the transcript level of *clpB* gradually increased and reached the maximal sevenfold increase within 60 min after heat shock. This result demonstrated that the *Meh. portucalensis clpB* is involved in heat-shock response as are the *clpB* genes from bacteria and eukaryotes. The transcription level of *clpB* in response to heat shock reached a maximum after 1 h then gradually decreased to 4.2-fold at 2 h. The effect of temperature (from 37 °C to 55 °C) on *groEL/ES* transcription was also tested with a culture of *Meh. portucalensis*

Fig. 5. (a) Time-course of *clpB* transcription under 45 °C heat shock. Total RNA (36 µg) was subjected to denaturing agarose gel electrophoresis. After transfer to nylon membranes, the RNA was hybridized with the *clpB*-2.6 probe. (b) Total RNA (3.6 µg) was subjected to denaturing agarose gel electrophoresis then stained with ethidium bromide and detected directly by UV light. (c) Relative transcript levels calculated with TINA software. (d) Temperature effects on *clpB* transcription. (e) Transcript levels of *clpB* at different temperatures with or without 1 mM betaine for 15 min. Grey bars, with betaine; white bars, without betaine.

grown at the optimal temperature (37 °C) as a control. The *groEL* and *groES* genes were co-transcribed as a 1.9 kb transcript (Fig. 6a). The transcript levels of *groEL/ES* were increased upon increasing the temperature, by 2.2-fold at 45 °C and by 2.6-fold at 55 °C (Fig. 6a). This result indicated that, as with *Meh. portucalensis clpB* (Fig. 6b), *groEL/ES* also respond to heat shock.

The effects of temperature (from 15 °C to 55 °C) on *clpB* transcription were also tested with a culture of *Meh.*

Fig. 6. Transcription levels of *Meh. portucalensis* *groEL/ES* and *clpB* during heat shock shown by Northern blotting. Total RNA (36 µg) were subjected to denaturing agarose gel electrophoresis. After transfer to nylon membranes, RNA was hybridized with (a) the *groEL*-0.6 probe and (b) the *clpB*-2.6 probe. (c) Total RNA (3.6 µg) was subjected to denaturing agarose gel electrophoresis then stained with ethidium bromide and detected by using UV light.

portucalensis grown at the optimal temperature 37 °C as control. The transcript levels of *clpB* were increased by raising the temperature: by fivefold at 45 °C and by 10-fold at 55 °C (Fig. 5d). Under cold shock, *clpB* transcript levels increased fourfold at around 30 °C but there were no obvious effects at 25 °C or below (data not shown). These results suggest that transcription of *clpB* gene was stimulated under both heat and cold shock for *Meh. portucalensis*, but not at the optimal temperature range (37–40 °C), or temperatures below 25 °C.

Addition of the osmolyte betaine rescues the temperature stress effect and reduces *clpB* transcription

The halophilic methanoarchaeon *Meh. portucalensis* strain FDF1 can transport the osmolyte betaine through a high-affinity specific betaine transporter (Lai *et al.*, 2000). The effect of betaine on *clpB* transcription was tested by Northern hybridization. During heat stress, the *clpB* transcript level decreased 2.5-, 0.6- and 1.4-fold at 30 °C, 37 °C and 55 °C, respectively, after the addition of betaine (1 mM) (Fig. 5e). These results demonstrate that the addition of betaine could rescue the temperature-stress effect and reduce *clpB* transcription.

DISCUSSION

The complete *Meh. portucalensis* *clpB* was cloned, sequenced and analysed. The deduced amino acid sequence of *clpB* shares high identity with the known molecular chaperone ClpBs. Although stress-response genes and molecular chaperones among Archaea have long been discussed (Albers *et al.*, 2000; Cannio *et al.*, 2000; Macario *et al.*, 1999, 2004; Maruyama & Furutani, 2000; Roberts, 2000; Scandurra *et al.*, 2000), archaeal ClpBs have never been defined and published. Three archaeal ClpA/B homologues were identified through bioinformatic surveys, *Mes. hungatei* YP_50 2751, *Mec. burtonii* YP_566814 (Saunders *et al.*, 2003) and the distant ClpA/B-type homologue of *Met. thermoautotrophicus* AAB84790 (Neuwald *et al.*, 1999; Laksanalamai *et al.*, 2004). The

length of the thermophilic archaeon *Met. thermoautotrophicus* ClpA/B homologue, 616 aa, is shorter than all known ClpB/Hsp100 proteins and it possesses only one NBD, which does not conform to the typical ClpB features. According to the amino acid sequence identity comparison, the ClpA/B homologue of *Met. thermoautotrophicus* is distant from *MpClpB* and the known ClpB/Hsp100 proteins. In this study, we describe preliminary research on the archaeal *clpB* from the halophilic methanogen *Meh. portucalensis* strain FDF1. Based on the preliminary search for phylogenetic relationships, *MpClpB* and *MbATPase* were clustered with homologues from bacteria and were closely related to those from the low-G + C Gram-positive bacteria, *B. cereus* and *Lactococcus lactis*, but not with those from the high-G + C Gram-positive organisms *C. glutamicum* and *Mycobacterium tuberculosis* (Fig. 2).

Amino acid sequence analysis revealed that *MpClpB* possesses the characteristics of the molecular chaperone ClpB, with two conserved nucleotide-binding domains, NBD1 and NBD2, which are separated by a long middle region that was predicted to form a coiled-coil structure. There were 6.5% amino acid substitutions in the NBD1 and NBD2 of *MpClpB* compared with *BcClpB*. The substrate-binding site of *EcClpB* was reported to be located at the central pore of NBD1 and the conserved Tyr251 of *EcClpB* directly binds to the oligopeptides (Schlieker *et al.*, 2004). Based on the amino acid alignment, a hydrophobic amino acid, Phe253, is suggested as the substrate-binding site in *MpClpB*, *MbATPase* and *BcClpB* (Supplementary Figure S1). The middle region of *MpClpB* (124 residues) represents a predicted long coiled-coil structure. Although the overall identity among pairwise comparisons of the ClpB/Hsp100/Hsp101/Hsp104 sequences from different organisms is high, the percentage identity of pairwise comparisons within the predicted coiled-coil region is surprisingly low (data not shown), given the amount of conservation at the secondary structural level. The protein structure of *MpClpB* was predicted by using the SWISS-MODEL web server (<http://swissmodel.expasy.org>) and the comparison of the predicted *MpClpB* 3D structure with the known ClpB 3D structure from *Thermus thermophilus* (Lee *et al.*, 2003) indicated that the 3D structure of this domain,

but not the sequence per se, is conserved. The long coiled-coil structure in the middle region in *MpClpB* contains three fragments of periodic seven-residue repeats (Fig. 3b), which is different from the continuous-type periodic seven-residue repeats among ClpB of bacteria and eukaryotes. This particular coiled-coil structure of archaeal ClpB needs further study.

The group I chaperonin genes *groEL* and *groES* occur in the genomes of *Methanosarcina* species and *Mes. hungatei* but they were not found in any of the other archaea whose genomes have been sequenced (Deppenmeier *et al.*, 2002; Klunker *et al.*, 2003; Macario *et al.*, 2004). In this study, analysis of the upstream sequence of *MpclpB* revealed a complete GroEL gene (1611 bp) and a complete GroES gene (276 bp), whereas a complete CDP-diacylglycerol-serine *O*-phosphatidyltransferase gene (684 bp) and a partial phosphatidylserine decarboxylase gene were located downstream of *MpclpB*. This is the first finding showing that the group I chaperonin GroEL and GroES genes and the chaperone ClpB gene coexist in Archaea. Also, the gene order with *groEL* located in front of *clpB* is novel and has never been found in any other organisms (Fig. 1). In *The. thermophilus*, *clpB* is located behind the *dnaK* operon (Osipiuk & Joachimiak, 1997) and they form a chaperone network (Ben-Zvi & Goloubinoff, 2001; Zolkiewski, 1999). Under heat shock at 45 and 55 °C, the transcription of both *groEL/ES* and *clpB* of *Meh. portucalensis* was stimulated (Fig. 6). This result suggests that, in *Meh. portucalensis*, ClpB may function together with GroEL and GroES in the stress response.

Northern analysis indicated that *Meh. portucalensis clpB* responds to temperature stress. In addition, this *clpB* gene responds to both hyper- and hypo-osmotic stress. Different osmolytes have different effects on chaperone genes. Low physiological concentrations of proline, glycerol and glycine betaine activated the molecular chaperones (Diamant *et al.*, 2001). High osmolyte concentrations, especially trehalose, strongly inhibited DnaK-dependent chaperone networks (Diamant *et al.*, 2001). Glycine betaine can strongly and specifically activate ClpB, resulting in an increased efficiency of chaperone-mediated protein disaggregation (Diamant *et al.*, 2003). *Meh. portucalensis* strain FDF1 can *de novo* synthesize betaine as a compatible solute (osmolyte) in response to increasing salt stress through the methylation of glycine, sarcosine and dimethylglycine with the novel glycine sarcosine dimethylglycine *N*-methyltransferase (Lai *et al.*, 1991, 1999, 2006). During salt up-shock to 3.1 M NaCl, the transcript level of *clpB* was decreased 0.74-fold by the addition of betaine (data not shown). This result suggested that the addition of betaine (1 mM) may rescue the salt damage to proteins and reduce transcription of *clpB*. Similar results were obtained with cultures grown with 2.1 M NaCl. In contrast, the transcript level of *clpB* was increased 1.55-fold in salt down-shock cultures (with 0.9 M NaCl) by the addition of betaine (data not shown). This result suggests that the addition of betaine at salt down-shock may actually be harmful and the transcription

of *clpB* increased to rescue the effect. For temperature effects, the addition of 1 mM betaine slightly repressed the transcription of *clpB* at the tested temperatures (30, 37 and 55 °C). The interplay among osmolytes, chaperone networks and stresses is of interest for further investigation.

REFERENCES

- Albers, S.-V., van de Vossen, J. L. C. M., Driessen, A. J. M. & Konings, W. N. (2000). Adaptations of the archaeal cell membrane to heat stress. *Front Biosci* 5, D813–D820.
- Balch, W. E., Fox, G. E., Woese, C. R. & Wolfe, R. S. (1979). Methanogens: reevaluation of a biological group. *Microbiol Rev* 43, 260–296.
- Barnett, M. E., Zolkiewska, A. & Zolkiewski, M. (2000). Structure and activity of ClpB from *Escherichia coli*: role of the amino- and carboxyl terminal domains. *J Biol Chem* 275, 37565–37571.
- Ben-Zvi, A. P. & Goloubinoff, P. (2001). Mechanism of disaggregation and refolding of stable protein aggregates by molecular chaperone. *J Struct Biol* 135, 84–93.
- Boone, D. R., Mathrani, I. M., Liu, Y., Menaia, J. A. G. F., Mah, R. A. & Boone, J. E. (1993). Isolation and characterization of *Methanohalophilus portucalensis* sp. nov. and DNA reassociation study of the genus *Methanohalophilus*. *Int J Syst Evol Microbiol* 43, 430–437.
- Boonyaratankornkit, B. B., Simpson, A. J., Whitehead, T. A., Fraser, C. M., El-Sayed, N. M. A. & Clark, D. S. (2005). Transcriptional profiling of the hyperthermophilic methanarchaeon *Methanococcus jannaschii* in response to lethal heat and non-lethal cold shock. *Environ Microbiol* 7, 789–797.
- Cannio, R., Fiorentino, G., Morana, A., Rossi, M. & Bartolucci, S. (2000). Oxygen: friend or foe? Archaeal superoxide dismutases in the protection of intra- and extracellular oxidative stress. *Front Biosci* 5, D768–D779.
- Celerin, M., Gilpin, A. A., Schisler, N. J., Ivanov, A. G., Miskiewicz, E., Krol, M. & Laudenbach, D. E. (1998). ClpB in a cyanobacterium: predicted structure, phylogenetic relationships, and regulation by light and temperature. *J Bacteriol* 180, 5173–5182.
- Conway de Macario, E., Maeder, D. L. & Macario, A. J. L. (2003). Breaking the mould: archaea with all four chaperoning systems. *Biochem Biophys Res Commun* 301, 811–812.
- Deppenmeier, U., Johann, A., Hartsch, T., Merkl, R., Schmitz, R. A., Martinez-Arias, R., Henne, A., Wiezer, A., Bäumer, S. & other authors (2002). The genome of *Methanosarcina mazei*: evidence for lateral gene transfer between bacteria and archaea. *J Mol Microbiol Biotechnol* 4, 453–461.
- Diamant, S., Eliahu, N., Rosenthal, D. & Goloubinoff, P. (2001). Chemical chaperones regulate molecular chaperones *in vitro* and in cells under combined salt and heat stresses. *J Biol Chem* 276, 39586–39591.
- Diamant, S., Rosenthal, D., Azem, A., Eliahu, N., Ben-Zvi, A. P. & Goloubinoff, P. (2003). Dicarboxylic amino acids and glycine-betaine regulate chaperone-mediated protein-disaggregation under stress. *Mol Microbiol* 49, 401–410.
- Dougan, D. A., Mogk, A., Zeth, K., Turgay, K. & Bukau, B. (2002). AAA+ proteins and substrate recognition, it all depends on their partner in crime. *FEBS Lett* 529, 6–10.
- Galagan, J. E., Nusbaum, C., Roy, A., Endrizzi, M. G., Macdonald, P., FitzHugh, W., Calvo, S., Engels, R., Smirnov, S. & other authors (2002). The genome of *M. acetivorans* reveals extensive metabolic and physiological diversity. *Genome Res* 12, 532–542.

- Jarrell, K. F., Faguy, D., Hebert, A. M. & Kalmokoff, M. L. (1992). A general method of isolating high molecular weight DNA from methanogenic archaea (archaeobacteria). *Can J Microbiol* **38**, 65–68.
- Johnson, J. L. (1985). DNA reassociation and DNA hybridization of bacterial nucleic acids. *Methods Microbiol* **18**, 33–74.
- Kagawa, H. K., Osipiuk, J., Maltsev, N., Overbeek, R., Quate-Randall, E., Joachimiak, A. & Trent, J. D. (1995). The 60 kDa heat shock proteins in the hyperthermophilic archaeon *Sulfolobus shibatae*. *J Mol Biol* **253**, 712–725.
- Klunker, D., Haas, B., Hirtreiter, A., Figueiredo, L., Naylor, G. D., Pfeifer, J., Müller, V., Deppenmeier, U., Gottschalk, G. & other authors (2003). Coexistence of Group I and Group II chaperonins in the Archaeon *Methanosarcina mazei*. *J Biol Chem* **278**, 33256–33267.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Lai, M.-C., Sowers, K. R., Robertson, D. E., Roberts, M. F. & Gunsalus, R. P. (1991). Distribution of compatible solutes in the halophilic methanogenic archaeobacteria. *J Bacteriol* **173**, 5352–5358.
- Lai, M.-C., Yang, D.-R. & Cuang, M.-J. (1999). Regulatory factors associated with synthesis of the osmolyte glycine betaine in the halophilic methanoarchaeon *Methanohalophilus portucalensis*. *Appl Environ Microbiol* **65**, 828–833.
- Lai, M.-C., Hong, T.-Y. & Gunsalus, R. P. (2000). Glycine betaine transport in halophilic methanogenic archaea, *Methanohalophilus portucalensis*. *J Bacteriol* **182**, 5020–5024.
- Lai, M.-C., Chen, S.-C., Shu, C.-M., Chiou, M.-S., Wang, C.-C., Chuang, M.-J., Hong, T.-Y., Liu, C.-C., Lai, L.-J. & Hua, J. J. (2002). *Methanocalculus taiwanensis* sp. nov., isolated from an estuarine environment. *Int J Syst Evol Microbiol* **52**, 1799–1806.
- Lai, M.-C., Lin, C.-C., Yu, P.-H., Huang, Y.-F. & Chen, S.-C. (2004). *Methanocalculus chunghsingensis* sp. nov., isolated from an estuary and a marine fishpond in Taiwan. *Int J Syst Evol Microbiol* **54**, 183–189.
- Lai, M.-C., Wang, C.-C., Chuang, M.-J., Wu, Y.-C. & Lee, Y.-C. (2006). The effects of substrate and potassium on betaine synthesizing enzyme glycine sarcosine dimethylglycine N-methyltransferase from a halophilic methanoarchaeon *Methanohalophilus portucalensis*. *Res Microbiol* **157**, 948–955.
- Laksanalamai, P., Whitehead, T. A. & Robb, F. T. (2004). Minimal protein-folding systems in hyperthermophilic archaea. *Nat Rev Microbiol* **2**, 315–324.
- Lee, S. & Tsai, F. T. F. (2005). Molecular chaperones in protein quality control. *J Biochem Mol Biol* **38**, 259–265.
- Lee, S., Sowa, M. E., Watanabe, Y.-H., Sigler, P. B., Chiu, W., Yoshida, M. & Tsai, F. T. F. (2003). The structure of ClpB: a molecular chaperone that rescues proteins from an aggregated state. *Cell* **115**, 229–240.
- Lee, S., Sowa, M. E., Choi, J.-M. & Tsai, F. T. F. (2004). The ClpB/Hsp104 molecular chaperone – a protein disaggregating machine. *J Struct Biol* **146**, 99–105.
- Lupas, A. (1996). Coiled coils: new structures and new functions. *Trends Biochem Sci* **21**, 375–382.
- Macario, A. J. L., Lange, M., Ahring, B. K. & Conway de Macario, E. (1999). Stress genes and proteins in the archaea. *Microbiol Mol Biol Rev* **63**, 923–967.
- Macario, A. J. L., Malz, M. & Conway de Macario, E. (2004). Evolution of assisted protein folding: the distribution of the main chaperoning systems within the phylogenetic domain archaea. *Front Biosci* **9**, 1318–1332.
- Macario, A. J. L., Brocchieri, L., Shenoy, A. R. & Conway de Macario, E. (2006). Evolution of a protein-folding machine: genomic and evolutionary analyses reveal three lineages of the archaeal *hsp70* (*dnaK*) gene. *J Mol Evol* **63**, 74–86.
- Martin, J., Gruber, M. & Lupas, A. N. (2004). Coiled coils meet the chaperone world. *Trends Biochem Sci* **29**, 455–458.
- Maruyama, T. & Furutani, M. (2000). Archaeal peptidyl prolyl *cis-trans* isomerases (PPIases). *Front Biosci* **5**, D821–D836.
- Mathrani, I. M. & Boone, D. R. (1985). Isolation and characterization of a moderately halophilic methanogen from a solar saltern. *Appl Environ Microbiol* **50**, 140–143.
- Neuwald, A. F., Aravind, L., Spouge, J. L. & Koonin, E. V. (1999). AAA+: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res* **9**, 27–43.
- Ogura, T. & Wilkinson, A. J. (2001). AAA+ superfamily ATPases: common structure – diverse function. *Genes Cells* **6**, 575–597.
- Osipiuk, J. & Joachimiak, A. (1997). Cloning, sequencing, and expression of *dnaK*-operon proteins from the thermophilic bacterium *Thermus thermophilus*. *Biochim Biophys Acta* **1353**, 253–265.
- Quate-Randall, E., Trent, J. D., Josephs, R. & Joachimiak, A. (1995). Conformational cycle of the archaeosome, a TCP1-like chaperonin from *Sulfolobus shibatae*. *J Biol Chem* **270**, 28818–28823.
- Queitsch, C., Hong, S. W., Vierling, E. & Lindquist, S. (2000). Heat shock protein 101 plays a crucial role in thermotolerance in *Arabidopsis*. *Plant Cell* **12**, 479–492.
- Roberts, M. F. (2000). Osmoadaptation and osmoregulation in archaea. *Front Biosci* **5**, D796–D812.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanchez, Y. & Lindquist, S. (1990). Hsp104 required for induced thermotolerance. *Science* **248**, 1112–1115.
- Saunders, N. F. W., Thomas, T., Curmi, P. M. G., Mattick, J. S., Kuczek, E., Slade, R., Davis, J., Franzmann, P. D., Boone, D. & other authors (2003). Mechanisms of thermal adaptation revealed from the genomes of the Antarctic Archaea *Methanogenium frigidum* and *Methanococcoides burtonii*. *Genome Res* **13**, 1580–1588.
- Scandurra, R., Consalvi, V., Chiaraluce, R., Politi, L. & Engel, P. C. (2000). Protein stability in extremophilic archaea. *Front Biosci* **5**, D787–D795.
- Schirmer, E. C., Glover, J. R., Singer, M. A. & Lindquist, S. (1996). HSP100/Clp proteins: a common mechanism explains diverse functions. *Trends Biochem Sci* **21**, 289–296.
- Schlieker, C., Weibezahn, J., Patzelt, H., Tessarz, P., Strub, C., Zeth, K., Erbse, A., Schneider-Mergener, J., Chin, J. W. & other authors (2004). Substrate recognition by the AAA+ chaperone ClpB. *Nat Struct Mol Biol* **11**, 607–615.
- Singh, S. K., Grimaud, R., Hoskins, J. R., Wickner, S. & Maurizi, M. R. (2000). Unfolding and internalization of proteins by ATP-dependent protease ClpXP and ClpAP. *Proc Natl Acad Sci U S A* **97**, 8898–8903.
- Singh, S. K., Rozycki, J., Ortega, J., Ishikawa, T., Lo, J., Steven, A. C. & Maurizi, M. R. (2001). Functional domains of the ClpA and ClpX molecular chaperones identified by limited proteolysis and deletion analysis. *J Biol Chem* **276**, 29420–29429.
- Squires, C. & Squires, C. L. (1992). The Clp proteins: proteolysis regulators or molecular chaperones? *J Bacteriol* **174**, 1081–1085.
- Squires, C. L., Pedersen, S., Ross, B. M. & Squires, C. (1991). ClpB is the *Escherichia coli* heat shock protein F84.1. *J Bacteriol* **173**, 4254–4262.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Waldmann, T., Lupas, A. N., Kellermann, J., Peters, J. & Baumeister, W. (1995). Primary structure of the thermosome from *Thermoplasma acidophilum*. *Biol Chem Hoppe Seyler* **376**, 119–126.

Woo, K. M., Kim, K. I., Goldberg, A. L., Ha, D. B. & Chung, C. H. (1992). The heat-shock protein ClpB in *Escherichia coli* is a protein-activated ATPase. *J Biol Chem* **267**, 20429–20434.

Zolkiewski, M. (1999). ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. A novel multi-chaperone system from *Escherichia coli*. *J Biol Chem* **274**, 28083–28086.

Edited by: J. van der Oost