

RESEARCH ARTICLE

Characterization of the molecular chaperone ClpB from the pathogenic spirochaete *Leptospira interrogans*

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Abstract

Leptospira interrogans is a spirochaete responsible for leptospirosis in mammals. The molecular mechanisms of the *Leptospira* virulence remain mostly unknown. Recently, it has been demonstrated that an AAA⁺ chaperone ClpB (a member of the Hsp100 family) from *L. interrogans* (ClpB_{LI}) is not only essential for survival of *Leptospira* under the thermal and oxidative stresses, but also during infection of a host. The aim of this study was to provide further insight into the role of ClpB in the pathogenic spirochaetes and explore its biochemical properties. We found that a non-hydrolysable ATP analogue, ATPγS, but not AMP-PNP induces the formation of ClpB_{LI} hexamers and stabilizes the associated form of the chaperone. ADP also induces structural changes in ClpB_{LI} and promotes its self-assembly, but does not produce full association into the hexamers. We also demonstrated that ClpB_{LI} exhibits a weak ATPase activity that is stimulated by κ-casein and poly-lysine, and may mediate protein disaggregation independently from the DnaK chaperone system. Unexpectedly, the presence of *E. coli* DnaK/DnaJ/GrpE did not significantly affect the disaggregation activity of ClpB_{LI} and ClpB_{LI} did not substitute for the ClpB_{EC} function in the *clpB*-null *E. coli* strain. This result underscores the species-specificity of the ClpB cooperation with the co-chaperones and is most likely due to a loss of interactions between the ClpB_{LI} middle domain and the *E. coli* DnaK. We also found that ClpB_{LI} interacts more efficiently with the aggregated G6PDH in the presence of ATPγS rather than ATP. Our results indicate that ClpB's importance during infection might be due to its role as a molecular chaperone involved in reactivation of protein aggregates.

Introduction

Bacterial ClpB is a molecular chaperone belonging to the Hsp100 subfamily of AAA⁺ ATPases (ATPases associated with a variety of cellular activities) that cooperates with the DnaK

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chaperone system in solubilization and reactivation of aggregated proteins [1–4]. There are a number of observations indicating that the cooperation of ClpB and DnaK in protein disaggregation is species-specific [5–8].

Like other Hsp100 chaperones, ClpB forms barrel-shaped hexamers in the presence of nucleotides [9]. Each ClpB protomer is composed of an N-terminal domain (ND), two AAA⁺ ATP-binding modules (NBD1, NBD2), and a coiled-coil middle domain (MD) inserted at the end of NBD1 (Fig 1A). ND of ClpB is important for binding and recognition of protein substrates [10], whereas MD determines functional interactions with the DnaK chaperone system (including the apparent species-specificity of ClpB/DnaK) required for an efficient protein disaggregation *in vivo* and *in vitro* [6,7]. The mechanism of the ClpB-mediated protein disaggregation couples the ATP hydrolysis with the translocation of substrate polypeptides through the central channel of the hexameric ring [11].

ClpB plays a crucial role in survival of bacteria under stressful conditions [12,13] and is also involved in supporting virulence of some bacterial pathogens [14–17], including a pathogenic spirochaete *Leptospira interrogans* [18] responsible for leptospirosis in mammals. Leptospirosis is considered the most widespread bacterial zoonosis of global importance. More than 1 million human cases of severe leptospirosis occur worldwide each year, with up to 20% mortality rate [19]. The sources of pathogenic leptospires are mainly infected and sick animals (or asymptomatic carriers), which excrete the bacteria with urine into the environment where they can survive even for several months. Thus, water and soil contaminated with infected urine may facilitate the spread of pathogenic *Leptospira*. In moderate-climate countries, the environment is a strong risk factor for *Leptospira* infections. In many regions, including the EU, there are significant economic losses due to reproductive disorders in cattle, sheep, pigs and horses linked to leptospirosis. The disease in these species often has a latent, chronic nature. Reproductive disorders and ocular inflammation in horses are the only symptoms of the disease, which hampers diagnosis and proper treatment and generates economic losses. Many serological and microbiological studies indicate a high rate of infections in domestic animals [20–23]. Despite a severity of leptospirosis and its global importance, the molecular mechanisms of the disease pathogenesis are not well understood. Thus, identification of the *Leptospira* virulence factors and characterization of their activity is particularly important for understanding the mechanisms of the disease.

A molecular chaperone ClpB is among the few known leptospiral virulence factors [18]. However, its role in pathogenic leptospires and biochemical activity have not been investigated so far. In this study, we explored for the first time structural and biochemical properties of ClpB from *L. interrogans* (ClpB_{Li}). As reported earlier [24], ClpB_{Li} shows a multi-domain organization similar to that of the well-characterized ClpB from *Escherichia coli* (ClpB_{Ec}) (Fig 1A) and the total sequence identity between ClpB_{Li} and ClpB_{Ec} is 52%. In this study, we found that the recombinant ClpB_{Li} can assemble into hexamers in a nucleotide-dependent manner, like other well-characterized bacterial ClpBs, and shows the aggregate-reactivation activity that may support the survival of *L. interrogans* under the host-induced stresses. Interestingly, ClpB_{Li} may mediate disaggregation of some aggregated proteins without the assistance of the DnaK system. Furthermore, the *E. coli* DnaK chaperone system does not potentiate the ClpB_{Li} activity and ClpB_{Li} does not rescue the survival of *E. coli* Δ clpB mutant under heat shock. The apparent lack of cooperation between ClpB_{Li} and the *E. coli* DnaK chaperone system during aggregate reactivation *in vivo* and *in vitro* demonstrates species-specificity among the chaperones, which could have evolved to address different types of stress affecting survival of different microorganisms.

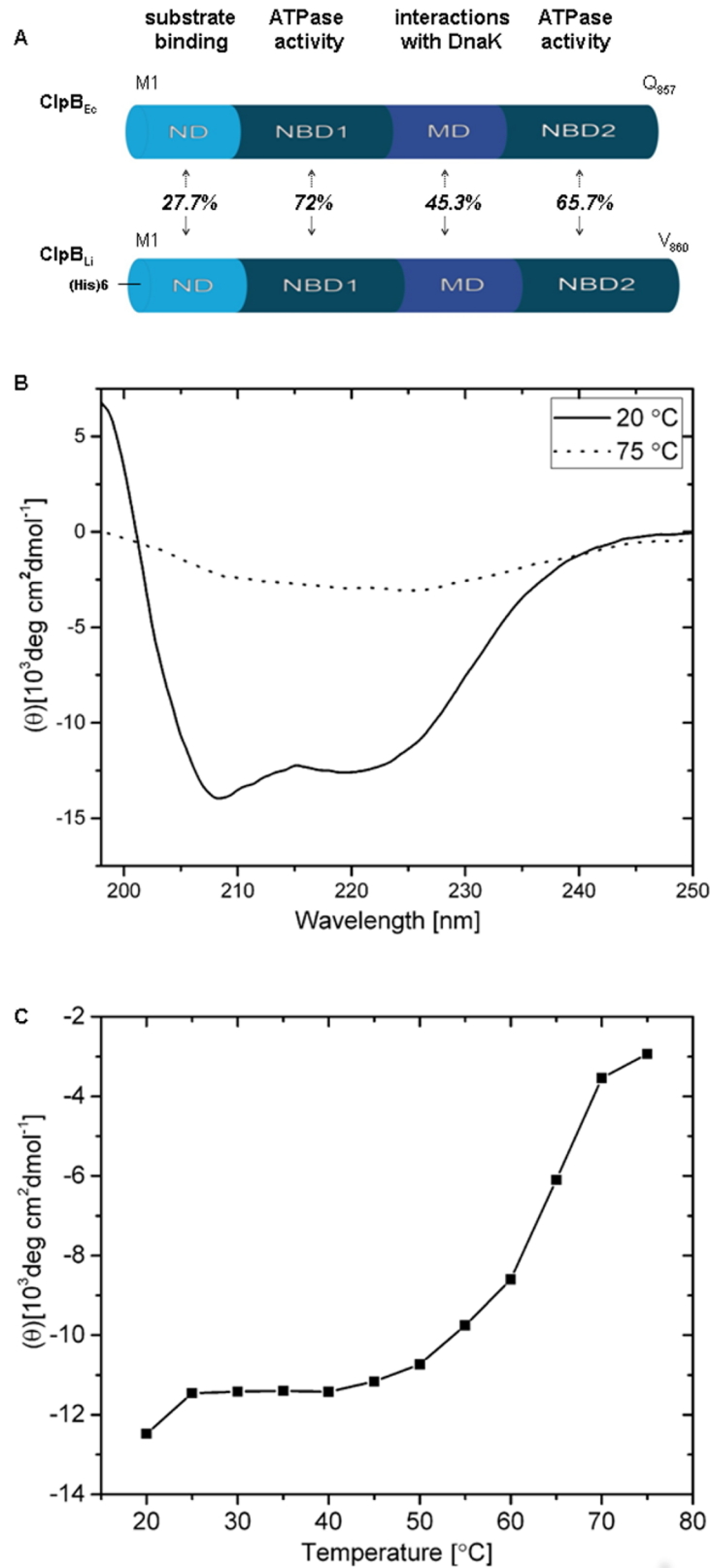


Fig 1. Structural characteristics of ClpB_{Li} used in this study. (A) Comparison of the domain organization of ClpB from *L. interrogans* and *E. coli*. Bacterial ClpB proteins are composed of the following domains: N-

terminal domain (ND), nucleotide binding domain 1 (NBD1), middle coiled-coil domain (MD), and nucleotide binding domain 2 (NBD2). The functions of the domains are indicated at the top. The amino acid residue numbers are shown for each chaperone and the amino acid sequence identity between ClpB_{Ec} and ClpB_{Li} is indicated for each domain. (B) CD spectra of ClpB_{Li} at 20°C (folded form) and 75°C (unfolded form) are shown. The CD signal was expressed as mean molar residue ellipticity (θ). (C) Temperature-induced changes in the CD signal at 222 nm for ClpB_{Li}.

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Materials and methods

Proteins

ClpB_{Li} was successfully overproduced as an N-terminal hexahistidine fusion protein in *E. coli* BL21(DE3) strain (Novagen) and then purified by immobilized metal affinity chromatography (IMAC) using Co-NTA agarose (Qiagen) and gel filtration chromatography (Superdex 200, Sigma-Aldrich) as previously reported [24]. After purification, the protein was extensively dialyzed against appropriate buffers (as described below in [Materials and methods](#)). The N-terminal histidine tag was removed by proteolytic digestion using the Thrombin Cleavage Capture Kit (Novagen) according to the manufacturer's protocol.

E. coli chaperones (ClpB_{Ec}, DnaK_{Ec}, DnaJ_{Ec}) were produced as previously described [25–27]. GrpE from *E. coli* (GrpE_{Ec}) was obtained from K. Liberek (Intercollegiate Faculty of Biotechnology of UG and MUG, Gdańsk, Poland). A Zn²⁺-dependent *E. coli* fructose-1,6-bisphosphate aldolase (Fda) was overproduced in XL1-Blue [pKEN8 (*fda*⁺, *amp*^R)] cells purchased from the American Type Culture Collection (ATCC 77472) and purified as described earlier [28]. Glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides*, κ -casein and poly-lysine were obtained from Sigma. Protein concentrations were estimated by the Bradford method [29] with bovine serum albumin (BSA) as a standard or from absorption at 280 nm using the extinction coefficient of ClpB_{Li} $\epsilon^{0.1\%} = 0.445 \text{ (mg/ml)}^{-1} \text{ cm}^{-1}$ calculated from the amino acid composition by ProtParam [30].

Circular dichroism (CD) spectroscopy

The far-UV CD spectra (200–250 nm) of ClpB_{Li} at a concentration of 0.15 mg/ml were recorded in a 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 20 mM MgCl₂, 200 mM KCl, 10% glycerol buffer, in 1-mm path-length cells, using a Jasco J-815 spectropolarimeter (Japan) equipped with Jasco Peltier element for temperature control. The mean residue ellipticity was calculated according to [31]. To assess the thermal stability of ClpB_{Li}, far-UV CD signals at 222 nm were recorded between 20 and 75°C with a scan rate of 0.5°C/min. A transition mid-point temperature (T_m) was calculated by fitting the sigmoidal Boltzman curve to the ellipticity data using the program OriginPro 9.1 (OriginLab Corp., USA, www.originlab.com).

Sedimentation velocity analytical ultracentrifugation

Analytical ultracentrifugation was performed at 20°C with Beckman Optima XL-I analytical ultracentrifuge equipped with a four- or eight-position An-Ti rotor and UV absorption detection at 290 nm in double-sector 1.2 cm cells with charcoal-filled epon centerpieces and sapphire windows. ClpB_{Li} was dialyzed twice against 50 mM Tris-HCl pH 7.5 buffer containing 0.2 M (or 30 mM) KCl, 20 mM MgCl₂, 1 mM EDTA, 2 mM β -mercaptoethanol, 5% glycerol, and 400 μ l of the dialysate was loaded into reference sectors of the cells. Samples (390 μ l) contained ClpB_{Li} (at concentrations of 1.2 or 3 mg/ml) alone or with 2 mM nucleotide: ATP γ S (adenosine-5'-(γ -thio)-triphosphate); Sigma), AMP-PNP (adenosine 5'-(β,γ -imido)-triphosphate; Sigma) or ADP (Sigma). ATP analogues at the same concentrations were also added to the reference sectors. Sedimentation velocity experiments were performed at 50,000 rpm and

radial absorption scans of protein-concentration profiles were measured at 4.5- or 5-min intervals. The data were analyzed using the SEDFIT program with continuous sedimentation coefficient distribution $c(s)$ model based on Lamm equation [32]. Integration of the $c(s)$ peaks provided the signal-weighted average sedimentation coefficients (s) and the corresponding standard sedimentation coefficients $s_{20,w}$ (referring to water solvent at 20°C). Partial specific volume of ClpB_{Li} (from the amino acid composition) as well as density and viscosity of the buffer were calculated using Sednterp program [33].

Proteolytic sensitivity assay

ClpB_{Li} or ClpB_{Ec} (1 μM) was preincubated in 50 mM Tris-HCl pH 7.5 buffer containing 200 mM KCl, 20 mM MgCl₂, 1 mM EDTA, 1 mM DTT and 10% glycerol for 10 min on ice without or with 5 mM nucleotides: ATP, ATPγS, ADP, AMP-PNP. Trypsin (Sigma) prepared in 1 mM HCl (at a concentration of 1 mg/ml) was then added to the reaction mixtures to a final concentration of 0.2 ng/μl, and the samples were incubated at 37°C for the indicated periods (from 0 to 60 min). The reactions were quenched by the addition of Laemmli SDS-PAGE buffer and samples were analyzed by 0.1%SDS-12.5%PAGE. The gels were stained with Coomassie blue dye.

ClpB ATPase assay

ClpB_{Li} and ClpB_{Ec} were incubated in assay buffer (100 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM EDTA, 10 mM MgCl₂ and 5 mM ATP) at 37°C for 30 min without or with 0.1 mg/ml κ-casein or 0.04 mg/ml poly-lysine, or 2.1 μM aggregated G6PDH. The concentration of ClpB was 0.05 mg/ml for determination of the basal activity and in the presence of κ-casein, and G6PDH or 0.005 mg/ml in the presence of poly-lysine. Inorganic phosphate concentration was determined using the malachite green dye-based colorimetric assay [34] and detection at A₆₄₀.

All absorbance measurements in this study were performed using a model U-1900 Hitachi UV-VIS spectrophotometer.

Aggregate reactivation assays

The purified Fda (2 μM, in buffer A: 100 mM Tris-HCl pH 7.5, 10 mM MgCl₂ and 0.3 mM ZnCl₂) was incubated at 55°C for 10 min. Subsequently, ATP (5 mM) and chaperones: ClpB_{Li} or ClpB_{Ec} (0.65 μM), DnaK_{Ec} (1 μM), DnaJ_{Ec} (0.2 μM) and GrpE_{Ec} (0.1 μM) were added. The total volume of a reaction mixture was 50 μl. The Fda activity was determined as described by Sigma Quality Test Procedure [35], and the decrease in A₃₄₀ was measured after 60- and 120-minute incubation at 25°C using a spectrophotometer. The aggregated Fda in buffer A with 5 mM ATP, but without the chaperones was used as control.

Aggregates of G6PDH were prepared as described earlier [36]. The stock protein (420 μM) was diluted 2-fold with the unfolding buffer (10 mM urea, 16% glycerol, 40 mM DTT) and incubated at 47°C for 5 min. Subsequently, the mixture was diluted 10-fold by the addition of refolding buffer B (50 mM Tris-HCl pH 7.5, 20 mM Mg(OAc)₂, 30 mM KCl, 1 mM EDTA, and 1 mM β-mercaptoethanol), incubated at 47°C for 15 min and then on ice for 2 min (stabilization of aggregates). Aggregated G6PDH (21 μM) was further diluted 10-fold with refolding buffer B. Subsequently, chaperones: ClpB_{Li} or ClpB_{Ec} (0.65 μM), DnaK_{Ec} (1 μM), DnaJ_{Ec} (0.2 μM), GrpE_{Ec} (0.1 μM) and 5 mM ATP were added. The G6PDH activity was determined as described before [36], and A₃₄₀ was measured after 30-, 60-, and 80-minute incubation at 30°C using a spectrophotometer. Aggregates diluted with the refolding buffer B without the chaperones were used as control.

The chaperone-mediated reactivation of aggregated G6PDH and Fda was monitored in the absence of an ATP regenerating system. Therefore, to avoid significant ATP depletion and ADP accumulation, we limited the measurements to an initial stage of the reaction.

The inclusion bodies (IBs) of β -galactosidase (VP1LAC protein; *E. coli* β -galactosidase fused to the aggregation-prone VP1 capsid protein of the foot-and-mouth disease virus) over-produced from pJVP1LAC [37] in *E. coli* strains MC4100 Δ clpB::kan or MC4100clpB⁺ (used as wild-type control; wt) and purified as described before [38] were resuspended in Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β -mercaptoethanol, pH 7.0), mixed and pipetted up and down. Subsequently, 5 mM ATP and, in the case of IBs isolated from Δ clpB mutant, also 1 μ M ClpB_{Li} or ClpB_{Ec} were added. The β -galactosidase activity was determined after 60-minute incubation at 30°C according to Miller's method [39]. For the calculation of units of β -galactosidase, A₄₂₀ was measured using a spectrophotometer and the enzyme activity was calculated as follows: β -galactosidase (Units/ml) = (A₄₂₀)/(0.0045) x (1) x (15) [40], where 0.0045, 1 and 15 indicate, the molecular extinction coefficient of *o*-nitrophenol, cuvette pathlength (cm), and the reaction time (min), respectively.

E. coli strain MC4100 (SG20250) (*araD139*, Δ (*argF-lac*)U169, *rpsL150*, *relA1*, *deoC1*, *ptsF25*, *rpsR*, *flbB53010*) was obtained from S. Gottesman (National Cancer Institute, Bethesda, MD), and its derivative MC4100 Δ clpB::kan was supplied by A. Toussaint (Université Libre de Bruxelles, Brussels, Belgium). Plasmid pJVP1LAC was kindly provided by García-Fruitós (Universitat Autònoma de Barcelona, Spain).

ClpB-aggregate interaction assay

The filtration assay was performed as reported earlier [36]. Aggregated G6PDH (21 μ M) was diluted 10-fold by the addition of the refolding buffer B containing ClpB_{Li} (0.65 μ M) and 5 mM nucleotide: ADP, ATP, ATP γ S, or AMP-PNP. The mixtures were incubated with shaking at 30°C for 10 min and then applied to the filter devices (Millipore Ultrafree-MC Centrifugal Filter Unit with the membrane pore size 0.1 μ m). After 5 min incubation at room temperature, the filter devices were centrifuged at 7,500 g for 5 min to get the flow-through fractions, then washed with the refolding buffer containing an appropriate nucleotide at 30°C for 5 min and re-centrifuged. Next, SDS-loading buffer (2x) was added to the filter devices and they were incubated at 50°C for 5 min with shaking. Then, the filter devices were centrifuged to obtain the eluate fractions, which were separated by 0.1%SDS-10%PAGE and stained with Coomassie blue dye. The stained gels were scanned and analyzed with 1Dscan EX, Scanalytics Inc. Sigma program.

Heat-shock survival assay

The *clpB_{Li}* gene was cloned into a low-copy pGB2 plasmid together with the native *E. coli* *clpB* heat-shock promoter (i.e. the σ^{32} -dependent promoter). The nucleotide sequence of *clpB_{Li}* was amplified from genomic DNA of *L. interrogans* by PCR using AccuTaq LA polymerase MIX (Sigma) with the following PCR primers: CATATGAAATTAATAAACTTACATCCAAATT with the NdeI restriction site underlined, and AAGCTTTTAAACTACAACAACCTTCTCC CTwith the HindIII restriction site underlined. The *E. coli* σ^{32} promoter was amplified from pGB2-ClpB_{Ec} [41] using the following primers: CCCGGGTTCCTCGCCTGGTTAGGGC with the XmaI site underlined, and CATATGAACTCCTCCATAACGGATCwith the NdeI site underlined. First, the PCR products were cloned into pJET1.2 blunt vector (Fermentas), then digested with NdeI, HindIII, and XmaI, and ligated with the linearized pGB2/XmaI-HindIII vector to produce pGB2-ClpB_{Li}. The *E. coli* MC4100 Δ clpB cells were transformed with the empty pGB2, pGB2-ClpB_{Ec} [41], or pGB2-ClpB_{Li} and bacterial survival during heat-shock was

determined as described earlier [41]. To detect ClpB in *E. coli* cultures, Western blotting was performed according to [42] using anti-ClpB_{L_i}158-334 serum [18], that recognized both ClpB_{L_i} and ClpB_{E_c}, a peroxidase-coupled goat anti-rabbit secondary antibody (Sigma), and visualized with the substrate chromogen, 3,3'-diaminobenzidine tetrachloride (DAB, Sigma) and 30% H₂O₂.

Results

Secondary structure and thermal stability of ClpB_{L_i}

First, we estimated the secondary structure of the recombinant ClpB_{L_i} and its thermal stability by performing CD spectroscopy, which was a prerequisite for further characterization of the chaperone. As shown in Fig 1B, the CD spectrum recorded at 20°C showed local minima at 208 and 222 nm, which indicates that the recombinant ClpB_{L_i} is folded into a structure that is dominated by α -helices. This result is in agreement with the secondary structure of ClpB_{E_c} obtained from spectroscopic measurements [25] and that observed in the crystal structure of ClpB from *T. thermophilus* [3]. Furthermore, the structure of ClpB_{L_i} is thermodynamically stable at the assay temperatures used in this study, as shown by a cooperative unfolding transition (Fig 1C), with T_m of approx. 67°C. The thermal unfolding of ClpB_{L_i} is accompanied by a loss of the α -helical structure (see dotted line in Fig 1B).

Nucleotide-induced oligomerization of ClpB_{L_i}

It has been shown that the self-association of ClpB from *E. coli* (ClpB_{E_c}) into hexameric ring-shaped structures is tightly regulated by protein concentration and enhanced by the presence of nucleotides [9,43]. It has been also shown that hexamerization of ClpB_{E_c} is necessary for its ATPase activity and the biological function [25]. Therefore, we decided to study the self-assembly of the recombinant ClpB_{L_i} and answer the question whether it forms nucleotide-induced oligomers. For this purpose, we carried out sedimentation velocity experiments (Figs 2 and 3).

As shown in Fig 3A, ClpB_{L_i} in the absence of nucleotides sedimented as a single predominant species with the standard sedimentation coefficient $s_{20,w} = 4.5$ S, which agreed with the previously determined sedimentation coefficient of the monomeric ClpB_{E_c} [25]. The addition of a weakly hydrolyzed ATP analogue, ATP γ S (Fig 3B), but not AMP-PNP (Fig 3D) induced efficient self-association of ClpB_{L_i}, manifested by the presence of a major peak at 14.1 S in the sedimentation coefficient distribution. The maxima of the distribution are broad, indicating that several types of oligomers are in rapid equilibrium in the presence of ATP γ S. In such cases, the peaks appear at intermediate positions, which do not correspond to the s -values of the sedimenting species [44]. The value of 14.1 S is lower but close to the sedimentation coefficient of the hexameric ClpB_{E_c} [9]. Thus, we conclude, that in the presence of ATP γ S, the hexamer of ClpB_{L_i} is most likely the largest oligomeric species. As shown in Fig 3C, in the presence of ADP, two major components are observed with sedimentation coefficients ~ 4.6 S and ~ 12.1 S. However, the position of the fastest sedimenting peak is about 2 S lower than for the distribution in the presence of ATP γ S. This result suggests that ADP does not support full association of ClpB_{L_i}. Since a high salt concentration is known to promote dissociation of subunits in oligomeric proteins, we tested whether a low salt concentration may stabilize ClpB hexamers without nucleotides present. Fig 3E shows the sedimentation velocity result obtained for ClpB_{L_i} (at a concentration of 3 mg/ml) in a buffer with 30 mM KCl (vs. 200 mM KCl in panels A-D). We found that low salt concentration and an increased ClpB_{L_i} concentration did not result in efficient self-association of the chaperone, because ClpB_{L_i} sedimented primarily at 5.6 S.

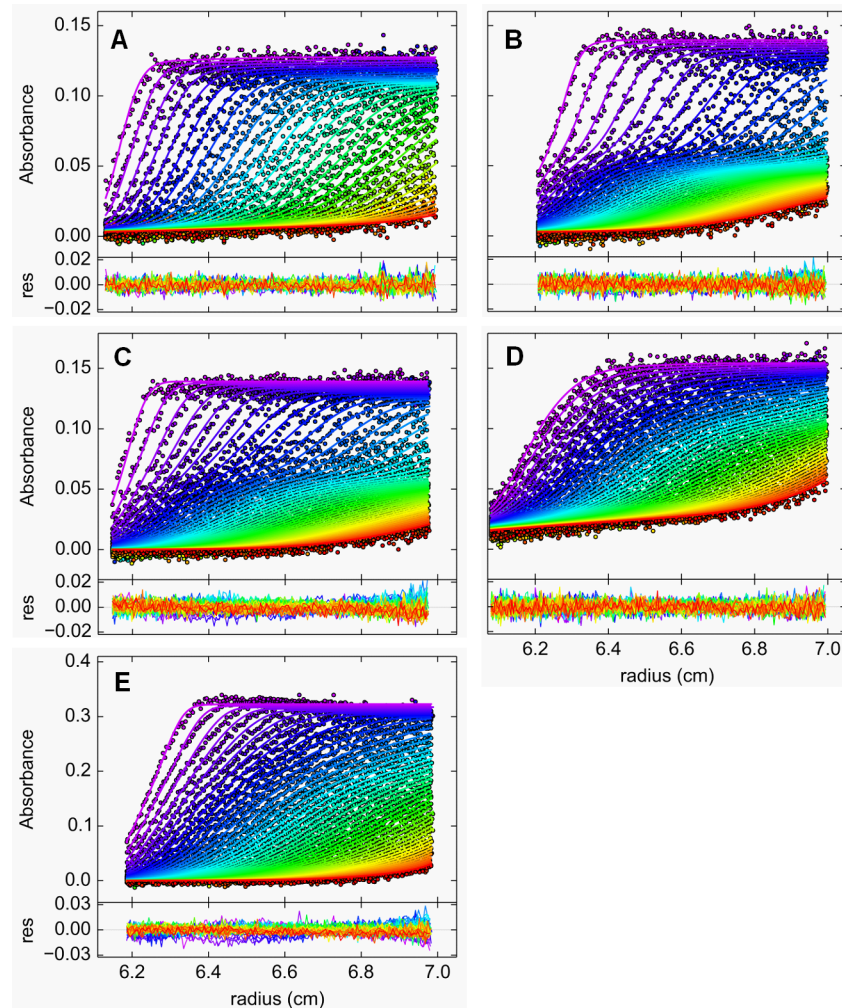


Fig 2. Sedimentation velocity experiments of ClpB_{Li}. Radial absorption profiles at 290 nm (•) with the best fits of SEDFIT *c(s)* model (—) are shown for 1.2 mg/ml (A) and 3 mg/ml ClpB_{Li} (E) without nucleotides, and for 1.2 mg/ml ClpB_{Li} with 2 mM nucleotide: ATPγS (B), ADP (C) or AMP-PNP (D). Ultracentrifugation was performed at 50,000 rpm and 20°C. Radial profiles were measured at 4.5-min (A, B, C, E) or 5-min (D) intervals in 50 mM Tris-HCl buffer pH 7.5 containing 20 mM MgCl₂, 2 mM β-mercaptoethanol, 1 mM EDTA, 5% glycerol and 200 mM (A-D) or 30 mM KCl (E). For (A) every second profile is shown for clarity. Bottom panels present the fitting residuals. The time evolution of radial distributions was plotted as colored curves in the order of purple-blue-green-yellow-red.

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Taken together, our observations (Fig 3 and Table 1) indicate that the ATP analogue, ATPγS, but not ADP, induces an efficient self-association of ClpB_{Li} into hexamers. ADP does induce oligomerization of ClpB_{Li}, but less efficiently than ATPγS.

We also investigated the effect of nucleotides on the structure of ClpB_{Li} by monitoring changes in its proteolytic degradation (Fig 4A). We compared the proteolytic pattern obtained for ClpB_{Li} with that found for ClpB_{Ec} (Fig 4B). The ClpB proteins were digested with trypsin in the absence of nucleotides and in the presence of ATP, ATPγS, ADP and AMP-PNP. Subsequently, the degradation products were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue dye (Fig 4A and 4B). We found that in the absence of nucleotides, ClpB_{Li} was digested into several fragments in the ~25- to ~60-kDa range with a complete loss of the intact ~100-kDa ClpB_{Li} after 20 min of incubation with trypsin. In the presence of

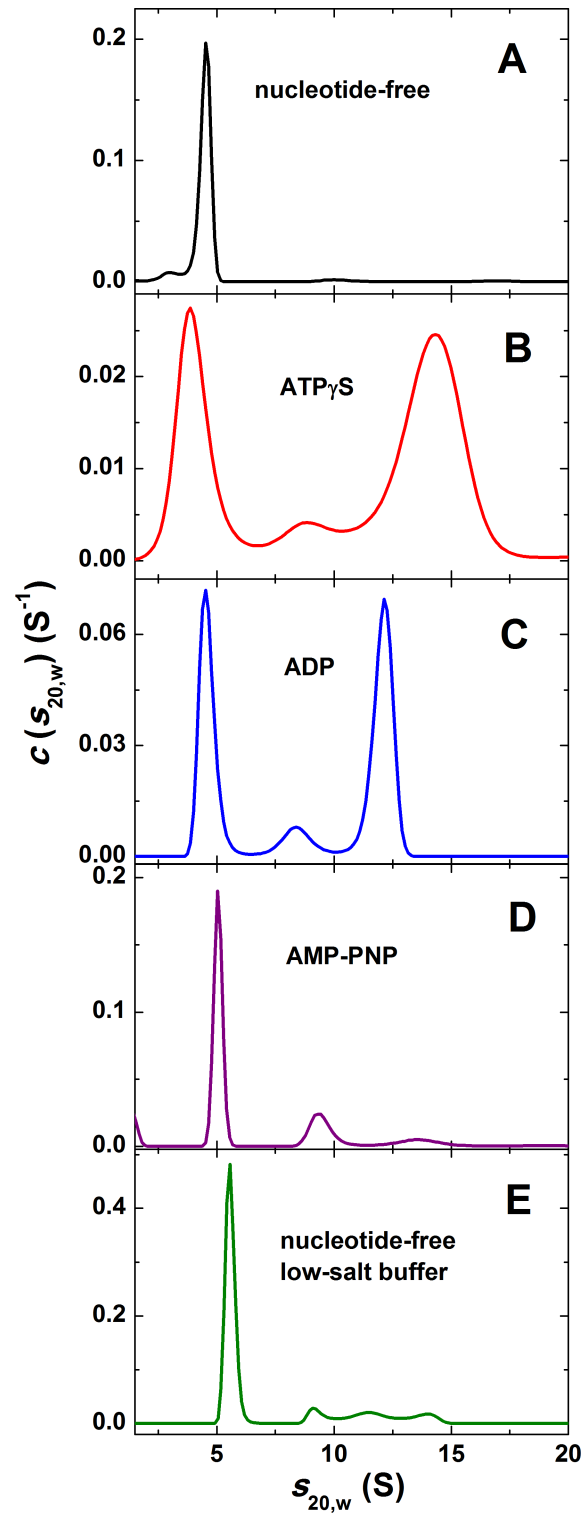


Fig 3. Nucleotide-induced oligomerization of ClpB_{LI}. Shown are the sedimentation coefficient distributions $c(s_{20,w})$ for 1.2 mg/ml ClpB_{LI} in the absence of nucleotides (A), in the presence of the indicated nucleotide at 2 mM concentration (B-D), and in the low-salt buffer without nucleotides for 3 mg/ml ClpB_{LI} (E). Sedimentation velocity data presented in Fig 2 were analyzed with a continuous sedimentation coefficient distribution $c(s)$ model. The distributions were transformed to standard conditions.

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Table 1. Summary of sedimentation coefficients for ClpB_{Li}.

ATP analogue [2 mM]	ClpB _{Li} [mg/ml]	s _{20,w} [S] (population %)			
		peak 1	peak 2	peak 3	peak 4
–	1.2	4.5 ± 0.3 (86%)	10.2 ± 0.7 (2%)	–	16.9 ± 1.0 (<1%)
–	3 ^a	5.6 ± 0.2 (74%)	9.5 ± 0.4 (9%)	11.6 ± 0.6 (10%)	13.7 ± 0.4 (7%)
γ-S	1.2	4.1 ± 0.8 (33%)	8.8 ± 1.0 (8%)	–	14.1 ± 1.4 (55%)
ADP	1.2	4.6 ± 0.4 (41%)	8.4 ± 0.7 (9%)	12.1 ± 0.5 (50%)	–
AMP-PNP	1.2	5.1 ± 0.2 (61%)	9.5 ± 0.5 (19%)	–	13.7 ± 1.0 (8%)

The s_{20,w}-values (mean ± S.E.) were determined from the data shown in Figs 2 and 3. Signal-weighted average sedimentation coefficients obtained by integration of the c(s) distribution peaks were corrected to standard conditions to provide the standard sedimentation coefficients s_{20,w} (corresponding to the density and viscosity of water at 20°C).

^a, The values determined for the low concentration of KCl (30 mM instead of 200 mM) in 50 mM Tris-HCl buffer pH 7.5 containing 20 mM MgCl₂, 2 mM β-mercaptoethanol, 1 mM EDTA, 5% glycerol.

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nucleotides, ClpB_{Li} showed varying degrees of protection against trypsin digestion, which indicates that ClpB_{Li} undergoes structural changes upon binding of all tested nucleotides. Interestingly, ClpB_{Li} in the presence of either ATPγS or ADP was more resistant to proteolysis than ClpB_{Ec}, (compare Fig 4A and 4B), as shown by a lack of prominent digestion fragments in ClpB_{Li} with ATPγS and ADP and their presence in ClpB_{Ec}.

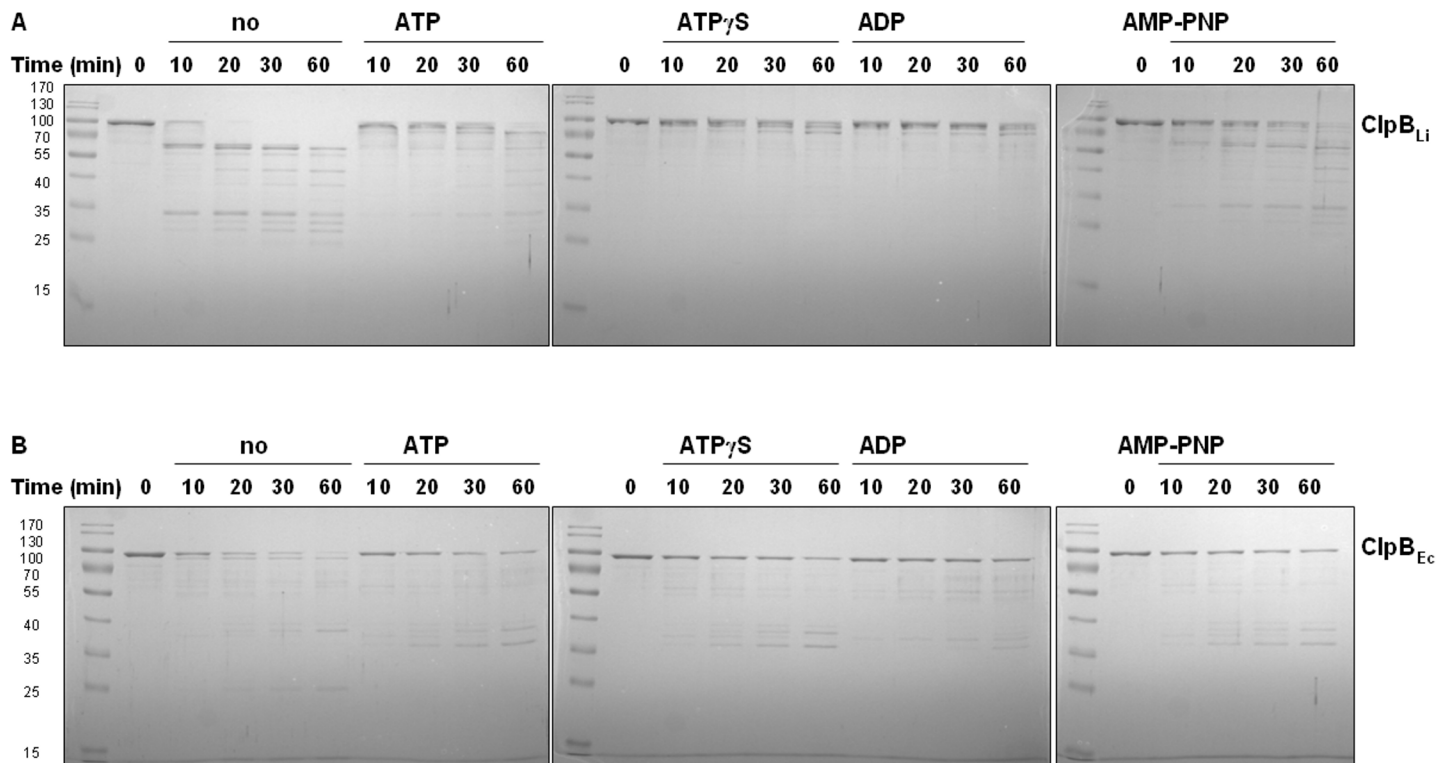


Fig 4. Proteolytic sensitivity of ClpB_{Li} and ClpB_{Ec} in the absence and presence of nucleotides. ClpB (1 μM) was incubated at 37°C for the indicated periods with 5 ng of trypsin in the absence or presence of 5 mM nucleotides. The degradation products were resolved by 0.1%SDS-12.5%PAGE and visualized by Coomassie-blue staining. Representative results from three experiments are shown. The positions of standard molecular mass markers (M) (in kDa), PageRuler prestained Protein Ladder (ThermoScientific), are shown on the left.

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A protective effect of nucleotides on ClpB_{Li} was weaker in the presence of ATP or AMP-PNP than with ATP γ S or ADP, as shown by low-molecular weight fragments appearing after 10–20 min of incubation with trypsin. A partial loss of protection against trypsin for ClpB_{Li} with ATP, as compared to the ATP γ S-bound state, can be due to a fraction of nucleotide-free ClpB_{Li} populated during the ATP turnover. ADP does induce oligomerization of ClpB_{Li}, but not as efficiently as ATP γ S (Fig 3B and 3C). Apparently, an incomplete oligomerization induced by ADP provides ClpB_{Li} with a significant protection against trypsin, comparable to that of ATP γ S. As for AMP-PNP, a lower extent of the ClpB_{Li} protection can be explained by a low population of the ClpB_{Li} oligomers induced by that ATP analogue (Fig 3D). Overall, the extent of the ClpB_{Li} protection against trypsin in the presence of different nucleotides (Fig 4) correlates with the capability of those nucleotides to stabilize the ClpB_{Li} oligomers (Fig 3).

ATPase activity of ClpB_{Li}

We determined the ATPase activity of ClpB_{Li} under the same conditions as the previously tested ClpB_{Ec} [25,36] using a malachite green phosphate-detection assay. As shown in Fig 5, ClpB_{Li} exhibits a slightly lower basal ATPase activity than ClpB_{Ec}. The presence of unstructured polypeptides: κ -casein or poly-lysine, which are known to enhance the ATPase activity of ClpB_{Ec} up to 5- and 25-fold, respectively [45], caused an increase in the ATPase activity of ClpB_{Li}. Thus, the ATPase of ClpB_{Li} resembles in this respect the ATPase of ClpB_{Ec}. Aggregated glucose-6-phosphate dehydrogenase (G6PDH) did not significantly affect the ATPases of ClpB_{Li} and ClpB_{Ec}.

Chaperone activity of ClpB_{Li}

It is known that ClpB_{Ec} efficiently reactivates aggregated proteins in cooperation with DnaK/DnaJ/GrpE [1,25]. Therefore, we tested the ability of ClpB_{Li} to reactivate aggregated protein substrates in the presence of the *E. coli* DnaK chaperone system. In the reactivation assays, we used chemically denatured G6PDH that was previously tested *in vitro* as a ClpB substrate [4,36] and two new model substrates: thermally aggregated FBP aldolase (Fda) and inclusion bodies (IBs) of VP1- β -galactosidase (VP1LAC). Using the pull-down strategy coupled with the mass spectrometry (MS) analysis, we identified Fda as a putative substrate for ClpB_{Li} (unpublished data). As we reported earlier, ClpB_{Ec} significantly increased the reactivation yield of β -galactosidase aggregated in the form of IBs [38,46].

The reactivation yield for the aggregated G6PDH (Fig 6A) in the presence of ClpB_{Li} was significantly higher than for ClpB_{Ec} or the *E. coli* DnaK/DnaJ/GrpE system. In the case of reactivation of the aggregated Fda (Fig 6B), ClpB_{Li} was again more effective than ClpB_{Ec} and equally potent as DnaK/DnaJ/GrpE. These results demonstrate an intrinsic disaggregase activity of ClpB_{Li}, which, for the selected substrates, exceeds that of ClpB_{Ec}. However, the aggregate reactivation yields in Fig 6A and 6B obtained with ClpB_{Li} in the presence of the *E. coli* DnaK/DnaJ/GrpE were similar to those obtained with ClpB_{Li} alone. This result suggests that ClpB_{Li} does not interact with the *E. coli* DnaK chaperone system during aggregate reactivation *in vitro*. The reactivation of β -galactosidase from IBs produced in *E. coli* (Fig 6C) occurred more efficiently with ClpB_{Li} than in the absence of ClpB, but did not reach the efficiency of ClpB_{Ec}. This observation, again, suggests that ClpB_{Li} is capable of reactivating protein aggregates, but does not cooperate with the *E. coli* co-chaperones. Altogether, these properties of ClpB_{Li} are similar to the previously investigated ClpB from a zoonotic bacterium *Ehrlichia chaffeensis* [36].

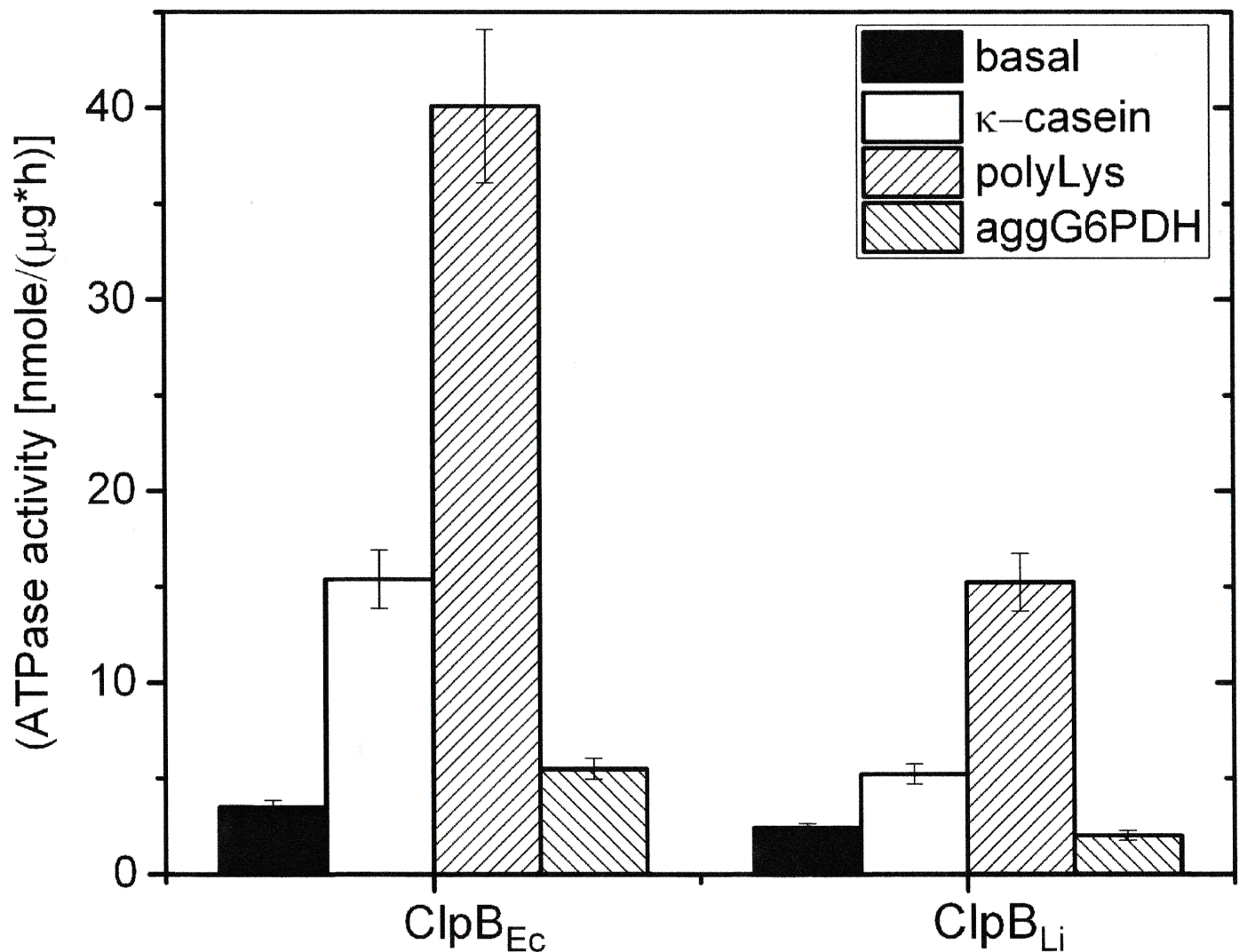


Fig 5. ATPase activity of ClpB_{Li} and ClpB_{Ec}. The rate of ATP hydrolysis was determined at 37°C in the absence of other proteins (basal activity), in the presence of κ-casein (0.1 mg/ml), poly-lysine (0.04 mg/ml) (polyLys), or aggregated G6PDH (2.1 μM) (aggG6PDH). The average values from three independent experiments are shown with the standard deviations.

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Interaction of ClpB_{Li} with aggregated protein substrates

Previous studies demonstrated that nucleotides regulate interactions of bacterial ClpBs with the aggregated substrates. It has been shown that only ATP γ S promotes significant binding of ClpB_{Ec} to the aggregates [13,36], while ClpB from *E. chaffeensis* interacts more efficiently with aggregates in the presence of the hydrolysable ATP [36]. Other nucleotides, such as ADP and AMP-PNP did not induce significant binding of ClpB_{Ec} to protein aggregates. We tested whether binding of ClpB_{Li} to the aggregates is more efficiently stimulated by ATP γ S (which induces a “frozen” ATP-like ClpB state) or by the hydrolysable ATP. We incubated ClpB_{Li} with the aggregated G6PDH in the absence and presence of the tested nucleotides and then determined the amount of ClpB_{Li} bound to aggregates (Fig 7) by performing a filtration assay (see Materials and methods). Only background amounts of ClpB_{Li} were detected in the

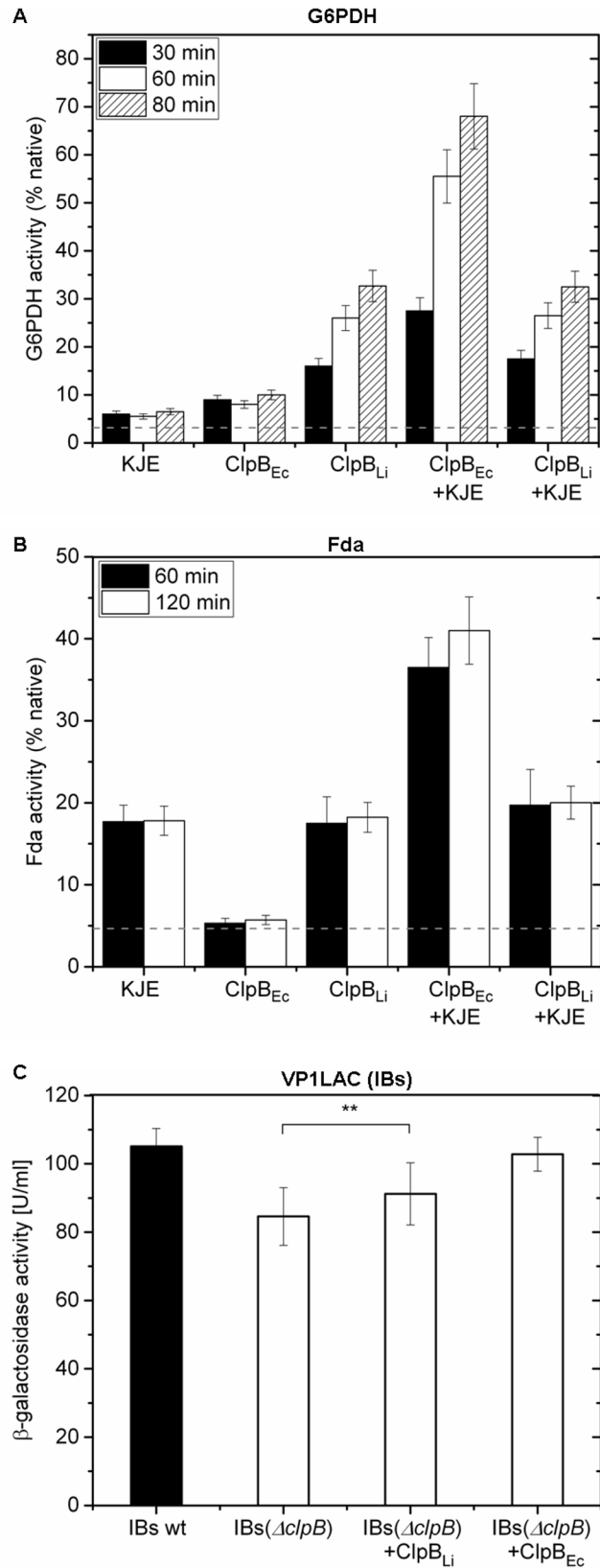


Fig 6. Reactivation of the aggregated substrates in the presence of ClpB_{Li} and ClpB_{Ec}. The reactivation of aggregated enzymes, G6PDH (A) and Fda (B) in the presence of DnaK/DnaJ/GrpE (KJE) from *E. coli* without ClpB and with ClpB_{Ec} or ClpB_{Li}. The native activity of G6PDH or Fda determined before the chemical denaturation or the heat treatment at 55°C, respectively, corresponds to 100%; the fraction of the enzyme activity remaining after the denaturation and also corresponding to the reactivation extent in the absence of chaperones (control) is marked by the broken line. (C) The effect of ClpB_{Li} and ClpB_{Ec} on the reactivation of β -galactosidase sequestered into IBs (VP1LAC) isolated from *E. coli* $\Delta clpB$ mutant cells. A statistically significant difference in the β -galactosidase activity regain in the absence and presence of ClpB_{Li} assessed by the paired t-test (using GraphPad Prism software) is indicated as **, $p < 0.01$. The results are presented as the average of three (A, C) or four (B) independent experiments with the standard deviations indicated.

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absence of the aggregates. We found that interactions of ClpB_{Li} with the aggregated G6PDH were more effective in the presence of ATP γ S than in the presence of ATP, similar to ClpB_{Ec}. Under the conditions of ATP turnover, ClpB_{Li} appears to lose the capability of binding stably to protein aggregates.

Heat-shock survival of the *clpB*-null *E. coli* in the presence of ClpB_{Li}

In *E. coli*, ClpB is necessary for bacterial survival under heat shock [12]. It was demonstrated that the lack of a functional ClpB decreased the growth rate of *E. coli* at 45°C and inhibited cell survival at 50°C. We investigated whether ClpB_{Li} can function in the *E. coli* cells and substitute for ClpB_{Ec}. We cloned the *clpB_{Li}* gene into a low-copy pGB2 plasmid together with the native *E. coli* σ^{32} -dependent *clpB* heat shock promoter. Next, the resulting plasmid pGB2-ClpB_{Li} and the control plasmids, pGB2 and pGB2-ClpB_{Ec} were introduced into the *E. coli* $\Delta clpB$ mutant and a heat-shock survival assay was performed (see [Materials and methods](#)). As shown in [Fig 8A](#), the heat-inducible expression of pGB2-ClpB_{Li} produced a in the ~100-kD protein detectable by Western blotting using anti-ClpB_{Li158-334} serum [18]. We observed, however, that ClpB_{Li} was unable to functionally substitute for ClpB_{Ec} and consequently, it did not rescue *E. coli* $\Delta clpB$ mutant under heat shock conditions ([Fig 8B and 8C](#)). In contrast, the expression of the *clpB_{Ec}* gene from pGB2 complemented the effect of the $\Delta clpB$ mutation. With ClpB_{Ec}, ~80% of the bacteria survived a severe heat shock at 50°C for 90 minutes, which is consistent with the previous results [12].

Discussion

To date, little is known about the structure and biological role of molecular chaperones from *Leptospira* spp., including ClpB. It has been demonstrated that the *L. interrogans* ClpB is essential for the pathogen survival under stress conditions and also during infection of the host [18]. Moreover, a recently reported immunoreactivity of ClpB_{Li} with the sera collected from *Leptospira*-infected animals [24] and the fact that *clpB_{Li}* is up-regulated in leptospiral cells [18] suggest that the ClpB activity may be important for pathogenicity of *Leptospira*. In this work, we described the biochemical and structural properties of ClpB from *L. interrogans*.

As demonstrated by the sedimentation velocity experiments ([Figs 2 and 3](#)), ClpB_{Li} forms hexamers in the presence of the ATP analogue, ATP γ S ([Fig 3B](#)), while it exists as a monomeric protein in the absence of nucleotides ([Fig 3A](#)). In contrast to ATP γ S, ADP induces partial self-association of ClpB_{Li} ([Fig 3C](#)). Insofar as ATP γ S-induced hexamerization is rather typical for bacterial ClpB homologues, the effect of ADP on their oligomerization *in vitro* appears species-dependent. Specifically, ADP stabilized the hexameric forms of the yeast ClpB orthologue, Hsp104 [47,48] or ClpB from the halophilic lactic acid bacterium *Tetragenococcus halophilus* [49], whereas ClpB_{Ec} [43] and the *T. thermophilus* ClpB [50] did not fully assemble into hexamers in the presence of ADP, similar to our results for ClpB_{Li}. Unexpectedly, another ATP

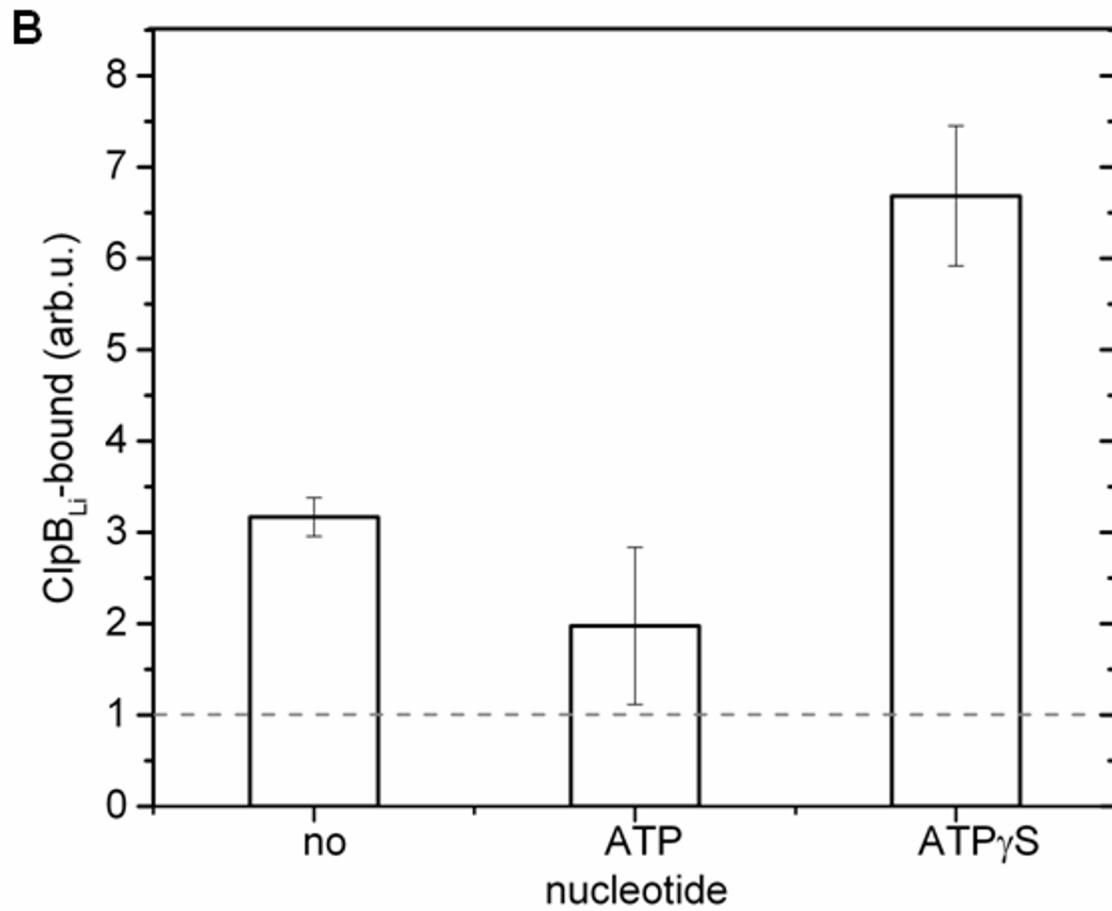
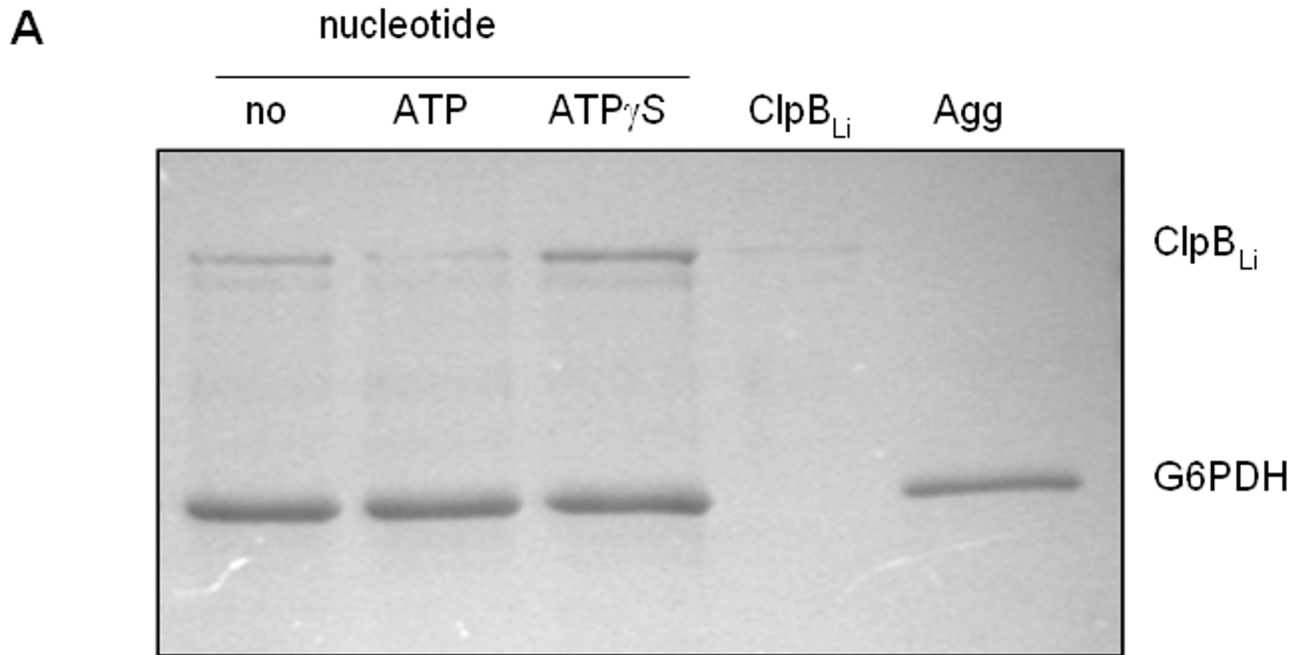


Fig 7. Interaction of ClpB_{Li} with the aggregated G6PDH. (A) ClpB_{Li} was incubated with aggregates of G6PDH (Agg) in the presence of 5 mM ATP or ATP γ S and without nucleotides. The solutions were passed through a 0.1- μ m filter. Subsequently, the fractions retained on the filters were solubilized with an SDS buffer and analyzed by the Coomassie blue-stained 0.1%SDS-10%PAGE gel. A representative result from three independent experiments is shown. (B) Bands corresponding to ClpB_{Li} were analyzed with Sigma Gel software. Results are presented as the average of three independent experiments with standard deviations indicated. The amount of ClpB_{Li} detected in the absence of the aggregates is indicated with the broken line.

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analogue, AMP-PNP, did not induce an effective assembly of oligomeric ClpB_{Li} (Fig 3D). This result indicates that AMP-PNP binding inhibits self-association of ClpB_{Li} or that the affinity of AMP-PNP towards ClpB_{Li} is low. A similar effect, where AMP-PNP did not induce oligomerization was observed for ClpB from *Tetragenococcus halophilus* [49]. We also tested the effect of a low salt concentration on the ClpB_{Li} oligomerization in the absence of nucleotide and at an increased concentration of the chaperone (Fig 3E). In contrast to ClpB_{Ec} [51], a decreased salt concentration did not stabilize the ClpB_{Li} oligomers. The results presented in Fig 4A show a protective effect of nucleotides on ClpB_{Li} during trypsin digestion with the strongest effect for ATP γ S and ADP and a weaker one with AMP-PNP, which correlates with the extent of oligomerization induced by these nucleotides (see Fig 3).

Like other previously characterized ClpB proteins, ClpB from *L. interrogans* catalyzes the hydrolysis of ATP (Fig 5), stably binds to aggregated substrates in the presence of an ATP analogue (Fig 7), and shows a disaggregase activity towards aggregated proteins: G6PDH, Fda and β -galactosidase trapped in IBs (Fig 6). It is worth noting that the reactivation yield of Fda (see Fig 6B) in the presence of ClpB_{Li} alone was similar to that obtained with the *E. coli* DnaK chaperone system, but ClpB_{Li} was significantly more efficient in mediating the aggregate reactivation than ClpB_{Ec}. It has been previously shown that the DnaK chaperone system can disaggregate some substrates, specifically small-size and low-complexity aggregates [28,52,53]. Thus, the intrinsic disaggregase activity of ClpB_{Li} in Fig 6A and 6B manifesting in the absence of the co-chaperones and exceeding that of ClpB_{Ec} suggests that the range of potential aggregated substrates of ClpB_{Li} may be broader than for ClpB_{Ec} and may overlap with that of DnaK.

Furthermore, the DnaK chaperone system from *E. coli* increased the efficiency of aggregate reactivation mediated by ClpB_{Ec}, but not ClpB_{Li} (see Fig 6A and 6B). A similar effect was observed before for ClpB from a parasite *Plasmodium falciparum* [8]. The apparent lack of cooperation between ClpB_{Li} and the *E. coli* co-chaperones *in vitro* (Fig 6) is consistent with the result of an *in vivo* assay presented in Fig 8 (see panels B and C), which shows that ClpB_{Li} is unable to restore the viability of *E. coli* Δ clpB cells after heat shock. This property of ClpB_{Li} resembles ClpB from *E. chaffeensis*, for which, however, the *E. coli* DnaK system potentiated the chaperone activity during reactivation of aggregated proteins *in vitro* [36]. The apparent lack of an efficient cooperation of ClpB_{Li} with *E. coli* co-chaperones during protein disaggregation both *in vivo* and *in vitro* is likely due to the species-specificity of multi-chaperone networks, as reported before [5,6,8]. As has been previously shown, the middle domain of ClpB is responsible for the species-specific cooperation among the chaperones [6]. The sequence identity between ClpB_{Li} and ClpB_{Ec} within the middle domain is only ~45% (Fig 1A), which is apparently insufficient to support a cooperation between ClpB_{Li} and the *E. coli* DnaK system. The lack of cooperation between ClpB_{Li} and the *E. coli* co-chaperones also possibly explains an inability of ClpB_{Li} to rescue the survival of *E. coli* under heat shock (Fig 8, panels B and C). However, the results in Fig 8 could be also explained by a lower potency of ClpB_{Li} towards proteins aggregated in heat-shocked *E. coli* vs. those accumulating in the chaperone's native environment of *Leptospira* during infection of the host.

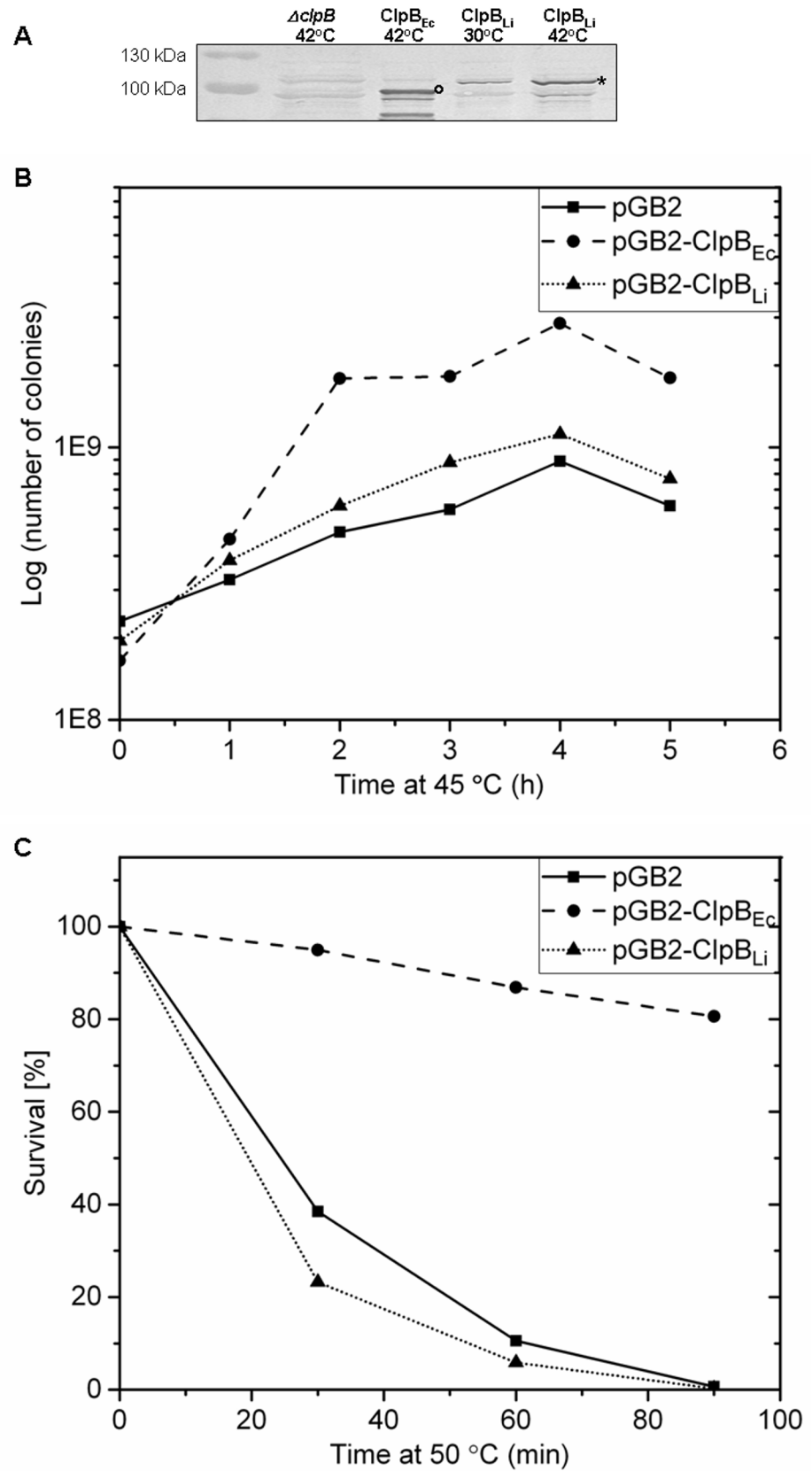


Fig 8. Effect of the *clpB_{Li}* gene expression on the growth and survival of *E. coli* Δ *clpB* mutant under heat shock. (A) Immunodetection of ClpB_{Li} with specific antibodies in *E. coli* Δ *clpB* cells grown at 30°C and after 2h of heat shock at 45°C. An asterisk indicates ClpB_{Li}. The position of ClpB_{Ec} (control of heat-inducible expression) was marked by a circle. (B) Growth curves of *E. coli* Δ *clpB* cells carrying empty pGB2 (control 1), pGB2-ClpB_{Ec} (control 2) or pGB2-ClpB_{Li} exposed to a mild heat shock at 45°C for the indicated times. (C) Survival of the same bacterial strains as in (B) after exposure to a severe heat shock at 50°C for the indicated times. The average values from three independent experiments are shown in (B) and (C).

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Conclusions

Our studies revealed several crucial structural and biochemical properties of the molecular chaperone ClpB from *Leptospira*, which may support its role in pathogenicity of spirochaetes. We showed that ClpB_{Li} forms hexameric assemblies that are stabilized and interact with protein aggregates in the ATP-bound state. Moreover, ClpB_{Li} exhibits a protein disaggregase activity that may contribute to the survival of *L. interrogans* under the host-induced proteotoxic stress. Our studies make a novel contribution to the largely uncharacterized biology of *Leptospira* and suggest that the *L. interrogans* and *E. coli* chaperones evolved differently to respond to the different nature of stress that the proteomes of either bacteria can be exposed to.

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Writing – review & editing: Michal Zolkiewski, Sabina Kędzierska-Mieszkowska.

References

1. Zolkiewski M. ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. *J Biol Chem*. 1999; 274: 28083–28086. PMID: [10497158](https://pubmed.ncbi.nlm.nih.gov/10497158/)
2. Mogk A, Tomoyasu T, Goloubinoff P, Rüdiger S, Röder D, Langen H, et al. Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. *EMBO J*. 1999; 18: 6934–6949. <https://doi.org/10.1093/emboj/18.24.6934> PMID: [10601016](https://pubmed.ncbi.nlm.nih.gov/10601016/)
3. Lee S, Sowa ME, Watanabe Y, Sigler PB, Chiu W, Yoshida M, et al. The structure of ClpB: a molecular chaperone that rescues proteins from an aggregated state. *Cell*. 2003; 115: 229–240. PMID: [14567920](https://pubmed.ncbi.nlm.nih.gov/14567920/)
4. Barnett ME, Nagy M, Kedzierska S and Zolkiewski M. The amino-terminal domain of ClpB supports binding to strongly aggregated proteins, *J Biol Chem*. 2005; 280: 34940–34945. <https://doi.org/10.1074/jbc.M505653200> PMID: [16076845](https://pubmed.ncbi.nlm.nih.gov/16076845/)
5. Schlee S, Beinker P, Akhrymuk A, Reinstein J. A chaperone network for the resolubilization of protein aggregates: direct interaction of ClpB and DnaK. *J Mol Biol*. 2004; 336: 275–285. PMID: [14741222](https://pubmed.ncbi.nlm.nih.gov/14741222/)
6. Miot M, Reidy M, Doyle SM, Hoskins JR, Johnston DM, Genest D, et al. Species-specific collaboration of heat shock proteins (Hsp) 70 and 100 in thermotolerance and protein disaggregation. *Proc Natl Acad Sci USA*. 2011; 108: 6915–6920. <https://doi.org/10.1073/pnas.1102828108> PMID: [21474779](https://pubmed.ncbi.nlm.nih.gov/21474779/)
7. DeSantis ME, Shorter J. The elusive middle domain of Hsp104 and ClpB: location and function. *Biochim Biophys Acta*. 2012; 1823: 29–39. <https://doi.org/10.1016/j.bbamcr.2011.07.014> PMID: [21843558](https://pubmed.ncbi.nlm.nih.gov/21843558/)
8. Ngansop F, Li H, Zolkiewska A, Zolkiewski M. Biochemical characterization of the apicoplast-targeted AAA+ ATPase ClpB from *Plasmodium falciparum*. *Biochem Biophys Res Commun*. 2013; 439: 191–195. <https://doi.org/10.1016/j.bbrc.2013.08.064> PMID: [23994135](https://pubmed.ncbi.nlm.nih.gov/23994135/)
9. Akoev V, Gogol EP, Barnett ME, Zolkiewski M. Nucleotide-induced switch in oligomerization of the AAA + ATPase ClpB. *Protein Sci*. 2004; 13: 567–74. <https://doi.org/10.1110/ps.03422604> PMID: [14978298](https://pubmed.ncbi.nlm.nih.gov/14978298/)
10. Rosenzweig R, Farber P, Velvis A, Rennella E, Latham MP, Kay LE. ClpB N-terminal domain plays a regulatory role in protein disaggregation. *Proc Natl Sci USA*. 2015; 112: E6872–81.
11. Weibezahn J, Tessarz P, Schlieker C, Zahn R, Maglica Z, Lee S, et al. Thermotolerance requires refolding of aggregated proteins by substrate translocation through the central pore of ClpB. *Cell*. 2004; 119: 653–665. <https://doi.org/10.1016/j.cell.2004.11.027> PMID: [15550247](https://pubmed.ncbi.nlm.nih.gov/15550247/)
12. Squires CL, Pedersen S, Ross BM, Squires C. ClpB is the *Escherichia coli* heat shock protein F84.1. *J Bacteriol*. 1991; 173: 4254–4262. PMID: [2066329](https://pubmed.ncbi.nlm.nih.gov/2066329/)
13. Nagy N, Guenther I, Akoyev V, Barnett ME, Zavodszky MI, Kedzierska-Mieszkowska S, et al. Synergistic cooperation between two ClpB isoforms in aggregate reactivation. *J Mol Biol*. 2010; 396: 697–707. <https://doi.org/10.1016/j.jmb.2009.11.059> PMID: [19961856](https://pubmed.ncbi.nlm.nih.gov/19961856/)
14. Kannan TR, Musatovova O, Gowda P, Baseman JB. Characterization of a unique ClpB protein of *Mycoplasma pneumoniae* and its impact on growth. *Infect Immun*. 2008; 76: 5082–5092. <https://doi.org/10.1128/IAI.00698-08> PMID: [18779336](https://pubmed.ncbi.nlm.nih.gov/18779336/)
15. Capestany CA, Tribble GD, Maeda K, Demuth DR, Lament RJ. Role of the Clp system in stress tolerance, biofilm formation, and intracellular invasion in *Porphyromonas gingivalis*. *J Bacteriol*. 2008; 190: 1436–1446. <https://doi.org/10.1128/JB.01632-07> PMID: [18065546](https://pubmed.ncbi.nlm.nih.gov/18065546/)
16. Chastanet A, Derre I, Nair S, Msadek T. *ClpB*, a novel member of the *Listeria monocytogenes* CtsR regulon, is involved in virulence but not in general stress tolerance. *J Bacteriol*, 2004; 186: 1165–1174. <https://doi.org/10.1128/JB.186.4.1165-1174.2004> PMID: [14762012](https://pubmed.ncbi.nlm.nih.gov/14762012/)
17. Meibom KL, Dubail I, Dupuis M, et al. The heat-shock protein ClpB of *Francisella tularensis* is involved in stress tolerance and is required for multiplication in target organs of infected mice. *Mol Microbiol*. 2008; 67: 1384–1401. <https://doi.org/10.1111/j.1365-2958.2008.06139.x> PMID: [18284578](https://pubmed.ncbi.nlm.nih.gov/18284578/)
18. Lourdault K, Cerqueira GM, Wunder EA Jr, Picardeau M. Inactivation of *clpB* in the pathogen *Leptospira interrogans* reduces virulence and resistance to stress conditions. *Infect Immun*. 2011; 79: 3711–3717. <https://doi.org/10.1128/IAI.05168-11> PMID: [21730091](https://pubmed.ncbi.nlm.nih.gov/21730091/)
19. Adler B, Lo M, Seemann T, Murray GL. Pathogenesis of leptospirosis: the influence of genomics. *Vet Microbiol*. 2011; 153: 73–81.
20. Ryan EG, Nola L, O'Grady L, More SJ, Doherty LM. Seroprevalence of *Leptospira* Hardjo in the Irish suckler cattle population. *Ir Vet J*. 2012; 65: 8. <https://doi.org/10.1186/2046-0481-65-8> PMID: [22546216](https://pubmed.ncbi.nlm.nih.gov/22546216/)
21. Arent Z, Kędzierska-Mieszkowska S. Seroprevalence study of leptospirosis in horses in northern Poland. *Vet Rec*. 2013; 172: 269.

22. Arent Z, Frizzell C, Gilmore C, Mackie D, Ellis WA. Isolation of leptospire from genital tract of sheep. *Vet Rec.* 2013; 173: 582.
23. Arent ZJ, Andrews S, Adamama-Moraitou K, Gilmore C, Pardali D, Ellis WA. Emergence of novel *Leptospira* serovars: a need for adjusting vaccination policies for dogs? *Epidemiol Infect.* 2013; 141: 1148–1153. <https://doi.org/10.1017/S0950268812002087> PMID: 22998981
24. Krajewska J, Arent Z, Więckowski D, Zolkiewski M, Kędzierska-Mieszkowska S. Immunoreactivity of the AAA+ chaperone ClpB from *Leptospira interrogans* with sera from *Leptospira*-infected animals. *BMC Microbiol.* 2016; 16: 151–158. <https://doi.org/10.1186/s12866-016-0774-8> PMID: 27421882
25. Barnett ME, Zolkiewska A, Zolkiewski M. Structure and Activity of ClpB from *Escherichia coli*: Role of the Amino- and Carboxy-terminal Domains. *J Biol Chem.* 2000; 275: 37565–37571. <https://doi.org/10.1074/jbc.M005211200> PMID: 10982797
26. Żmijewski M, Macario AJ, Lipińska B. Functional similarities and differences of an archaeal Hsp70 (DnaK) stress protein with the homologue from the bacterium *Escherichia coli*. *J Mol Biol.* 2004; 336: 539–54. PMID: 14757064
27. Żylicz M, Yamamoto T, McKittrick N, Sell S, Georgopoulos C. Purification and properties of the dnaJ replication protein of *Escherichia coli*. *J Biol Chem.* 1985; 260: 7591–7598. PMID: 3889001
28. Kędzierska S, Jezierski G, Taylor A. DnaK/DnaJ chaperones system reactivates endogenous *E. coli* thermostable FBP aldolase in vivo and in vitro; the effect is enhanced by GroE heat shock proteins. *Cell Stress Chaperones.* 2001; 6: 29–37. PMID: 11525240
29. Bradford MM. A rapid and sensitive method for quantitation of proteins utilizing the principles of protein-dye binding. *Anal Biochem.* 1976; 72: 248–254. PMID: 942051
30. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, et al. Protein Identification and Analysis Tools on the Expasy Server. In: Walker JM editor. *The proteomics protocols handbook*. Humana Press; 2005. pp. 571–607.
31. Kelly SM, Jess TJ, Price NC. How to study proteins by circular dichroism. *Biochim Biophys Acta.* 2005; 1751: 119–139. <https://doi.org/10.1016/j.bbapap.2005.06.005> PMID: 16027053
32. Schuck P. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. *Biophys J.* 2000; 78: 1606–1619. [https://doi.org/10.1016/S0006-3495\(00\)76713-0](https://doi.org/10.1016/S0006-3495(00)76713-0) PMID: 10692345
33. Laue TM, Shah BD, Ridgeway TM, Pelletier SL. Computer-aided interpretation of analytical sedimentation data for proteins. In: Harding SE, Rowe AJ, Horton JC, editors. *Analytical Ultracentrifugation in Biochemistry and Polymer Science*. The Royal Society of Chemistry, U.K; 1992. pp. 90–125. <https://doi.org/10.1007/s00249-009-0425-1>
34. Schacherl M, Waltersperger S, Baumann U. Structural characterization of the ribonuclease H-like type ASKHA superfamily kinase MK0840 from *Methanopyrus kandleri*. *Acta Crystallogr D Biol Crystallogr.* 2013; 69: 2440–2450. <https://doi.org/10.1107/S0907444913022683> PMID: 24311585
35. Bergmeyer HU. *Methods of Enzymatic Analysis*, vol. 4. Verlag Chemie, Weinheim; 1992.
36. Zhang I, Kędzierska-Mieszkowska S, Liu H, Cheng C, Ganta RR, Zolkiewski M. Aggregate-reactivation activity of the molecular chaperone ClpB from *Ehrlichia chaffeensis*. *PLoS One.* 2013; 8: e62454. <https://doi.org/10.1371/journal.pone.0062454> PMID: 23667479
37. Corchero JL, Viaplana E, Benito A, Villaverde A. The position of the heterologous domain can influence the solubility and proteolysis of β -galactosidase fusion proteins in *E. coli*. *J Biotechnol.* 1996; 48: 191–200. PMID: 8861998
38. Guenther I, Zolkiewski M, Kędzierska-Mieszkowska S. Cooperation between two ClpB isoforms enhances the recovery of the recombinant β -galactosidase from inclusion bodies. *Biochem Biophys Res Commun.* 2012; 426: 596–600. <https://doi.org/10.1016/j.bbrc.2012.08.135> PMID: 22982305
39. Miller JH. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; 1972.
40. Kyung-Hwan J. Enhanced enzyme activities of inclusion bodies of recombinant β -galactosidase via the addition of inducer analog after L-arabinose induction in the *araBAD* promoter system of *Escherichia coli*. *J Microbiol Biotechnol.* 2008; 18: 434–442. PMID: 18388459
41. Kędzierska S, Akoev V, Barnett ME, Zolkiewski M. Structure and function of the middle domain of ClpB from *Escherichia coli*. *Biochemistry.* 2003; 42: 4242–4248.
42. Harlow E, Lane D. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; 1988.
43. Zolkiewski M, Kessel M, Ginsburg A, Maurizi MR. Nucleotide-dependent oligomerization of ClpB from *Escherichia coli*. *Protein Sci.* 1999; 8: 1899–1903. <https://doi.org/10.1110/ps.8.9.1899> PMID: 10493591

44. Schuck P. On the analysis of protein self-association by sedimentation velocity analytical ultracentrifugation. *Anal Biochem.* 2003; 320: 104–124. PMID: [12895474](#)
45. Strub C, Schlieker C, Bukau B, Mogk A. Poly-L-lysine enhances the protein disaggregation activity of ClpB. *FEBS Lett.* 2003; 553: 125–130. PMID: [14550559](#)
46. Zblewska K, Krajewska J, Zolkiewski M, Kędzierska-Mieszkowska S. Role of the disaggregase ClpB in processing of proteins aggregated as inclusion bodies. *Arch Biochem Biophys.* 2014; 555–556: 23–7. <https://doi.org/10.1016/j.abb.2014.05.012> PMID: [24943258](#)
47. Parsell DA, Kowal AS, Lindquist S. *Saccharomyces cerevisiae* Hsp104 protein. Purification and characterization of ATP-induced structural changes. *J Biol Chem.* 1994; 269: 4480–4487. PMID: [8308017](#)
48. Schirmer EC, Queitsch C, Kowal AS, Parsell DA, Lindquist S. The ATPase activity of Hsp104, effect of environmental conditions and mutations. *J Biol Chem.* 1998; 273: 15546–15552. PMID: [9624144](#)
49. Sugimoto S, Yoshida H, Mizunoe Y, Tsuruno K, Nakayama J, Sonomoto K. Structural and functional conversion of molecular chaperone ClpB from the Gram-positive halophilic lactic acid bacterium *Tetragenococcus halophilus* mediated by ATP and stress. *J Bacteriol.* 2006; 188: 8070–8078. <https://doi.org/10.1128/JB.00404-06> PMID: [16997952](#)
50. Watanabe Y, Motohashi K, Yoshida M. Roles of the two ATP binding sites of ClpB from *Thermus thermophilus*. *J Biol Chem.* 2002; 277: 5804–5809. <https://doi.org/10.1074/jbc.M109349200> PMID: [11741950](#)
51. Lin J, Lucius AL. Examination of the dynamic assembly equilibrium for *E. coli* ClpB. *Proteins.* 2015; 83: 2008–2024. <https://doi.org/10.1002/prot.24914> PMID: [26313457](#)
52. Skowyra D, Georgopoulos C, Zyllicz M. The *Escherichia coli* dnaK gene product, the Hsp70 homologue, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner. *Cell.* 1990; 62: 939–944. PMID: [2203539](#)
53. Diamant S, Ben-Zvi AP, Bukau B, Goloubinoff P. Size-dependent disaggregation of stable protein aggregates by DnaK chaperone machinery. *J Biol Chem.* 2000; 275: 21107–21113. <https://doi.org/10.1074/jbc.M001293200> PMID: [10801805](#)