

Identification of a gene encoding Lon protease from *Brevibacillus thermoruber* WR-249 and biochemical characterization of its thermostable recombinant enzyme

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A gene encoding thermostable Lon protease from *Brevibacillus thermoruber* WR-249 was cloned and characterized. The *Br. thermoruber* Lon gene (*Bt-lon*) encodes an 88 kDa protein characterized by an N-terminal domain, a central ATPase domain which includes an SSD (sensor- and substrate-discrimination) domain, and a C-terminal protease domain. The *Bt-lon* is a heat-inducible gene and may be controlled under a putative *Bacillus subtilis* σ^A -dependent promoter, but in the absence of CIRCE (controlling inverted repeat of chaperone expression). *Bt-lon* was expressed in *Escherichia coli*, and its protein product was purified. The native recombinant *Br. thermoruber* Lon protease (Bt-Lon) displayed a hexameric structure. The optimal temperature of ATPase activity for Bt-Lon was 70 °C, and the optimal temperature of peptidase and DNA-binding activities was 50 °C. This implies that the functions of Lon protease in thermophilic bacteria may be switched, depending on

temperature, to regulate their physiological needs. The peptidase activity of Bt-Lon increases substantially in the presence of ATP. Furthermore, the substrate specificity of Bt-Lon is different from that of *E. coli* Lon in using fluorogenic peptides as substrates. Notably, the Bt-Lon protein shows chaperone-like activity by preventing aggregation of denatured insulin B-chain in a dose-dependent and ATP-independent manner. In thermal denaturation experiments, Bt-Lon was found to display an indicator of thermostability value, T_m of 71.5 °C. Sequence comparison with mesophilic Lon proteases shows differences in the rigidity, electrostatic interactions, and hydrogen bonding of Bt-Lon relevant to thermostability.

Keywords: AAA⁺ protein; chaperone-like activity; heat-shock protein; Lon protease; thermostability.

Lon protease (also called La) is the first ATP-dependent protease purified from *Escherichia coli* [1,2] that plays an important role in intracellular protein degradation (for reviews [3–5]). This enzyme degrades damaged/abnormal proteins and several short-lived regulatory proteins which are crucial for radiation resistance, cell division, synthesis of capsular oligosaccharides, and formation of biofilms [6]. In *E. coli*, Lon has been identified as a heat-shock protein (HSP) [7,8]. In bacilli, although the *Bacillus subtilis* *lon* gene (*Bs-lon*) is induced by heat shock [9], the heat-shock response has not been detected for the *Bacillus brevis* *lon* promoter [10]. Lon protease functions as a homo-oligomer, the subunit of which consists of an N-terminal central

ATPase and C-terminal protease domains [4,11]. In addition, *E. coli* Lon has been shown to act as a DNA-binding protein [12]. However, the biological functions of Lon protease resulting from DNA binding are still unclear. Lon protease and Clp/HSP100 are major ATP-dependent proteases in *E. coli*. They have been described as members of the AAA⁺ (ATPases associated with diverse cellular activities) superfamily that assist in the assembly, operation, and disassembly of DNA–protein complexes [13]. Clp/HSP100 proteins act as molecular chaperones and play a role in the unfolding of substrates and their translocation into the cavity of the cylinder of the proteins themselves [14]. In the past decade, although ATP-dependent proteases of the AAA⁺ superfamily have been shown to exhibit chaperone-like activity [15–17], the direct biochemical characterization of a chaperone-like activity of Lon has not been carried out.

The stability of proteins is highly important to the survival of thermophilic organisms at high temperatures [18]. Insights into the stabilizing interactions among the thermophilic proteins have been gained from comparisons of amino-acid sequences and 3D structures with the homologous mesophilic enzymes. The advantage of this approach is that the high sequence identity between the proteins compared minimizes the noise originating from phylogenetic differences [18,19]. Nevertheless, the lack of 3D structures for homologous pairs of proteins has hampered such detailed comparisons. So far, no

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Abbreviations: *Bt-lon*, *Br. thermoruber* Lon protease gene; Bt-Lon, *Br. thermoruber* Lon protease; Bs-Lon, *Bacillus subtilis* Lon protease; AAA⁺, superfamily of ATPases associated with diverse cellular activities; SSD domain, sensor-discrimination and substrate-discrimination domain; EMSA, electrophoretic mobility-shift assays; RBS, ribosome-binding site; HSP, heat-shock protein.

Enzyme: Lon protease (EC 3.4.21.53).

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single mechanism or general traffic rule responsible for the stability of thermophilic proteins has been proposed [18–21].

In this paper, we report the gene cloning and characterization of a thermostable Lon protease from *Brevibacillus thermoruber* WR-249. We show that the recombinant *Br. thermoruber* Lon protease (Bt-Lon) is a HSP and a thermostable enzyme. In addition, we confirm that Bt-Lon possesses chaperone-like activity toward denatured proteins in a dose-dependent and ATP-independent manner. We also discuss factors contributing to protein thermostability in conjunction with sequence comparison analyses of Bt-Lon and *B. subtilis* Lon protease (Bs-Lon).

Materials and methods

Bacterial identification and culture conditions

All biochemical tests and identification procedures were performed as specified previously [22]. In brief, samples of hot spring water, solfataric soil, and mud were collected from hot springs located in the Wu-rai area (E: 121°32'34"; N: 24°51'52"), Taipei County, Taiwan. All isolates purified by serial transfers were preserved in modified *Thermus* medium containing 15% glycerol at –70 °C. One isolate, designated WR-249, was chosen for this study. After the extraction of genomic DNA, PCR-mediated amplification, and sequencing of the purified PCR product, the 16S rDNA sequence was compared with the previously determined *Bacillus* sequences available from the EMBL database. The isolate was identified as thermophilic *Br. thermoruber* WR-249 and grown at 50 °C in a liquid-modified *Thermus* medium.

Bacterial strain, enzymes and chemicals

E. coli JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 Δ(lac-proAB)-F'(traD36 proAB lacI^q lacZΔM15)*], used in cloning experiments, and *E. coli* BL21 (DE3) [*F⁻ ompT hsdS_B (r_B⁻ m_B⁻) gal dcm* (DE3)] (Novagen, Madison, WI, USA), used for gene expression, were grown in Luria–Bertani medium, supplemented with ampicillin (50 µg·mL⁻¹). DNA ligation kits were obtained from Takara Shuzo (Kyoto, Japan). Fluorogenic peptides, succinyl-Phe-Leu-Phe-methoxynaphthylamide (Suc-FLF-MNA) and glutaryl-Ala-Ala-Phe-methoxynaphthylamide (Glt-AAF-MNA) were purchased from Bachem (Bubendorf, Switzerland). Insulin from bovine pancreas and dithiothreitol were purchased from Sigma.

DNA manipulation and sequence analysis

Plasmid DNA preparation, purification of DNA from agarose gel, and restriction enzyme analysis were performed by the standard methods [23]. DNA sequence analysis, translation, and alignment with related proteins were carried out using the BIOEDIT suite [24].

Molecular cloning of *Br. thermoruber* Lon gene (Bt-lon)

Based on the codon usage preference of thermophilic *Br. thermoruber* WR-249, the following two degenerate

oligonucleotide primers were used to amplify a part of the gene encoding the Lon protease by PCR. One of the primers, 5'-AATACC(C/G)CC(C/G)GG(C/G)GT(C/G)GG(C/G)AAGACGTCGCT-3' (forward), was based on the conserved nucleotide sequences around the ATP-binding site [25]. The other primer, 5'-CGTGAT(C/G)CCGGC(C/G)GA(C/G)GG(C/G)CCGTCTTTGG-3' (reverse), was based on the serine residue, which is the putative active site of Lon proteases reported to date [9,10,26,27]. A single 983-bp product was amplified and cloned into the pGEM-T-easy vector (Promega) for sequence determination. Sequence analysis of the PCR product revealed significant homology with the other known *lon* genes.

To obtain the full-length gene, the chromosome walking (CW) procedures were performed with *Br. thermoruber* genomic DNA using LA PCR *in vitro* Cloning Kit (Takara Shuzo, Kyoto, Japan). First, the genomic DNA was extracted from *Br. thermoruber* by standard methods [23] and digested with *Hind*III and *Sal*I. The digested DNA fragments were ligated with cassette adaptors and then used as a template for the following experiment. The primary PCR was performed using the Lon gene-specific primer: 5'-AATCGTATGCGTGCTGTTGGCCGTCGTGAT-3' (Send-CW-1) or 5'-AACCAGAATGACAAGTTCAGCC ACCATTACATCGA-3' (3'end-CW-1) and the cassette primer C1 provided in the kit. Finally, a nested primer pair including 5'-ACTTGTCATTCTGGTTGGGGTCCAGC ACTT-3' (5'end-CW-2) or 5'-ATGCTGAAGGTAATT CGTCATACACCAGAGAA-3' (3'end-CW-2) and the cassette primer C2 were used for the nested PCR. The amplified DNA fragments were cloned and sequenced to complete the Bt-*lon* sequence.

Heat-shock experiments and Northern blotting

Mid-exponential phase cultures of *Br. thermoruber* were heat-shocked by placing the culture vials in a water bath at 60 °C or 65 °C for 30 min. The cells were harvested in precooled plastic tubes at 4 °C for 3 min, and centrifuged at 10 000 g for 8 min.

Total RNA was extracted from *Br. thermoruber* using the Qiagen RNA kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Northern blotting was performed by standard procedures [23]. RNA gel blot hybridization was carried out using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics GmbH, Mannheim, Germany), and followed the manufacturer's instructions except for visualization with nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) as a substrate of alkaline phosphatase.

Preparation of Bt-*lon* expression constructs

The full-length Bt-*lon* flanked by the *Nde*I and *Xho*I sites was amplified by PCR with *Br. thermoruber* genomic DNA and two primers, sense (5'-AATGATGCATATG GCGAACGTTCCGGTAA-3') and antisense (5'-ATTA CTCGAGCGCCTGCGTCCAGGCCAG-3'). The underlined sequences indicate the *Nde*I site in the sense primer and the *Xho*I site in the antisense primer. The amplified DNA fragment was digested with *Nde*I and *Xho*I and

then ligated with the corresponding plasmid pET-21a(+) (Novagen) for the production of recombinant Bt-Lon.

Expression and purification of Bt-Lon

Bt-Lon was overexpressed in *E. coli* strain BL21(DE3). An overnight culture of fresh transformant was diluted 1 : 100 in fresh Luria–Bertani medium (containing ampicillin 50 $\mu\text{g}\cdot\text{mL}^{-1}$) and grown at 37 °C until the A_{600} value for the culture reached 0.5, followed by growth with the addition of 1.0 mM isopropyl β -D-thiogalactoside for an additional 3–4 h. The cells were harvested by centrifugation (6500 *g*), resuspended in 50 mM Tris/HCl (pH 8.0) containing 300 mM NaCl, 1% Triton X-100, 20% glycerol, 10 mM imidazole and 10 mM 2-mercaptoethanol, freeze-thawed, and disrupted by ultrasonication. The cell debris was removed by centrifugation at 8000 *g* for 15 min at 4 °C.

The lysate was mixed with Ni/nitrilotriacetic acid affinity agarose (Qiagen, Hilden, Germany) for 60 min at 4 °C with end-over-end mixing, and the resin was packed into an Econo-Pac column (Bio-Rad Laboratories, Hercules, CA, USA). The column was washed using 20 vol. buffer containing 10 mM Tris/HCl (pH 7.4)/300 mM NaCl/20 mM imidazole and then eluted with five volumes of the same buffer containing 200 mM imidazole. Affinity-purified Bt-Lon was concentrated using a Centriprep 30 concentrator (Amicon) and further purified on TSK HW-55F (Tosoh, Tokyo, Japan) gel-filtration column equilibrated in buffer containing 50 mM Tris/HCl (pH 8.0), 10 mM MgCl_2 and 150 mM NaCl. The protein concentration of the purified Bt-Lon was determined by the Bradford method (Bio-Rad Laboratories), and the homogeneity of the purified Bt-Lon was analyzed by SDS/PAGE. N-Terminal amino-acid sequence analysis was carried out by automated Edman degradation with a protein sequencer (model 477A; Applied Biosystems).

Analytical gel filtration chromatography

The gel filtration experiments were performed using fast protein liquid chromatography on a Superose 6 HR 10/30 column (Amersham Biosciences) equilibrated with buffer containing 50 mM Tris/HCl (pH 8.0), 10 mM MgCl_2 , 150 mM NaCl, and 10% glycerol with a flow rate of 0.5 $\text{mL}\cdot\text{min}^{-1}$. Blue dextran was used to determine the void volume, V_0 . Several proteins of known molecular mass (thyroglobulin, 669 kDa; apoferritin, 443 kDa; β -Amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; BSA, 66 kDa; carbonic anhydrase, 29 kDa; all from Sigma) were used as the standards and their elution volumes, V_e , were determined. The standard curve was plotted with the logarithm of molecular mass against V_e/V_0 of the standard protein.

Peptidase and ATPase assays

The peptidase activity of Bt-Lon was examined as described previously [4]. Peptidase assay mixtures contained 50 mM Tris/HCl (pH 8.0), 10 mM MgCl_2 , 1.0 mM ATP, 0.3 mM fluorogenic peptide, and 5–10 μg Bt-Lon in a total volume of 200 μL . Reaction mixtures were incubated for 60 min at 50 °C or at the indicated temperatures and stopped by the addition of 100 μL 1% SDS and 1.2 mL 0.1 M sodium

borate (pH 9.2). Fluorescence was measured in a Hitachi F4010 fluorescence spectrophotometer with excitation at 335 nm, and emissions were monitored at 410 nm for fluorogenic peptides containing 4MNA (4-methoxy- β -naphthylamide), Suc-FLF-MNA or Glt-AAF-MNA. One unit of peptidase activity was defined as the amount of enzyme required to release 1 pmol 4MNA per h. The amount of 4MNA released during peptidase assays was calibrated using the free compound (Sigma).

ATPase assays were performed for the detection of free inorganic phosphate as described previously [28]. Reaction mixtures were composed of 50 mM Tris/HCl (pH 8.0), 10 mM MgCl_2 , 1.0 mM ATP, and 2–5 μg Bt-Lon in a total volume of 100 μL and incubated for 30 min at 50 °C or at the indicated temperatures. The color of the reaction was developed by adding 800 μL malachite/molybdate solution and terminated by the addition of 100 μL 34% sodium citrate. The absorbance of the final reaction was determined at 660 nm. Absorbances were converted into phosphate concentrations using K_2HPO_4 standards. One unit of ATPase activity was defined as the amount of enzyme required to release 1 nmol $\text{P}_i\cdot\text{h}^{-1}$. The background values of hydrolysis were subtracted in each assay.

Electrophoretic mobility-shift assays (EMSA)

For plasmid mobility-shift assays, plasmid pET-21a(+) was used routinely. Bt-Lon (4 μg) was incubated in a total volume of 25 μL containing 50 mM NaCl, 10 mM MgCl_2 and 50 mM Tris/HCl, pH 8.0, for 20 min with plasmid DNA (500 ng) at the indicated temperatures. Analysis used standard 0.8% agarose gels, and DNA bands were visualized by ethidium bromide staining.

Assay of chaperone-like activity

The assay is based on preventing the aggregation of denatured insulin B-chain [29]. Insulin (0.3 $\text{mg}\cdot\text{mL}^{-1}$) in NaCl/ P_i buffer at pH 7.4 was unfolded by adding dithiothreitol to reach 20 mM as the final concentration at 37 °C, and aggregation was monitored by measuring the absorption due to light scattering at 360 nm in a spectrophotometer for 30 min in the absence or presence of various amounts of Bt-Lon. The ratios (w/w) of insulin to Bt-Lon were 6 : 1 and 3 : 1, respectively.

Circular dichroism

CD spectra were recorded on a Jasco J-715 spectropolarimeter with a 0.1-cm light path for far-UV CD measurements at 25 °C. Protein concentrations were 0.4 mM in NaCl/ P_i buffer, pH 7.4. The bandwidth was 1.0 nm, and ellipticity measurements were averaged for 3 s at each wavelength. All spectra reported are the average of five scanning accumulations.

Thermal denaturation and unfolding transition

The temperature dependence of the CD ellipticity at 222 nm was monitored using a 0.1-cm path length cuvette with a Jasco J-715 spectropolarimeter equipped with a temperature controller (model RTE-111; Nealab, Portsmouth, NH,

USA). Protein solutions ($\approx 35 \mu\text{g}\cdot\text{mL}^{-1}$) were heated from 20 °C to 90 °C at a rate of 60 °C/h. The native protein fraction was determined as $(\epsilon - \epsilon_D)/(\epsilon_N - \epsilon_D)$, where ϵ is the observed ellipticity, and ϵ_N and ϵ_D are the ellipticities of the native and denatured baselines, respectively. The temperature parameter, T_m , was derived from the CD denaturation curve on the basis of a two-state mechanism [30].

Nucleotide sequence accession number

The 16S rDNA sequence of the new isolate, strain WR-249, elucidated here has been deposited with GenBank/EBI under the following accession number: AY19600. The nucleotide sequence of *Bt-lon* reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number AY197372.

Results

Sequence identification and analysis of the Bt-lon

A thermophilic bacterium was isolated from hot springs located in Wu-rai, Taipei County, Taiwan and identified as *Br. thermoruber* WR-249 (data not shown). Using the strategy as described in Materials and methods, a 983-bp DNA fragment was purified and cloned from this thermophilic bacterium. Nucleotide sequence analysis of this fragment revealed a high homology with the Lon protease. To complete the gene sequence, we utilized the technique of chromosome walking (see Materials and methods) to obtain the entire *Bt-lon*, which is 2749 bp long and encoded as a protein of 779-amino acids with a predicted molecular mass of 87 787 Da. The nucleotide sequence from 174 bp to 180 bp (GGAGAGG) was found to be homologous to a putative ribosome-binding site (RBS) (Fig. 1), which was also homologous to the 3'-terminal sequence of *Br. thermoruber* 16S rDNA. In the light of this identity with the RBS, we found an initiation codon (TTG) from 9 bp downstream of RBS, which is followed by a long ORF of 2337 bp. Consequently, this codon most likely encodes the first Met residue of the nascent Bt-Lon. In fact, TTG is used as a start codon more frequently in *Brevibacillus brevis* than in *E. coli* [10,31].

Lon protease is highly conserved and has been identified from various organisms. The deduced amino-acid sequence of the *Bt-lon* revealed 88%, 67%, 55%, 51%, 47%, 41%, and 15% identity with those of *Br. brevis* [10], *Bacillus subtilis* [9], *E. coli* [26], *Thermus thermophilus* [27], *Mycococcus xanthus* [32], *Mycobacterium smegmatis* [11], and

Thermococcus kodakaraensis [33], respectively. Belonging to the AAA⁺ superfamily, Bt-Lon possesses one central AAA domain that comprises the Walker A and B motifs, sensor 1, and sensor 2 (SSD) [34]. The amino-acid sequences around the Walker A-motif GPPGVGKTS (residues 355–362) acting as an ATP-binding site and the putative proteolytic S⁶⁷⁸ active site PKDGPSAG (residues 673–680) of Bt-Lon are highly conserved (Fig. 2). A multiple alignment of various Lon proteases showed that the N-terminal, SSD, and protease domain of this family was highly variable (Fig. 2). In addition, it should be noted that the coiled-coil regions were located at N-terminal regions (residues 184–226 and 238–279) and the SSD domain (residues 495–605) (Fig. 2), which were analyzed and predicted by the COILS program [35]. The coiled-coil conformations are frequently solvent-exposed domains and are considered to be involved in protein–protein or protein–DNA interaction [36].

Analysis of promoter and heat-induced transcription of Bt-lon

The *lon* gene of *E. coli* and *B. subtilis* belongs to the heat-shock regulon, the transcription of which is increased on heat induction through the action of the heat-shock-specific sigma factors [37]. To characterize the promoter region, we searched for the upstream region of *Bt-lon* from nucleotides 1–180 bp and found a putative promoter sequence, TTAG ACA for the –35 region and TACAAT for the –10 region (Fig. 1), which had extensive homology with the consensus sequence of σ^A -dependent heat-shock promoters in *B. subtilis* and σ^{70} promoter in *E. coli* (Table 1). We also identified the TNTG motif at the –16 region [38], which is prominent in σ^A -dependent promoters of *B. subtilis* (Table 1). Interestingly, we noticed that an inverted repeat, but not the CIRCE (controlling inverted repeat of chaperone expression) in the typical σ^A -dependent promoter [39,40], is localized between the –10 region and RBS (Fig. 1), which is also found in the other gene of *B. subtilis* (Table 1). Because the *Br. brevis lon* gene is not induced by heat shock [10], we attempted to investigate whether transcription of *Bt-lon* is induced in response to elevated temperature. We conducted Northern-blot analysis with heat-shocked cells, and the result shows that transcription of *Bt-lon* is enhanced after a shift to higher temperatures (data not shown). However, the mechanisms of induction of *Bt-lon* require more study.

Characterization of Bt-Lon

To characterize Bt-Lon, the entire coding region of *Bt-lon* was expressed in *E. coli* and its product was purified. *Bt-lon*

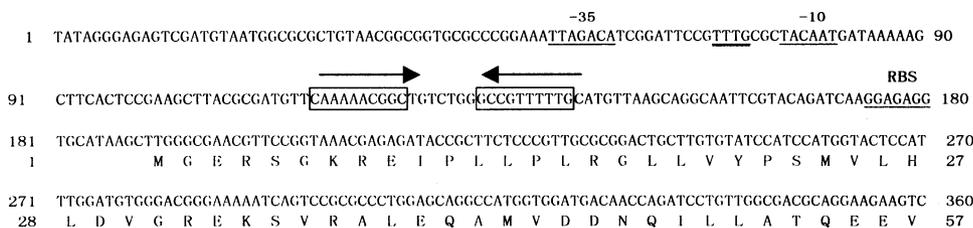


Fig. 1. Putative promoter region of Bt-lon. Potential –35 and –10 regions and the RBS are underlined. The –16 region is bold underlined. An inverted repeat of dyad symmetry is boxed and indicated by a pair of horizontal arrows.

<i>Br. thermoruber</i>	1	MG-ERSGKREIFLLPLRGLLVYPMVHLHDVGRKESVRALEQAMVDDNQILLATQEEVHI EEP SAEQI FSVGTVARVKQMLKLPNGTIRV	89
<i>B. brevis</i>	1	MG-ERSGKREIFLLPLRGLLVYPTMVLHLDVGRKESIRALEQAMVDDNKILLATQEEVHI EEP DAEQI YSIGTVARVKQMLKLPNGTIRV	89
<i>B. subtilis</i>	1	MA-EEL-KRSIPLRGLLVYPTMVLHLDVGRDKSVALEQAMHDHMFPLATQQDISIDEPGEDEIFTVGTYTKIKQMLKLPNGTIRV	88
<i>E. coli</i>	1	MNPFERSERIEIFVLPFRDVVYPHMYIPLFVGRKEISIRCLEAAMDHDKKIMLVAQKEASTDEPGVNDLFTVGTVASILQMLKLPDGTIVK	90
<i>T. thermophilus</i>	1	MK--DFLRLELFLVLPFRNTVVLPHHTTTGVGVGRKLSKRAVEEALSADRLLFLVTQKDPVEVDDPAPEDLYAVGTAVVQAMRLPDGTIQV	88
<i>Br. thermoruber</i>	90	LVEGLQARARIDEYIRQDDFFQVSIITYLEEKADENEVEALMRAVLSHFQYIKLSKKISPEALT--SVSDIEEPGRLADVIAHSLPLKMK	177
<i>B. brevis</i>	90	LVEGLQARAKIEEYLQKEDYFVVSITYLKEEKAEENEVEALMRSLLTHFQYIKLSKKVSPETLT--SVQDIEEPGRLADVIAHSLPLKMK	177
<i>B. subtilis</i>	89	LVEGLKRAHIVKYNEHEDYTSVDIQLIHEDDSKDTDEBALMRTLDDHFDQYIKISKKISAETYA--AVTDEIEEPGRMADTVIAHSLPLKLA	176
<i>E. coli</i>	91	LVEGLQARARISALSDNGEHFSAKAAYLESPTIDERQEVLVRTAISQFEGYIKLNKKIPPEVLT--SLNSIDDPARLADTIAAHMPLKLA	178
<i>T. thermophilus</i>	89	MVEARSRARLLSYVAAPYLRAVGEAIPPEPLKDPALARVLVNEVQEAPEFYRLQNHKTLRLDRYQQAFAVSKTRDPAILADLVAAHATWTL	178
<i>Br. thermoruber</i>	178	DKQEILETTNIKERLNILLDILNNEVELELERKISNRVKKQMERTQKEYLLREQMKAIQKELGKDGGRQSEVDELRAQLEKSDAPERIK	267
<i>B. brevis</i>	178	DKQEILETVNIQERLEILLTILNNEVELELERKIGNRVKKQMERTQKEYLLREQMKAIQKELGKDGGRQSEVDELRAQLEKSDAPERIK	267
<i>B. subtilis</i>	177	DKQDILETADVDRLNKVIDFINNEKEVLEIEKIGQRVKSMEERTQKEYLLREQMKAIQKELGKDGKGTGEVQTLTEKIEEAGMFDHVK	266
<i>E. coli</i>	179	DQKQSVLEMSDVNELEYLMAMMESEIDLLQVEKRIIRNRVKKQMEKSKREYLLNEQMKAIQKELGEMDDAPPDENALRRKIDAAKMPKEAK	268
<i>T. thermophilus</i>	179	EKQTILETEFEVEERLKRVLALLLDLLELRFELDKKAAARVKKQMQDQQRREYLLREQMKAIQKELGGGEFFLTFIEEELREERLEKKGMEPEVK	268
<i>Br. thermoruber</i>	268	NKIEKELERLEKMPPTSAGAVIRTYIDTLLSLPWTRRTVDNLDIHAEQVLNEDHYGLEKPKERVLEYLAVQKLVN--SMRG--PILCL	353
<i>B. brevis</i>	268	AKIEKELERLEKMPSTSAGSVIRTYIDTLLFALPWTKTEDNLDIKHAEVLDDEHYGLEKPKERVLEYLAVQKLVN--SMRG--PILCL	353
<i>B. subtilis</i>	267	ETALQELNRYEKIPSSSAESSVIRNYIDWLVALPWTDETDKDLKLEAGRLLEHHGLEKPKERILEYLAVQKLTK--SLKG--PILCL	352
<i>E. coli</i>	269	EKAERELQKLMMSFMSAEATVVRGYIDMWMVQPNARTKVKKDLRQAQELDLDTHYGLERVKDRILEYLAVQSRVN--KIKG--PILCL	354
<i>T. thermophilus</i>	269	<u>EKAELKELKLEPMQFSGPEATVSRTYLDWLLVVPWTEADPEVLDISVTKRVLDEDEHYGLEKPKERILEYLAVRQLTQGKEVKGHA</u> <u>PILCF</u>	358
<i>Br. thermoruber</i>	354	VGPPGVGKTSLSARLARALEREFRVIRISLGGVDRDEAIRGHRRTYVVALPGRIIQGMKQAGTINPVFLLEIDKLDASDFRGDPASALLEVL	443
<i>B. brevis</i>	354	VGPPGVGKTSLSARVARALGREFRVIRISLGGVDRDEAIRGHRRTYVVALPGRIIQGMKQAGTINPVFLLEIDKLDASDFRGDPASALLEVL	443
<i>B. subtilis</i>	353	AGPPGVGKTSLSAKSLGRKPFVIRISLGGVDRDESEIRGHRRTYVGMPPGRIIQGMKAGKLNVPVFLLEIDKMSDFRGDPSAMLEVL	442
<i>E. coli</i>	355	VGPPGVGKTSLGQSSIAKATGRKYVRMALGGVDRDEAIRGHRRTYIGSMPPGLIQMKAVGVVKNPLFLLEIDKMSDMRGDPASALLEVL	444
<i>T. thermophilus</i>	359	<u>VGPPGVGKTSLGKSIARSMNRREHRIISLGGVDRDEAIRGHRRTYIGALPGKIIQGMKQGVVNPVFLLEIDKLSDDWRGDPAAALLEVL</u>	448
		Walker A	Walker B
<i>Br. thermoruber</i>	444	DPNQNDKFSHDYIEETYDLTNVMFITANSTHTIPRPLLDLRMEVISIAGYTELEKLNLMRDYLLPKQMEEHGLGRDKLQMNDEAMKLVIR	533
<i>B. brevis</i>	444	DPNQNDKFSHDYIEETYDLTNVMFITANSLDTIPRPLLDLRMEVISISGYTELEKLNLRGYLLPKQMEDHGLGDKDKLMNEDAMKLVIR	533
<i>B. subtilis</i>	443	DPEQNSSFSDHYIEETFDLSKVLFIATANNLATIPGPLEDRMEIINEAGYTEIEKLEIVKDHLLPKQIKHGLKKSNDLQRQAILLDIIR	532
<i>E. coli</i>	445	DPEQNVAFSDHYLEVYDLSDMVFMVATSNMNIAPAPLLDRMEVIRISGYTEDEKLNIAKRHLPLKQIERNALKKGELTVDSDSAIIGIIR	533
<i>T. thermophilus</i>	449	DPEQNHPTDHYLDVVPYDLSKVFFITANTLSTIPRPLLDLRMEVIEIPGYTLHEKRAIARYFRWFQVKEAGLEG-RLIETDRAIERIVQ	537
		Sensor 1	
<i>Br. thermoruber</i>	534	QYTREAGVRNLRNREANI CRKAARLIVSGE-KKRVVVTPKTVESLLGKPRYRYGLAEREDQVGAVTGLAWTQAGGDTLNVVSVILPGK GK	622
<i>B. brevis</i>	534	LYTREAGVRNLRNREANVCRKAAKIIVGGE-KKRVVVTAKTLEALLGKPRYRYGLAEEKDQVGSVTGLAWTQAGGDTLNVVSVILAGK GK	622
<i>B. subtilis</i>	533	YYTREAGVRSLEQLAAICRKAAKIAVAEE-RRKITVTEKNLQDFIGKRIFRYQQAETEDQGVVVTGLAYTTVGGDLSIEVSLSPGK GK	621
<i>E. coli</i>	534	YYTREAGVRLEREISKLCKRAVKQLLDDKSLKHIEINGDNLHDYLVGQRFQYGRADNENRVGQVTGLAWTEVGGDLLTIETACVPGK GK	623
<i>T. thermophilus</i>	538	<u>FYTREAGVRNLDREL SKVARKAKDYLEKFMWGVVVDAPDL EAYLVGVVYRPPDRAEKEPQVGAOGLAWTPTVGGTLLTIEAVAVPGT GK</u>	627
		Sensor 2 (SSD)	
<i>Br. Thermoruber</i>	623	LTLTGQLGDVMKESAAQAFSYIRSRAAANNIDPEPHEKNDIHIHVPEGAIPKDGPSAGITMATALVSALTGIPVRKEVGMTGEITLRGRV	712
<i>B. brevis</i>	623	LTLTGQLGDVMKESAAQAFSYIRSRAEWGIDPEPHEKNDIHIHVPEGAIPKDGPSAGITMATALVSALTGIPVKEVGMTGEITLRGRV	712
<i>B. subtilis</i>	622	LILTGKLDVDMRESAAQAFSYVRSKTEELGIEPDFHEKYDIHIHVPEGAVPKDGPSAGITMATALVSALTGRAVSRVGMTGEITLRGRV	711
<i>E. coli</i>	624	LYTGTSLGEVMQESIQAALTVVRAAEKLGINDPFEYKRDHIVHVPEGATPKDGPSAGIAMCTALVSLTGNPVRADVAMTGEITLRQV	713
<i>T. thermophilus</i>	628	VNLTGNLGEVMKESAAHALTYLRAHREEWGLPEGFHKDYDLHIHVPEGATPKDGPSAGITMATALASALTGRPVRMDIAMTGEITLRGRV	717
<i>Br. thermoruber</i>	713	LPIGGLKEKALSAHRAGLTTIILPKNEKEDIEDIPESVRKELKLIIVHEMDEVLRHALTRQAVGETR-----	779
<i>B. brevis</i>	713	LPIGGLKEKMSAHRAGLTTIILPKNEKEDIEDIPESVREALTFYPVEHLDEVLRHALTKQVPGDK-----	779
<i>B. subtilis</i>	712	LPIGGLKEKALGAHRAGLTTIIPKNEKEDIEDIPESVREGLTFILASHLDEVLEHAL----VGEKK-----	774
<i>E. coli</i>	714	LPIGGLKEKLLAAHRGGIKTVLIPFNKRDLEEDPNVIADLDLHPVKRIEVEVLTALARNEPSGMQVVTA-----	784
<i>T. thermophilus</i>	718	LPIGGVKEKLLAAHQAGIHRVILPKENAAELKEVPEILKDLIEHFVEEVGVLKLLLPVPPPPAVQPPDRPQGVGA	795

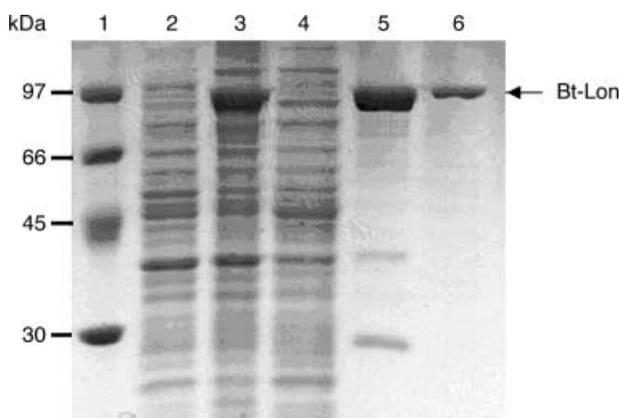
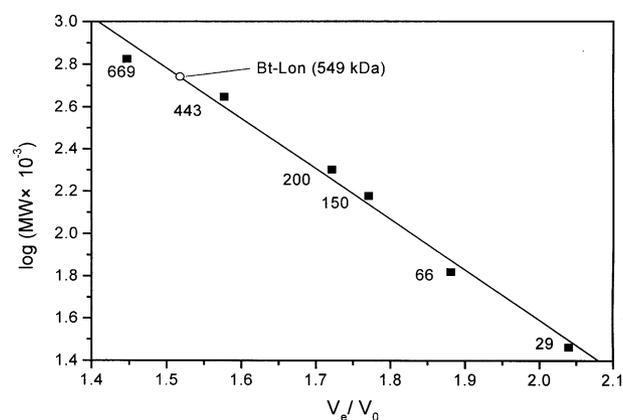
Fig. 2. Multiple alignments of amino-acid sequences of Bt-Lon and other Lon proteases. The sequence alignment was based on the CLUSTALW algorithm implemented in the BIOEDIT program. Identical amino-acid residues are shaded. The sequences with underlined and broken underlined characters indicate the conserved structural motifs in the ATPase domain (AAA⁺ module) and coiled-coil region, respectively. A filled circle shows the serine residue acting as the proteolytic active site of Lon proteases. SSD represents sensor and substrate discrimination [34]. The sources of Lon sequence include (GenBank/EMBL accession numbers in parentheses): *Br. thermoruber* (AY197372), *B. brevis* (D00863), *B. subtilis* (X76424), *E. coli* (J03896), and *T. thermophilus* (AF247974).

was specifically induced and overexpressed in *E. coli* BL21(DE3) after the addition of 1 mM isopropyl β-D-thiogalactoside (Fig. 3, lanes 2 and 3). SDS/PAGE analysis indicated that the recombinant protein was a single band of ≈ 90 kDa after purification by affinity and gel filtration chromatography (Fig. 3, lanes 4–6). The N-terminal amino-acid sequence of the recombinant protein as determined by Edman degradation was identical with the deduced sequence of Bt-Lon. The native molecular mass of recombinant Bt-Lon was estimated by analytical gel-filtration chromatography as 549 kDa (Fig. 4). This result shows that the recombinant Bt-Lon forms a hexamer in nature.

To characterize the peptidase activity of recombinant Bt-Lon, a fluorogenic peptide, Glu-AAF-MNA, was used as substrate. The optimum temperature for the Bt-Lon peptidase activity was determined to be 50 °C (Fig. 5A). Like ATP-dependent *E. coli* Lon proteases described previously [41], the proteolytic activity of Bt-Lon was greatly enhanced in the presence of 1 mM ATP (Fig. 6). The optimum temperature for the Bt-Lon ATPase activity, however, was determined to be 70 °C (Fig. 5A). The maximum specific activity of ATPase at 70 °C is (3.2 ± 0.16) × 10⁴ pmol P_i(μg Lon)⁻¹h⁻¹. The substrate specificity for the peptidase activity of Bt-Lon was also examined using the fluorogenic

Table 1. Compilation of *B. subtilis* σ^A -dependent promoter sequences compared with *Br. thermoruber lon* promoter region. +, present; -, absent.

Bacterial species	Gene	- 35	Spacer	- 16	- 10	Inverted repeat	Ref.
<i>Br. thermoruber</i>	<i>lon</i>	TTAGACA	17	TTTG	TACAAT	+	This work
<i>Br. brevis</i>	<i>lon</i>	TTAGACA	17	TTTG	TACAAT	-	[10]
<i>B. subtilis</i>	<i>lon</i>	TTGTACA	17	GTTG	TATAAT	-	[9]
<i>B. subtilis</i>	<i>clpX</i>	TTGTTAC	20	TATG	TAAAAT	+	[61]
<i>B. subtilis</i>	<i>fisH</i>	TTGTATT	17	TATG	TACTAT	-	[62]
<i>B. subtilis</i>	<i>spoIIG</i>	TTGACAG	21	CTTG	TATAAT	+	[63]
<i>B. subtilis</i>	<i>amyR</i>	TTGTTTT	16	TGTG	TAATTT	+	[38]
<i>B. subtilis</i>	σ^A consensus	TTGACA	16-18	TNTG	TATAAT	CIRCE	[64]
<i>E. coli</i>	σ^{70} consensus	TTGACA	16-18	-	TATAAT	-	[37]

**Fig. 3.** SDS/PAGE analysis of expression and purification of the recombinant Bt-Lon. Lane 1, molecular mass markers (in kDa): phosphorylase b (97), albumin (66), ovalbumin (45) and carbonic anhydrase (30); lanes 2 and 3, crude lysate from *E. coli* cells containing pET21a-Bt-Lon plasmid without and with isopropyl thiogalactoside induction, respectively; lanes 4 and 5, the unbound and bound fractions, respectively collected from the crude lysate eluted from a Ni/nitrilotriacetate affinity agarose column; lane 6, the fraction in lane 5 was further purified by gel filtration. The arrow shows the recombinant Bt-Lon.**Fig. 4.** Estimation of the molecular mass of native Bt-Lon by analytical gel filtration. The semilogarithmic plot of elution volume (V_e/V_0) vs. log (molecular mass) of standard proteins [thyroglobulin (669 kDa), apoferritin (443 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), and carbonic anhydrase (29 kDa)] is shown as the standard curve. The molecular mass of native Bt-Lon (O) was estimated from the standard curve based on the elution volume of native Bt-Lon and the molecular masses of the standard proteins (■). The analytical gel filtration was performed on a Superose 6 HR 10/30 column.

peptides under optimum conditions. Interestingly, the results indicate that Bt-Lon cleaves both fluorogenic peptides, but prefers Glt-AAF-MNA to Suc-FLF-MNA (Fig. 6). It showed a specific activity of 697.6 ± 34.9 and 267.68 ± 13.4 pmol for Glt-AAF-MNA and Suc-FLF-MNA, respectively. In other words, it cleaved Glt-AAF-MNA 2-3 times more efficiently than Suc-FLF-MNA. These results conflict with those for *E. coli* Lon [41] and suggest that the substrate preference of Bt-Lon is different from that of *E. coli* Lon.

The primary function of HSPs is to act as chaperones, preventing irreversible aggregation of misfolded proteins in the cell [42]. To test that Lon protease possesses chaperone-like activity, we examined whether Bt-Lon prevents the aggregation of dithiothreitol-induced denatured insulin by monitoring the kinetics of aggregation by light scattering. As shown in Fig. 7, curve 1, denatured insulin formed aggregates in the absence of Bt-Lon. In contrast, at the 3 : 1 (w/w) ratio of insulin to Bt-Lon, Bt-Lon almost completely

prevented the dithiothreitol-induced aggregation of insulin B-chain (Fig. 7, curve 4). At the 6 : 1 (w/w) ratio of insulin to Bt-Lon, Bt-Lon suppressed the dithiothreitol-induced aggregation of insulin B-chain to about 67% (Fig. 7, curve 2). The result indicates that Bt-Lon is efficient in preventing the aggregation of denatured insulin and in a dose-dependent manner. As described previously [42], ATP was critical for the activity of chaperones. The chaperone-like activity of Bt-Lon was also examined in the presence of ATP. The result shows that Bt-Lon prevents insulin aggregation in an ATP-independent manner (Fig. 7, curves 2 and 3).

Thermal stability

The thermostability of Bt-Lon was evaluated by measuring the residual activity as a function of temperature. Maximal ATP-dependent protease and ATPase activity were detected at 50 °C and 70 °C, respectively (Fig. 5A), higher than

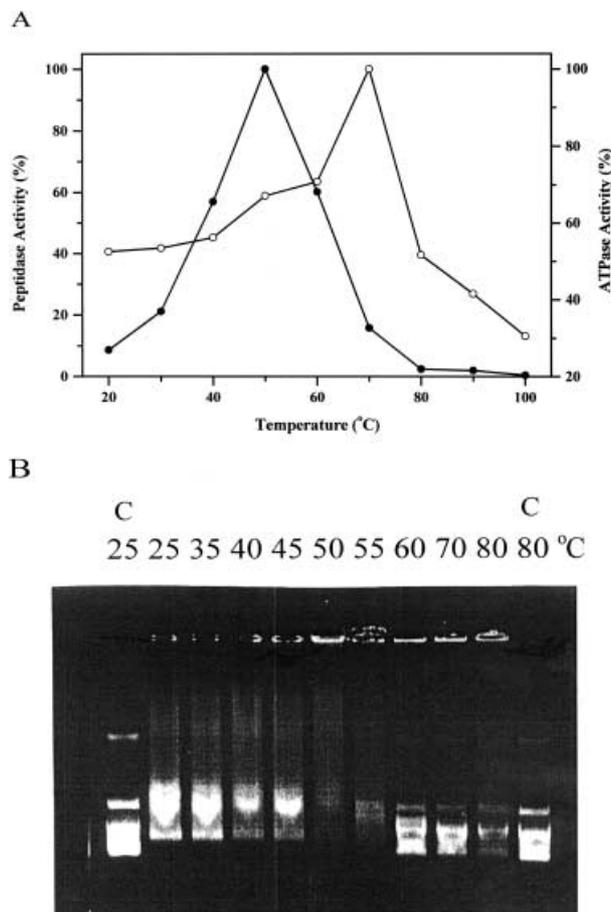


Fig. 5. Effects of temperature on the activities of Bt-Lon. (A) Effects of temperature on peptidase (●) and ATPase (○) activities of Bt-Lon. The peptidase and ATPase assays were performed at the indicated temperatures as described in Materials and Methods. Glt-AAF-MNA was used as a substrate for peptidase assay. (B) Effects of temperature on the DNA-binding activity of Bt-Lon. 25 μ L of the solution containing 4 μ g Bt-Lon and 500 ng plasmid DNA was incubated for 20 min at the indicated temperatures and then subjected to an EMSA as described in Materials and Methods. C, Control experiment, DNA only.

those of *E. coli* Lon (37 °C). In addition, the effect of temperature on the DNA-binding activity of Bt-Lon was examined by EMSA after 20 min of incubation at 25, 35, 40, 45, 50, 55, 60, 70, and 80 °C. Figure 5B shows that the DNA-binding activity of Bt-Lon was reduced after incubation at 55 °C and abolished after incubation at 60 °C. Compared with *E. coli* Lon, Bt-Lon is a relatively thermostable enzyme.

To examine the indicator of thermostability, heat-induced unfolding transition of Bt-Lon was monitored by CD in the far-UV region at 222 nm. This approach was used because the folded Bt-Lon showed a relative CD spectrum with maxima at 210 and 222 nm, suggesting a major α -helical secondary structure in itself (Fig. 8A). The deconvolution of this spectrum yielded \approx 40% α -helix, 30% β -sheet, and 30% random coil and was similar to that of *E. coli* Lon [43]. The result of the unfolding transition showed a midpoint of 71.5 °C, called the melting temperature (T_m), which is often

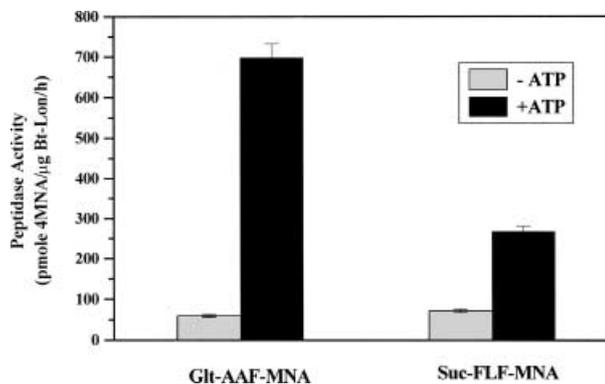


Fig. 6. ATP dependence and substrate specificity of peptidase activity of Bt-Lon. Assays were carried out in 0.2 mL of the solution containing 5–10 μ g of Bt-Lon, 50 mM Tris/HCl (pH 8.0), 10 mM $MgCl_2$, 0.3 mM fluorogenic peptides as substrates in the presence or absence of 1.0 mM ATP. Reaction mixtures were incubated for 60 min at 50 °C.

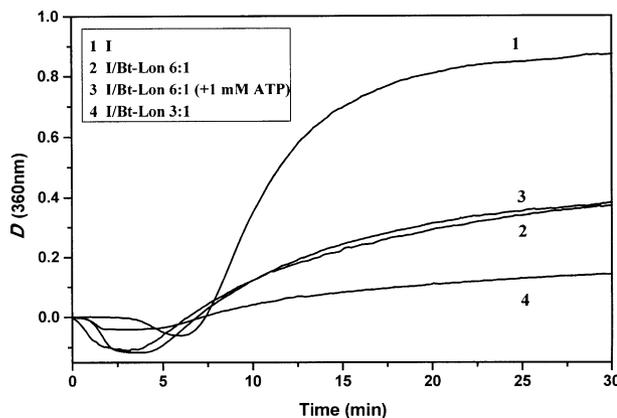


Fig. 7. Chaperone-like activities of Bt-Lon. The chaperone-like activities were measured as the aggregation of denatured insulin B-chain induced by the addition of 20 mM dithiothreitol in NaCl/ P_i buffer. Curve 1, insulin alone; curve 2, insulin + Bt-Lon (50 μ g); curve 3, insulin + Bt-Lon (50 μ g) plus 1 mM ATP; curve 4, insulin + Bt-Lon (100 μ g). The protein concentration of insulin was 300 μ g mL⁻¹ in NaCl/ P_i buffer (pH 7.4). The ratios (w/w) of insulin to Bt-Lon are given in the inset.

used as a measure of protein thermal stability (Fig. 8B) [18,19].

To obtain an insight into the mechanism of thermostability of this protein, we compared sequences of thermophilic Bt-Lon with those of mesophilic Bs-Lon. The G + C content of the protein-coding region of Bt-lon is 59.44% compared with 44.73% for the Bs-lon. Reflecting high G + C content of Bt-lon, this result is consistent with our (and the general) presumption that the thermophilic bacteria possess a high G + C content in DNA [44]. This presumption also guided the design for the experiments of gene cloning. In comparison with homologous proteins from thermophilic and mesophilic organisms, thermophilic proteins contain more hydrophobic and charged amino acids and fewer uncharged polar residues than mesophilic

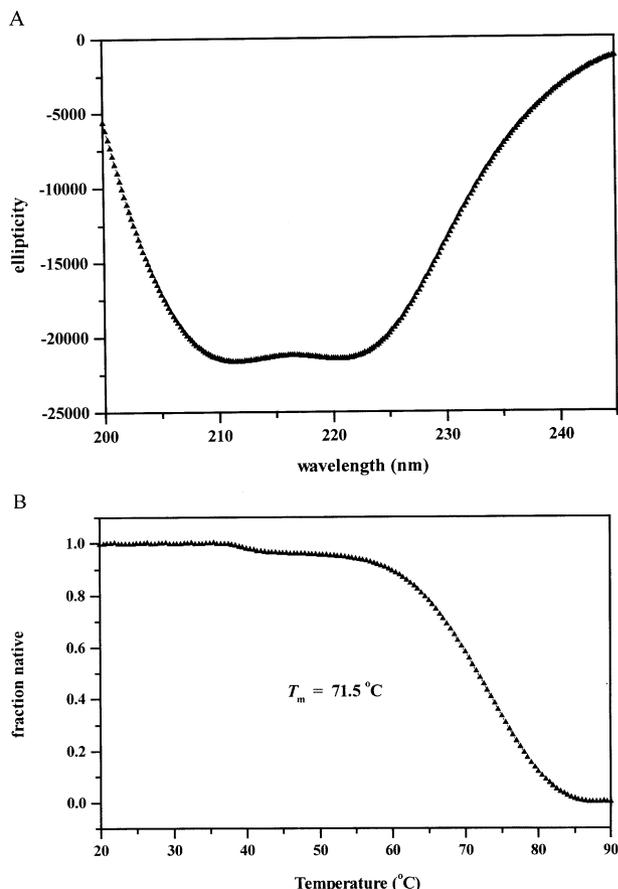


Fig. 8. Thermal denaturation of Bt-Lon by circular dichroism. (A) Far-UV CD spectra of Bt-Lon at 25 °C. (B) Thermal denaturation was monitored by the change in CD ellipticity at 222 nm. The fractions of native protein obtained after a two-state analysis of the data (see Materials and methods) are shown as a function of temperature. In (A) and (B), the units of ellipticity are degrees·cm⁻²·dmol⁻¹.

proteins [19,45]. The results, nevertheless, show that there are no significant changes in the contents of charged and uncharged polar residues and in the hydrophobicity value [46]. In spite of this, Bt-Lon displays a higher aliphatic index (100.13 vs. 98.53) [47], which is defined as the relative volume of a protein occupied by aliphatic side chain. On the other hand, Bt-Lon is characterized by a higher content of V (56 vs. 48), P (33 vs. 28), and E (84 vs. 76) and by a lower content of G (50 vs. 55) than Bs-Lon. We also found that the N+Q content of Bt-Lon is higher than that of Bs-Lon (54 vs. 39), which is in contrast with the criterion of the N+Q content as a general indicator of protein thermostability [19]. It is noteworthy that the ratio in R/(R+K) of Bt-Lon is higher than that of Bs-Lon (0.54 vs. 0.39). All together, more rigid, more electrostatic interactions or hydrogen bonding may confer the thermostability of Bt-Lon.

Discussion

The gene encoding the Lon protease from thermophilic *Br. thermoruber* has been isolated. Compared with other

Lon proteases, Bt-Lon also possesses a three-domain structure consisting of an N-terminal domain (≈ 310 residues), a central ATPase, and a C-terminal protease domain (Fig. 2). The phenomenon of highly variable N-terminal and SSD domains is in agreement with the finding that they are responsible for the discriminatory recognition of specific substrates [34,48].

In *E. coli*, HSPs are primarily induced at the level of transcription, and the activation of HSP gene is enhanced as a result of increased activity of transcription factors – σ^{32} [37]. HSPs include chaperones and ATP-dependent proteases (e.g. ClpAP, Lon). Nevertheless, regulatory strategies for HSP synthesis in Gram-positive bacteria differ markedly from those in *E. coli*. In *B. subtilis*, four classes of HSP genes can be distinguished according to their regulatory strategies [40]. For example, Class IV includes HSP genes such as *lon*, *ftsH*, and *ahpCF*, not belonging to Classes I through III. Although the mechanism of induction of Bt-*lon* is still unknown, Bt-*lon* has been confirmed to be a HSP gene, and it has been predicted that it may be induced by heat utilizing a putative σ^A -dependent promoter in the absence of CIRCE [9] (Fig. 1, Table 1). Interestingly, an atypical inverted repeat was found in the promoter of Bt-*lon*, which is not a transcription terminator of any genes. Whether this inverted repeat is related to the mechanism of induction remains to be studied.

The catalytic activities (including peptidase and ATPase) of Lon proteases are dependent on their tertiary and quaternary structures [49–51]. The different optimal temperatures for the enzymatic activities of protease (≈ 50 °C) and ATPase (≈ 70 °C) imply that the active site of the peptidase domain is situated in a more fragile region responding to the temperature increase than that of the ATPase domain. In general, the enzyme activity is more readily affected than the overall conformational integrity of the protein, because the active site of the enzyme is usually situated in a limited region that is more flexible than the molecule as a whole [52,53]. Therefore, it is not surprising that a subtle change in the tertiary structure around the active-site region could not be detected by CD (Fig. 8), but was manifested by a change in enzymatic activity. Bt-Lon is a hexamer in its quaternary structure. Consequently, as an alternative explanation, the different optimal temperatures of peptidase and ATPase may be attributed to different oligomerization geometry at different temperatures that affect the enzymatic activities. The discrepancy in optimum temperature between peptidase and ATPase was also observed in the thermophilic Lon protease from *Thermococcus kodakaraensis* KOD1 [33]. The substrate specificity and catalytic mechanism of Lon protease is still unclear. The substrate preference shown by Bt-Lon between Glt-AAF-MNA and Suc-FLF-MNA is different from that shown by *E. coli* (Fig. 6) [41]. Therefore, it is believed that the substrate specificity of Bt-Lon is different from that of *E. coli* Lon. In *E. coli*, many physiological substrates (e.g. SulA, RcsA, and CcdA) of Lon have been identified so far, but no consensus features in the primary or higher-order structures of these substrates have been reported [6]. In *B. subtilis*, however, only one specific substrate of Lon, the developmental σ^G factor, has been reported [54]. Therefore, identification of more target substrates or interactive partners of Bs-Lon using a proteomic approach may

provide more information on the molecular basis of substrate specificity.

Lon is an ATP-dependent protease and belongs to the AAA⁺ superfamily of ATPases, which have been shown to have chaperone-like activity [16,17]. Based on this, Lon proteases may have chaperone-like activity as well. This is the first report providing direct biochemical evidence for the chaperone-like behavior of Lon proteases. ATP-dependent proteases and chaperones are involved not only in general protein quality control but also in the regulation and management of specific protein-protein or protein-DNA interaction [13]. According to their modes of action, the chaperones can be divided into three distinct groups: holders, folders and unfolders [55]. For instance, bacterial Clp/HSP100 proteins do not refold protein substrates but rather unfold them in preparation for their subsequent degradation or refolding (by a folder cochaperone) [14]. Clp/HSP100 and Lon protease are proposed as members of the AAA⁺ superfamily sharing considerable sequences that are homologous with AAA proteins [13]. In this work, we confirmed that Lon protease possesses chaperone-like activity similar to that of the Clp/HSP100 family. On the one hand, the results may explain the fact that DnaJ, a folder cochaperone, is not necessary for folding or preventing PhoA aggregation in Lon-dependent degradation [56], despite the fact that DnaK is involved in Lon-dependent degradation [57]. On the other hand, the results suggest a role for Lon protease in the degradation of DNA-binding proteins such as ResA and σ^G transcription factor [6,54] via chaperone-like (Fig. 7) and DNA-binding activity (Fig. 5B) under normal conditions. Bt-Lon was shown to have chaperone-like activity by using denatured insulin as a substrate in an ATP-independent manner (Fig. 7). According to the current model of ATP-dependent protein degradation, the energy-dependent processes are only unfolding and translocation of substrate, but not degradation [14]. Thus, the results may be explained by the fact that the denatured insulin B-chain did not require energy to be unfolded initially and then did not proceed with translocation into a compartment of Bt-Lon. This property is similar to that of *E. coli* Lon, which cleaves the denatured CcdA without ATP hydrolysis [15]. We can also exclude the possibility that decreased turbidity or light scattering of the insulin B-chain is caused by degradation by Bt-Lon, as the insulin is not degraded by Lon proteases under normal conditions [58]. In addition, these phenomena are consistent with the binding of the Lon or Clp protease to a substrate that may not be sufficient to trigger degradation because one or more additional signals are required [34].

The Bt-Lon possesses multiple functions such as DNA-binding, protease, ATPase and chaperone-like activities. These different biological functions in cells will be regulated or manipulated depending on the conditions of cell growth. The optimum temperature for the peptidase and DNA-binding activity of Bt-Lon is 50 °C, which is the optimum temperature for cell growth. This implies that specific proteins such as transcription factors are degraded by Bt-Lon at optimum temperature (50 °C) to regulate cell growth. At higher temperatures, the cell growth of *Br. thermoruber* is much slower and most enzymes or

proteins become denatured or inactivated. Thus, to survive under these harsh conditions, either Bt-Lon disassociates DNA and protects proteins from denaturation by acting as a chaperone-like molecule (or cochaperone) or unfolds and degrades the damaged proteins coupling with ATPase activity. This hypothesis is supported by the fact that the DNA-binding ability of Lon was reduced by the denatured protein substrates and heat shock [59] and that the degradation of Lon became independent of ATP hydrolysis when its substrate lost secondary structure at elevated temperatures [15]. However, the factors causing Bt-Lon to switch from protease activity to chaperone-like activity have not been identified.

Although Lon proteases have been identified from two thermophilic organisms [27,33], none of the reports dealt with their properties or mechanisms of thermal stability. As shown in Fig. 5, Bt-Lon is a thermostable ATP-dependent peptidase and DNA-binding protein. Results of thermal denaturation and unfolding transition experiments show that the melting temperature (T_m) of Bt-Lon could be estimated at 71.5 °C (Fig. 8B). As expected, the T_m is higher than the optimal temperature for growth of the organism (50 °C). In addition, maximal ATPase activity was detected at 70 °C (Fig. 5A), which is consistent with the T_m . To obtain an insight into the mechanism of thermostability of this protein, we compared the properties of thermophilic Bt-Lon with those of mesophilic Lon. As shown in Fig. 4, Bt-Lon is a homohexamer of 88 kDa subunits, which is distinct from the homotetrameric structure of *E. coli* Lon [4]. This result is consistent with the previous statement that thermophilic proteins have a higher oligomerization state than their mesophilic homologues [19]. It remains a mystery how amino-acid substitutions contribute to the thermostability of a thermophilic protein [20,21]. The higher N+Q content of Bt-Lon may enhance electrostatic interactions or increase hydrogen bonding [60]. The ratio R/(R+K) is often higher in thermophilic enzymes than in their mesophilic counterparts [19]. Although the charged amino acids in thermophilic Bt-Lon are roughly the same as in mesophilic Bs-Lon, more R and E residues are found in Bt-Lon than in Bs-Lon, at the expense of K (52 vs. 68) and D (39 vs. 52) residues, respectively. Several properties of R residues reveal that they would be better adapted to high temperatures than K residues [19]. However, more information through a structure-mutation approach is needed to verify the stabilizing factors associated with thermostability.

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