

Stand-alone ClpG disaggregase confers superior heat tolerance to bacteria

Changhan Lee^{a,1}, Kamila B. Franke^{b,2}, Shady Mansour Kamal^{a,c,2}, Hyunhee Kim^{a,1,2}, Heinrich Lünsdorf^d, Jasmin Jäger^b, Manfred Nitzte^e, Janja Trček^f, Lothar Jänsch^e, Bernd Bukau^b, Axel Mogk^{b,3}, and Ute Römling^{a,3}

^aDepartment of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, 17177, Sweden; ^bCenter for Molecular Biology of the University of Heidelberg (ZMBH) and German Cancer Research Center (DKFZ), DKFZ-ZMBH Alliance, Heidelberg, 69120, Germany; ^cDepartment of Microbiology and Immunology, Faculty of Pharmaceutical Sciences & Pharmaceutical Industries, Future University in Egypt, Cairo, 11835, Egypt; ^dCentral Facility for Microscopy, The Helmholtz Centre for Infection Research, Braunschweig, 38124, Germany; ^eCellular Proteomics, The Helmholtz Centre for Infection Research, Braunschweig, 38124, Germany; and ^fFaculty of Natural Sciences and Mathematics, Department of Biology, University of Maribor, Maribor, 2000, Slovenia

Edited by Susan Gottesman, National Institutes of Health, Bethesda, MD, and approved November 27, 2017 (received for review July 6, 2017)

AAA+ disaggregases solubilize aggregated proteins and confer heat tolerance to cells. Their disaggregation activities crucially depend on partner proteins, which target the AAA+ disaggregases to protein aggregates while concurrently stimulating their ATPase activities. Here, we report on two potent ClpG disaggregase homologs acquired through horizontal gene transfer by the species *Pseudomonas aeruginosa* and subsequently abundant *P. aeruginosa* clone C. ClpG exhibits high, stand-alone disaggregation potential without involving any partner cooperation. Specific molecular features, including high basal ATPase activity, a unique aggregate binding domain, and almost exclusive expression in stationary phase distinguish ClpG from other AAA+ disaggregases. Consequently, ClpG largely contributes to heat tolerance of *P. aeruginosa* primarily in stationary phase and boosts heat resistance 100-fold when expressed in *Escherichia coli*. This qualifies ClpG as a potential persistence and virulence factor in *P. aeruginosa*.

protein disaggregation | AAA+ protein | heat tolerance | mobile genetic element | Hsp100

Maintenance of protein homeostasis is essential for all living organisms from bacteria to humans. Crucial components of the protein quality control system are diverse and include degradation and refolding factors that are preventing the accumulation of misfolded proteins (1, 2). Severe stress conditions can overwhelm these protective activities, leading to massive aggregation of cellular proteins. The loss of proteins by aggregation impedes multiple cellular processes and can ultimately lead to cell death. Additionally, protein aggregates might cause toxicity by depleting cellular factors, including molecular chaperones. Cellular survival during aggregation causing adverse growth conditions therefore relies on the solubilization of aggregated proteins (3). This specific protein quality control activity is particularly important for bacteria, fungi, and plants as they are routinely exposed to heat (4, 5).

The major front line against severe heat stress is formed by ring-forming, hexameric AAA+ disaggregases that exert an ATP-fueled translocation activity enabling them to extract single polypeptides from the aggregate by threading them through their central pore. Tandem AAA+ domains, mediating ATP binding and hydrolysis, provide the energy for the disaggregation process. Notably, all characterized AAA+ disaggregases do not function on their own but strictly require cooperation with partner proteins. These partners (also called adaptors) target AAA+ disaggregases to the aggregate surface and concurrently stimulate AAA+ ATPase and threading activities. Variable N-terminal (N) and inserted middle (M) domains of AAA+ disaggregases mediate specific binding partners and are therefore essential for protein disaggregation.

The central disaggregation machinery of most bacteria, fungi, and plants is formed by an aggregate-binding Hsp70 protein (DnaK in bacteria) that acts as a partner of central homologous disaggregases: ClpB (bacteria), Hsp104 (fungi), or Hsp101 (plants). This bichaperone system is most important for conferring heat tolerance (4–8).

Some Gram-positive bacteria (e.g., *Bacillus subtilis*) lack a ClpB homolog and protein disaggregation is taken over by the AAA+ chaperone ClpC (9, 10). ClpC activity essentially depends on cooperation with adaptor proteins, which function mechanistically similar to Hsp70 by recruiting substrates and stimulating ClpC ATPase activity (11, 12). The role of Clp disaggregases extends beyond heat tolerance though, being involved in e.g., virulence, persistence, oxidative stress, and antibiotic resistance (6, 7, 13–15). Although the molecular basis of virulence phenotypes mainly remains to be unraveled, ClpB has been shown to be required for type VI secretion system functionality in *Francisella tularensis* (16).

Pseudomonas aeruginosa is a highly successful opportunistic pathogen, which causes a broad range of infections (17). A worldwide dominating group of *P. aeruginosa* strains is the clone C cluster, with members prevalent in the environment and in a broad spectrum of acute and chronic diseases (18–22). Although extensive inter- and intracolon genome variability has been observed, the genetic determinants underlying the successful spread of *P. aeruginosa* clone C strains are poorly understood.

The *P. aeruginosa* core genome and a transmissible locus for protein quality control 1 (TLPQC-1) present in clone C strains code for two horizontally transferred AAA+ disaggregases, termed ClpG/ClpG_{GI} (this work and ref. 23). TLPQC-1 and ClpG homologs have previously been shown to confer heat resistance in various environmental and clinically relevant bacteria (23, 24). Here we show that ClpG/ClpG_{GI} work independently

Significance

Severe heat stress causes massive protein loss by aggregation ultimately causing cell death. Cellular survival relies on protein disaggregation mediated by the Hsp70-ClpB (Hsp100) bichaperone system in most bacteria. *Pseudomonas aeruginosa* additionally codes for two stand-alone ClpG disaggregases, which had been acquired by horizontal gene transfer by the species and most abundant clone C strains, respectively. These ClpG disaggregases largely contribute to the resolution of protein aggregates to confer superior heat tolerance partially replacing the DnaK-ClpB system.

Author contributions: C.L., A.M., and U.R. designed research; C.L., K.B.F., S.M.K., H.K., H.L., J.J., M.N., A.M., and U.R. performed research; C.L., H.L., M.N., J.T., L.J., B.B., A.M., and U.R. analyzed data; and C.L., A.M., and U.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Published under the PNAS license.

¹Present address: Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109.

²K.B.F., S.M.K., and H.K. contributed equally to this work.

³To whom correspondence should be addressed. Email: Ute.Romling@ki.se or a.mogk@zmbh.uni-heidelberg.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1712051115/-DCSupplemental.

from partner proteins in contrast to previously characterized AAA+ disaggregases. ClpG/ClpG_{GI} exhibit high disaggregation activity in vitro, confer superior heat tolerance to *P. aeruginosa* clone C strains, and upon expression in *Escherichia coli* boost protein disaggregation and survival during severe heat stress independent of aggregate-binding Hsp70. Our work leads to the hypothesis that horizontal transfer of ClpG accompanied the development of the species *P. aeruginosa* to increase resistance toward adverse stress conditions, and acquisition of ClpG_{GI} contributed to the worldwide transmission of the clone C population in patients and the aquatic habitat.

Results

***P. aeruginosa* ClpG, a AAA+ Protein, Was Acquired by Horizontal Gene Transfer.** *P. aeruginosa* clone C strains harbor the so far uncharacterized AAA+ proteins ClpG and ClpG_{GI}, which are phylogenetically distant from other functionally characterized AAA+ chaperones of Gram-negative and -positive bacteria (SI Appendix, Fig. S1). ClpG is encoded on the core genome of *P. aeruginosa*, while ClpG_{GI} is encoded by TLPQC-1, a part of the *Pseudomonas aeruginosa* clone C genomic island 1 (PACGI-1) (Fig. 1A) (25).

ClpG and ClpG_{GI} exhibit 63% sequence identity and 76% sequence homology and share the same domain organization (Fig. 1B and SI Appendix, Fig. S2). They harbor two AAA+ domains, an N1 N-terminal domain, an M domain inserted into the first AAA+ domain, and a C-terminal extension (Fig. 1B and SI Appendix, Fig. S2). The N1 N-terminal domain can be further divided into two subdomains. The N2 N-terminal subdomain is unique to ClpG/ClpG_{GI} and includes a putative zinc binding motif containing three conserved cysteines and a single histidine residue. The N-terminal subdomain N shows sequence and structural homology to N domains of other Hsp100 protein family members (ClpA, ClpB, and ClpC). The ClpG/ClpG_{GI} M domains are predicted to form a coiled-coil structure, similar to ClpB and ClpC M domains (26, 27). ClpG/ClpG_{GI} lack the conserved tripeptide sequence L/I/V-G-F/L in the second AAA+ domain, which mediates interaction with the peptidase ClpP in ClpC (Fig. 1B and SI Appendix, Fig. S2) (28) and are therefore expected to work independently from ClpP, similar to ClpB, the major disaggregase in *E. coli* (29). A ClpB homolog is also encoded on the *P. aeruginosa* core genome with 45/62% and 47/64% sequence identity/similarity to ClpG_{GI} and ClpG, respectively.

The phylogenetic clustering of ClpG and ClpG_{GI} is different from the major bacterial disaggregase ClpB and, in contrast to ClpB, does not reflect the species relationship represented by 16S rRNA alignment (Fig. 1C and SI Appendix, Fig. S3A). This suggests that *P. aeruginosa* acquired *clpG* and *clpG_{GI}* by horizontal gene transfer. Accordingly, close homologs of ClpG present in individual *Pseudomonas* species strains are located at a different position on the genome and such is ClpG of *E. coli* strain KTE154 located on a genomic island (SI Appendix, Fig. S3B), indicating frequent acquisition of ClpG homologs by horizontal gene transfer in proteobacteria.

The phylogenetic distribution of ClpG and ClpG_{GI} is restricted, compared with widespread ClpB, as these proteins are only found in selected Gram-negative bacteria (Fig. 1C and SI Appendix, Fig. S3A). Furthermore, ClpG is specific to the species *P. aeruginosa* within the *Pseudomonas* genus, while ClpG_{GI} seems to be a clone rather than a species-specific protein present in isolates of unrelated genera of Gram-negative bacteria, suggesting its recent acquisition. Summing up, ClpG/ClpG_{GI} proteins form a subgroup of bacterial AAA+ chaperones that have been acquired by a set of Gram-negative bacteria via horizontal gene transfer.

ClpG Confers Superior Heat Tolerance to *P. aeruginosa* Strains. To elucidate physiological functions of ClpG and ClpG_{GI}, we constructed single and double mutants in the *P. aeruginosa* SG17M clone C strain. As the most closely related AAA+ chaperones ClpB, ClpC, and the biochemically uncharacterized ClpG homolog

ClpK contribute to survival during nonlethal heat stress (4, 9, 24, 30), we tested the mutants for alteration in heat tolerance. For comparison, we additionally generated a *P. aeruginosa* Δ clpB mutant. As the *dna-shsp20_{GI}-clpG_{GI}* operon of TLPQC-1 of the aquatic isolate SG17M is largely expressed during stationary phase (25), we compared survival of cells in logarithmic and stationary growth phases upon lethal heat shock from 20 °C to 50 °C (Fig. 1D and E).

Both ClpG_{GI} and ClpB contribute to heat tolerance in logarithmic growth, while enhanced heat sensitivity is observed in the Δ clpB Δ clpG_{GI} double deletion strain. Subsequent deletion of *clpG* further increased heat sensitivity in the triple mutant. Low levels of ClpG_{GI} and ClpB production can be detected in the logarithmic growth phase, consistent with the moderate approximately 10-fold decrease in heat tolerance phenotype of the single mutants (Fig. 1F). In contrast, in stationary phase, only Δ clpG Δ clpG_{GI} cells showed pronounced 100-fold reduced survival 60 min after heat shock, indicating a dominant role of ClpG/ClpG_{GI} proteins (Fig. 1D). Additional deletion of *clpB* in Δ clpG Δ clpG_{GI} cells, however, further increased heat sensitivity, suggesting that ClpB can partially compensate for ClpG function. Western blot analysis indicated substantial production of ClpG_{GI} during stationary phase and >10-fold lower expression of ClpG, but barely detected ClpB production (Fig. 1F). These findings are in agreement with the pronounced and redundant roles of ClpG_{GI} and ClpG in stationary phase heat tolerance. Together these findings indicate distinct, but overlapping contributions of ClpG/ClpG_{GI} and ClpB proteins to bacterial heat tolerance in logarithmic and stationary growth phase. Inducing the expression of either ClpG or ClpG_{GI} in single copy from the *araC* promoter at the chromosomal Tn7 site was sufficient to restore heat shock tolerance to wild-type levels in the Δ clpB Δ clpG Δ clpG_{GI} triple deletion mutant (SI Appendix, Fig. S4); thus, the function of ClpB and ClpG chaperones in thermotolerance is interchangeable.

Since various bacterial AAA+ chaperones cooperate with the peptidase ClpP in protein quality control, we tested whether ClpG/ClpG_{GI} function in heat tolerance depends on ClpP (SI Appendix, Fig. S5). *P. aeruginosa* SG17M Δ clpP cells were by far not as heat sensitive as Δ clpG Δ clpG_{GI} mutant cells. Additionally, single copy Tn7-based genomic expression of either *clpG* or *clpG_{GI}* was sufficient to restore heat tolerance in the Δ clpG Δ clpG_{GI} Δ clpP triple deletion mutant to Δ clpP cell levels (SI Appendix, Fig. S5). This demonstrates that ClpG/ClpG_{GI} function independently from ClpP in thermotolerance, consistent with the absence of the ClpP interaction motif L/I/V-G-F/L in ClpG/ClpG_{GI} chaperones (Fig. 1B and SI Appendix, Fig. S2).

To generalize the roles of ClpG and ClpB in heat tolerance, we constructed respective Δ clpB and Δ clpG mutants in the well-investigated nonclone C *P. aeruginosa* reference strain PAO1, harboring ClpG, but not ClpG_{GI}. Equally as in SG17M, the contribution of ClpB to heat tolerance was dominant during logarithmic growth, while *clpG* did not contribute (Fig. 1E). Notably, heat shock survival of the SG17M strain 30 min after heat shock is more than 10³-fold higher compared with PAO1 (Fig. 1D and E). As the survival rate of the SG17M Δ clpG_{GI} mutant is still superior compared with wild-type PAO1, components in addition to ClpG_{GI} must contribute to survival of clone C SG17M wild-type cells.

Analysis of heat tolerance of strain PAO1 during stationary phase revealed a major contribution of ClpG as deletion of *clpG*, but not *clpB*, enhanced heat sensitivity (Fig. 1E). Simultaneous deletion of *clpB* and *clpG* substantially increased heat sensitivity, again revealing overlapping and independent contributions of ClpB and ClpG chaperones to heat tolerance in *P. aeruginosa* cells.

Δ clpG/ Δ clpG_{GI} Mutant Cells Exhibit Increased Protein Aggregation and Defects in Protein Disaggregation. Thermotolerance in bacteria and fungi is linked to protein disaggregation processes (31, 32) and any defect in this process will therefore cause increased

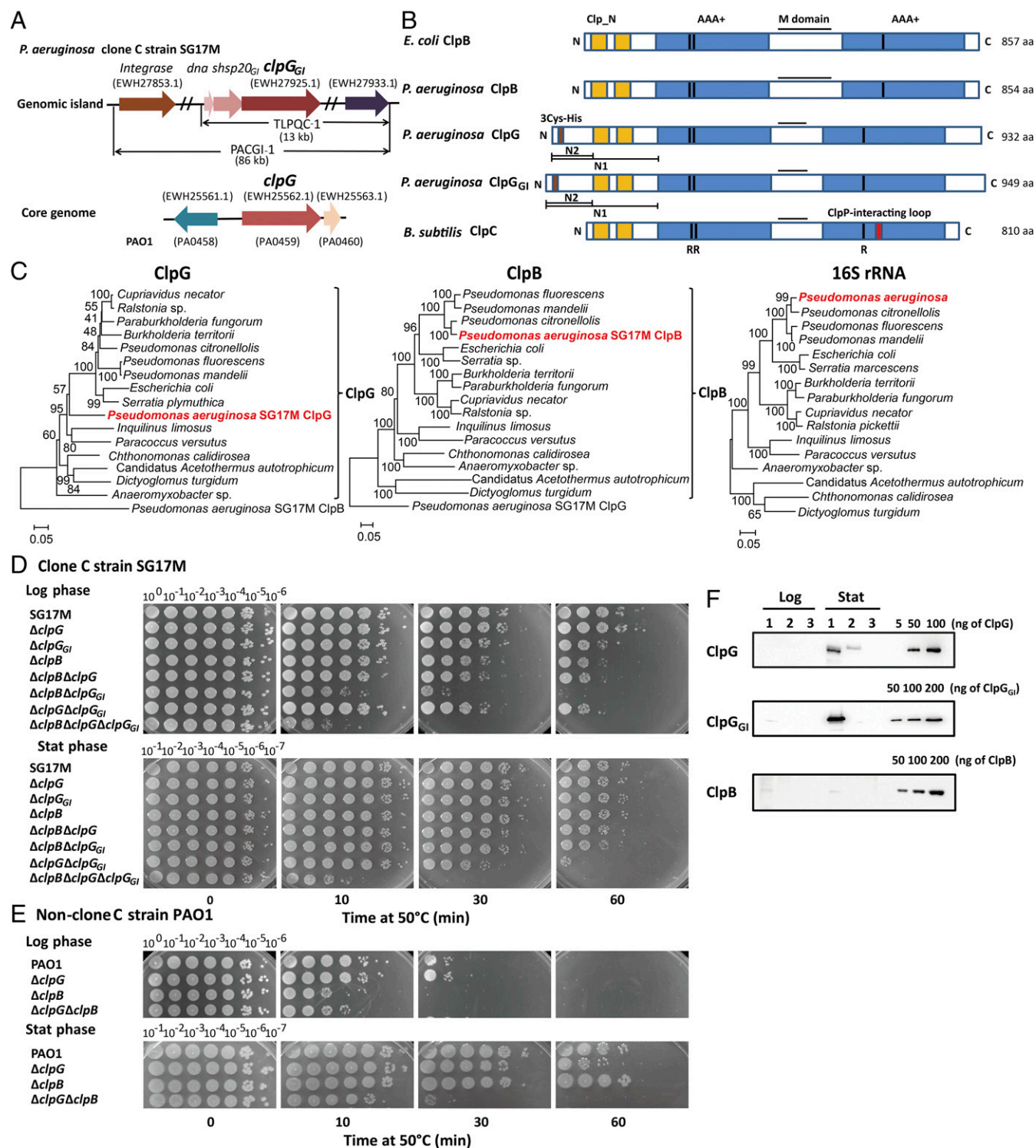


Fig. 1. The Hsp100 protein ClpG substantially contributes to heat tolerance of *P. aeruginosa*. (A) Genomic context of ClpG and ClpG_{GI} located on the core genome and on the TLPOC-1 part of PACGI-1 in *P. aeruginosa* SG17M (23). Locus: *dna* (EWH27923.1), *shsp20G1* (EWH27924.1). (B) Domain organization of *E. coli* ClpB, *B. subtilis* ClpC, and *P. aeruginosa* ClpG/ClpG_{GI} proteins. The Hsp100 proteins are composed of two ATPase domains (AAA-1 and AAA-2), an N-terminal domain (Clp_N), and an inserted middle (M) domain. Clp_N2 of ClpG/ClpG_{GI} includes a potential Zn²⁺-binding motif composed of three cysteines and a histidine residue. (C) ClpG of *P. aeruginosa* has a unique position within the phylogenetic tree of ClpG homologs deviating from the phylogenetic position of ClpB, which is in congruence with the 16S rRNA phylogenetic tree. (D and E) Heat tolerance of clone C *P. aeruginosa* SG17M (D) or nonclone C *P. aeruginosa* PAO1 (E) and indicated mutant cells in logarithmic (log) and stationary (stat) growth phase. Cells were grown at 20 °C and subjected to lethal heat shock at 50 °C for 10, 30, and 60 min. Cellular viabilities were determined by spotting serial dilutions (10^0 – 10^{-6}) on LB plates. (F) Western blot analysis of ClpG, ClpG_{GI}, and ClpB protein levels in logarithmic and stationary growth phase. 1: SG17M wild type; 2: $\Delta clpG$, $\Delta clpG_{GI}$, or $\Delta clpB$ deletion mutant; and 3: $\Delta clpB\Delta clpG\Delta clpG_{GI}$ triple deletion mutant. Purified proteins serve as standards for protein amount.

protein aggregation at elevated temperatures. To test whether reduced heat tolerance can be correlated to increased protein aggregation, we first compared the amount and pattern of aggregated proteins in *P. aeruginosa* SG17M wild-type and AAA+ chaperone mutant cells after growth at the elevated temperature of 42 °C for 24 h (Fig. 2A and *SI Appendix*, Fig. S6A). We observed slightly increased protein aggregation in $\Delta clpB$ and $\Delta clpG_{GI}$ cells; however, a significant increase in protein aggregation required simultaneous deletion of $clpG/clpG_{GI}$ copies and $clpB$ correlating with the high heat sensitivity of the triple mutant (Fig. 1D). Isolated protein aggregates included a vast variety of cellular proteins. A 15-kDa protein particularly elevated in the aggregate fraction of the $\Delta clpB \Delta clpG \Delta clpG_{GI}$ triple mutant was identified by mass spectrometry as the small heat shock protein IbpA (*SI Appendix*, Table S1). sHsps coaggregate with misfolded proteins thereby representing sensitive aggregation reporters to underline the aggregation phenotype. Similarly, we found the bacterial Hsp90 chaperones HtpG to be enriched in the aggregate fraction of triple mutants (Fig. 2A and *SI Appendix*, Table S1). These findings indicate that ClpB and ClpG/ClpG_{GI} have overlapping functions in preventing and/or reversing protein aggregation. Notably, we also observed single protein species that already strongly aggregated in $\Delta clpB$ (LasI) or $\Delta clpG_{GI}$ (NuoCD) single deletion mutants (Fig. 2A and *SI Appendix*, Table S1). This suggests that DnaK-ClpB and ClpG_{GI} exhibit, to a certain degree, substrate-specific activities.

In an alternative approach, we shifted *P. aeruginosa* SG17M wild-type, $\Delta clpG \Delta clpG_{GI}$, and $\Delta clpB \Delta clpG \Delta clpG_{GI}$ mutant cells from 30 °C to 42 °C for 60 min and allowed for recovery upon subsequent incubation at 30 °C (Fig. 2B and *SI Appendix*, Fig. S6B). Protein aggregates were enhanced in $\Delta clpG \Delta clpG_{GI}$ and $\Delta clpB \Delta clpG \Delta clpG_{GI}$ cells and subsequent heat shock increased the amount of aggregated proteins in all cells. Upon return to 30 °C, protein aggregates were partially removed and this process occurred with similar relative speed, although $\Delta clpB$

$\Delta clpG \Delta clpG_{GI}$ cells still contained a high aggregate load after a 120-min incubation. These findings support complementary activities of ClpB and ClpG/ClpG_{GI} chaperones in *P. aeruginosa* protein quality control and point to a function of ClpG/ClpG_{GI} in handling of protein aggregates.

ClpG Is a Potent Disaggregase Without Any Accessory Factor. As ClpB and ClpG proteins seem to exert overlapping activities in *P. aeruginosa* cells, we wanted to compare their biochemical properties. As ClpG_{GI} is more widespread than ClpG, and $\Delta clpG_{GI}$ cells are more heat sensitive compared with $\Delta clpG$ (Fig. 1D), we focused our analysis largely on ClpG_{GI} and subsequently confirmed key features for the ClpG counterpart.

We purified ClpG_{GI} and first tested for nucleotide-driven hexamer formation as a common feature of AAA+ chaperones. ClpG_{GI} oligomerization was analyzed by size exclusion chromatography (SEC) (*SI Appendix*, Fig. S7) and transmission electron microscopy (TEM) (*SI Appendix*, Fig. S8). In the absence of ATP, ClpG_{GI} is mainly present as a monomer (*SI Appendix*, Fig. S7). ClpG_{GI} oligomers formed upon addition of ATP (2 mM), although oligomerization was not complete under the given experimental conditions (*SI Appendix*, Fig. S7). Partial oligomer formation of ClpG_{GI} was also observed in TEM (+ATP γ S), although less efficient compared with *E. coli* or *P. aeruginosa* ClpB (*SI Appendix*, Fig. S8A). The electron density map of ClpG_{GI} oligomeric particles revealed the formation of a hexameric ring structure composed of two layers, likely formed by AAA-1 and AAA-2 domains (*SI Appendix*, Figs. S8B and C and S9). While ClpG_{GI} has an extended N-terminal domain, the electron density map of ClpG_{GI} is flattened in the N-terminal region and the map is likely restricted by a high mobility of the N-terminal extension. Fitting of the ClpG_{GI} map with a ClpB structural model (Protein Data Bank ID code 4D2Q) (33) revealed missing density for ClpB M domains, which can be explained by the reduced size of the ClpG_{GI} M domain, but also by the limited resolution of the map. Together these findings show that ClpG_{GI} forms hexamers in an ATP-dependent manner as reported before for other AAA+ chaperones.

Next, we tested whether the complementary functions of ClpB and ClpG_{GI} observed *in vivo* can be documented as complementary chaperone activities *in vitro* and therefore tested for disaggregation activity of ClpG_{GI}. We monitored the disaggregation of heat-aggregated malate dehydrogenase (MDH), which can be efficiently rescued by the DnaK-ClpB bichaperone system. To couple the fast solubilization of MDH aggregates with fast MDH refolding, all assays were performed in the additional presence of the *E. coli* GroEL/GroES chaperone system. The GroEL/GroES system on its own does not exhibit any disaggregation activity and only accelerates refolding of soluble, unfolded MDH after its removal from protein aggregates (34). *E. coli* ClpB (*EcClpB*) did not exhibit disaggregation activity on its own and essentially required cooperation with the *E. coli* DnaK chaperone system (DnaK, DnaJ, GrpE; *EcKJE*). The *EcClpB-EcKJE* bichaperone system reactivated 80% of aggregated MDH in 60 min (Fig. 3A). A comparable disaggregation activity was determined for the *P. aeruginosa* bichaperone system (*PaClpB-PaDnaK-EcJE*) (*SI Appendix*, Fig. S10A). Also, *PaDnaK* and *PaClpB* were interchangeable with their *E. coli* counterparts (*SI Appendix*, Fig. S10A), demonstrating full disaggregation potential of *P. aeruginosa* ClpB and DnaK. Strikingly and in contrast to *EcClpB* and *PaClpB*, *P. aeruginosa* ClpG_{GI} exhibited high disaggregation potential on its own and rescued heat-aggregated MDH with an activity comparable to the *EcClpB-EcKJE* bichaperone system (Fig. 3A).

To substantiate this stand-alone disaggregation activity of ClpG_{GI}, we used heat-aggregated luciferase as alternative substrate. ClpG_{GI} was again highly active without assistance by additional factors and even showed a twofold increased yield of disaggregation/refolding of aggregated luciferase compared with

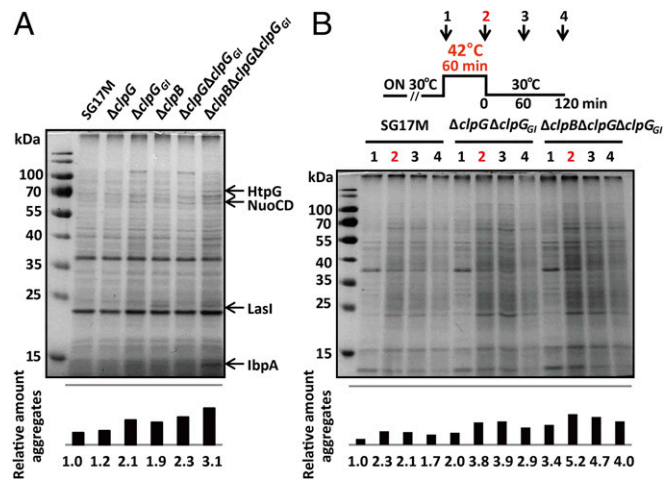


Fig. 2. Enhanced protein aggregation in *P. aeruginosa* SG17M cells lacking ClpB and ClpG/ClpG_{GI}. (A) *P. aeruginosa* SG17M and mutants were cultured at 42 °C for 24 h and protein aggregates were isolated. Proteins that specifically aggregate in $\Delta clpG_{GI}$ (NuoCD) and $\Delta clpB$ (LasI) cells are indicated. Aggregation of the HtpG and IbpA chaperones is most pronounced in the $\Delta clpB \Delta clpG \Delta clpG_{GI}$ triple mutant. (B) *P. aeruginosa* SG17M and mutants were grown at 30 °C overnight (1) and incubated at 42 °C for 60 min (2). Next, cells were shifted to 30 °C for 60 min (3) and 120 min (4) for recovery. Protein aggregates were isolated at indicated steps and analyzed by SDS-PAGE. For semiquantitative comparison, the density of the entire lane was estimated by ImageJ with SG17M (A) or lane 1 of SG17M (B) arbitrarily set as 1. These experiments were repeated twice with similar results and one representative shown.

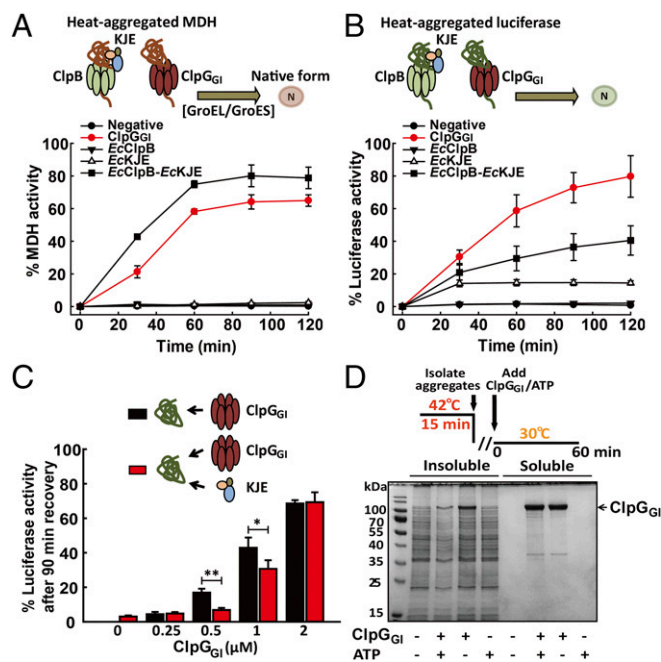


Fig. 3. ClpG_{GI} shows potent disaggregating activity in vitro and ex vivo. (A and B) Refolding of heat-aggregated malate dehydrogenase (MDH) and luciferase by the *E. coli* DnaK-ClpB bichaperone system (EcClpB and EcKJE) or ClpG_{GI}. The activities of native enzymes were set as 100%. (C) Refolding of aggregated luciferase was monitored in the absence or presence of EcKJE and increasing concentrations of ClpG_{GI} (**P* < 0.05; ***P* < 0.01). (D) Resolubilization of heat-aggregated *P. aeruginosa* proteins by ClpG_{GI} ex vivo. Soluble *P. aeruginosa* SG17M crude extract from cells grown at 30 °C for 24 h was heat treated at 42 °C for 15 min. Insoluble protein aggregates were isolated and subsequently subjected to resolubilization by ClpG_{GI}/ATP (2 mM) for 60 min. Insoluble and soluble protein fractions were separated and analyzed by SDS-PAGE.

EcClpB-EcKJE (Fig. 3B). A robust, stand-alone luciferase reactivation activity was also determined for *P. aeruginosa* ClpG, confirming the potent and independent disaggregation activity of this AAA+ chaperone (SI Appendix, Fig. S10B).

EcKJE initiates protein disaggregation by the bichaperone system by recruiting and activating EcClpB at the aggregate surface. As ClpG can act on protein aggregates on its own, we tested whether DnaK and ClpG can cooperate at the aggregate surface or whether they compete for binding to the aggregated substrate. To this end, we compared the luciferase disaggregation activity of ClpG_{GI} at different concentrations (0.25–2 μM) either alone or in the presence of EcKJE (1 μM DnaK, 0.2 μM DnaJ, 0.1 μM GrpE), which exhibits only low level disaggregation activity in the absence of EcClpB (Fig. 3C). EcKJE reduced reactivation of heat-aggregated luciferase by ClpG_{GI} if ClpG_{GI} was present at lower concentration (0.5 μM) and a comparable inhibitory effect was noticed in the presence of PaKEcJE (Fig. 3C and SI Appendix, Fig. S10C). Increasing the ClpG_{GI} concentration to 2 μM restored efficient luciferase reactivation in the presence of EcKJE and proceeded with the same disaggregation rate as determined in the absence of EcKJE (Fig. 3C). These findings indicate that the DnaK chaperone system and ClpG_{GI} do not cooperate in protein disaggregation but instead compete for binding to the aggregate surface. The DnaK-ClpB bichaperone system and ClpG/ClpG_{GI} therefore form independent disaggregase systems operating on the same substrate.

Next, we tested whether the uncovered disaggregation activity of ClpG_{GI} is promiscuous and active toward aggregated proteins from *P. aeruginosa* SG17M. For this purpose, a lysate of soluble proteins was derived from *P. aeruginosa* stationary phase cells, subjected to heat denaturation and protein aggregates were

isolated (Fig. 3D). Aggregates were incubated with and without ClpG_{GI} in the absence and presence of ATP. We observed a spectrum of solubilized protein species for ClpG_{GI} + ATP, although some minor bands were also observed without ATP, likely representing ClpG_{GI} degradation products present in the ClpG_{GI} sample. The ATP-dependent disaggregation activity of ClpG_{GI} was most evident from the significant reduction in protein aggregate levels compared with control reactions (Fig. 3D and SI Appendix, Fig. S10D). We infer that ClpG_{GI} resolubilizes aggregates of heat-denatured proteins with a broad specificity.

ClpG_{GI} Can Replace DnaK-ClpB and Hsp104 Disaggregase Functionality.

To demonstrate that the identified ClpG/ClpG_{GI} disaggregation activity is operative in diverse genetic backgrounds (35), we tested whether ClpG_{GI} can replace ClpB and Hsp104 activities in *E. coli* and *Saccharomyces cerevisiae* cells. We first expressed ClpG and ClpG_{GI} in *E. coli* wild-type and Δ clpB mutant cells and tested for heat tolerance upon temperature upshift from 30 °C to 50 °C (Fig. 4A). *E. coli* Δ clpB cells were less heat tolerant compared with wild-type cells and viability was 10⁴-fold reduced 60 min after heat shock, in agreement with literature (4). Expressing either ClpG or ClpG_{GI} in Δ clpB cells fully restored thermotolerance, demonstrating that ClpG/ClpG_{GI} can entirely take over ClpB disaggregase function in vivo. Notably, expressing ClpG_{GI} in *E. coli* wild-type cells conferred superior thermotolerance, increasing the fraction of viable cells 100-fold after heat shock (120 min) compared with wild-type cells harboring an empty control plasmid (Fig. 4A).

In a complementary approach we assessed the ability of ClpG_{GI} to reactivate aggregated proteins in *E. coli* Δ clpB cells using thermolabile YFP-luciferase as disaggregation reporter (Fig. 4B). YFP-luciferase produced at 30 °C in *E. coli* wild-type and Δ clpB cells additionally expressed ClpG_{GI}. After a heat shock to 45 °C in the presence of the protein synthesis inhibitor erythromycin and subsequent incubation at 30 °C, *E. coli* wild-type cells recovered 15% of luciferase activity within 90 min, whereas Δ clpB cells only allowed for 3% luciferase refolding. This ClpB-specific resolubilization defect of Δ clpB cells could be rescued by ClpG_{GI} expression (Fig. 4B). Here, 30% of YFP-luciferase was refolded within 90 min, exceeding the reactivation potential of *E. coli* wild-type cells. This confirms that the presence of the alternative ClpG_{GI} disaggregation system increases the ability of *E. coli* cells to rescue aggregated proteins.

As ClpB cooperates with the accessory chaperone DnaK, we tested the effect of clpG_{GI} expression in *E. coli* dnaK103 mutant cells, which synthesize a truncated nonfunctional DnaK protein (36). clpG_{GI} expression restored the temperature-sensitive growth phenotype of dnaK103 mutant cells at 42 °C (Fig. 4C). Furthermore, clpG_{GI} expression reestablished heat tolerance upon 50 °C heat shock to dnaK103 mutant cells, which otherwise lost viability within 10 min (Fig. 4D), again with an efficiency similar to plasmid-encoded DnaK wild type. The presence of ClpG_{GI} in dnaK103 cells reduced the amount of protein aggregates isolated at 30 °C and allowed for aggregate removal during a subsequent recovery period after heat shock to 45 °C, in contrast to dnaK103 control cells harboring an empty vector (Fig. 4E and SI Appendix, Fig. S11). Together these findings substantiate a DnaK-independent disaggregation activity of ClpG_{GI}, which can compensate for the broad protein folding defects associated with loss of functionality of the DnaK (Hsp70) partner chaperone.

In an additional approach, we analyzed whether ClpG_{GI} can replace Hsp104 function in the propagation of amyloid-like protein aggregates, prions, in yeast cells. Prion fibril fragmentation, which leads to the production of smaller propagons that are transmitted to daughter cells, is executed by the Hsp104 disaggregase. To monitor propagation of the yeast prion states [PSI⁺] and [URE3], originating from amyloid-formation of the Sup35 and Ure2 proteins, we made use of the yeast reporter strains 779-6A and 1075. In these strains, the production of the

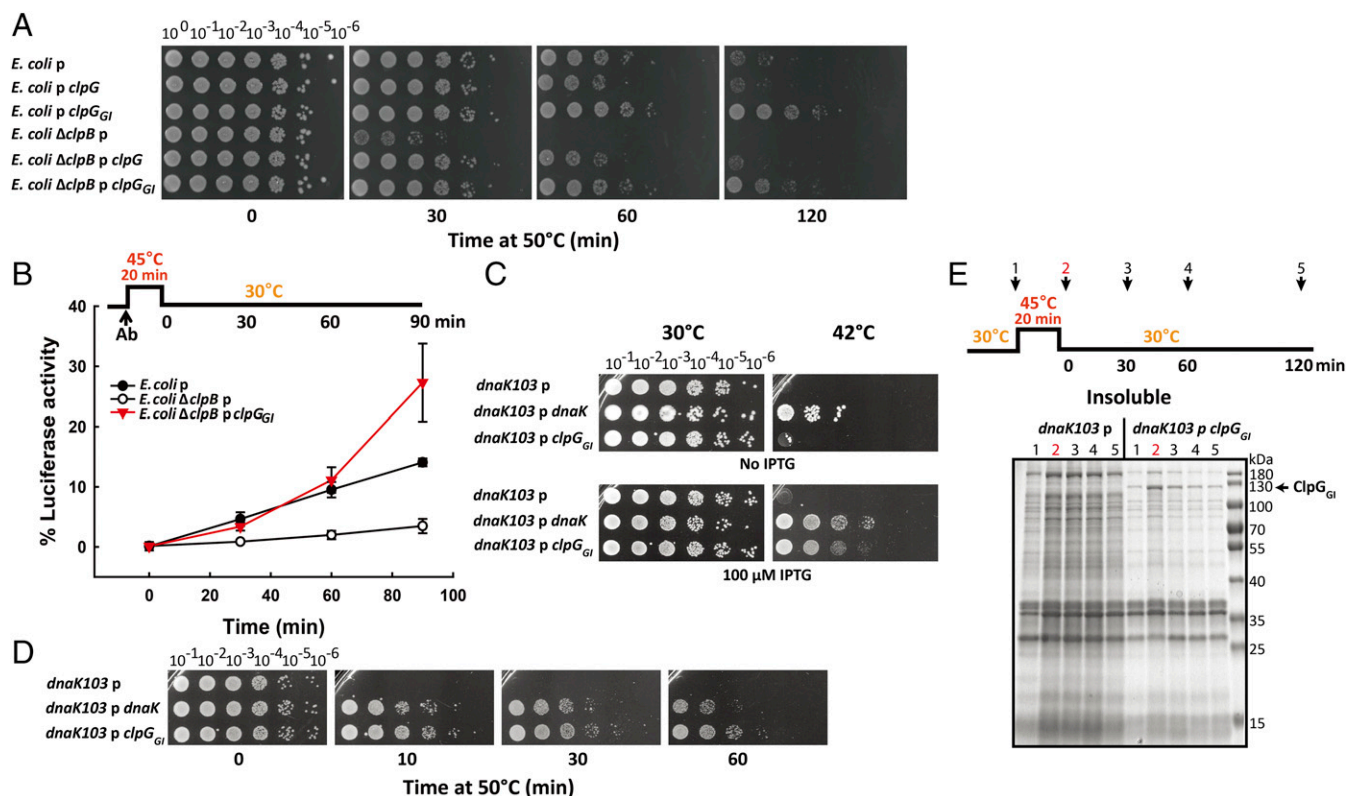


Fig. 4. ClpG_{GI} restores disaggregase function in the absence of *E. coli* ClpB and DnaK. (A) *E. coli* wild-type and Δ *clpB* cells expressing *clpG* or *clpG_{GI}* were grown at 30 °C and shifted to 50 °C. Cellular viabilities were determined by spotting serial dilutions (10^0 – 10^{-6}) of cells on LB plates. (B) *E. coli* wild-type and Δ *clpB* cells (with and without *clpG_{GI}*) expressing YFP-luciferase were grown to midlogarithmic growth phase at 30 °C. Protein synthesis was stopped by addition of erythromycin and cells were shifted to 45 °C for 20 min followed by a recovery period at 30 °C. Luciferase activity was determined prior to (set to 100%) and post heat shock and at the indicated time points during cellular recovery. p = pJN105; p *clpG*, p *clpG_{GI}* = respective genes cloned in pJN105 (A and B). (C) Serial dilutions of *E. coli* *dnaK103* mutant cells expressing *dnaK* or *clpG_{GI}* were spotted on LB plates with and without 100 μ M isopropyl β -D-1-thiogalactopyranoside (IPTG) and incubated at 30 °C and 42 °C. (D) *E. coli* *dnaK103* mutant cells expressing *dnaK* or *clpG_{GI}* were grown at 30 °C in the presence of 250 μ M IPTG and shifted to 50 °C. Cellular viabilities were determined by spotting serial dilutions (10^{-1} – 10^{-6}) of cells on LB plates. (E) *E. coli* *dnaK103* mutant cells harboring an empty vector (p) or expressing *clpG_{GI}* (p *clpG_{GI}*) were grown at 30 °C in the presence of 250 μ M IPTG and shifted to 45 °C for 20 min followed by a recovery period at 30 °C. Soluble and insoluble protein fractions were isolated at the indicated time points and analyzed by SDS-PAGE (SI Appendix, Fig. S11). p = pUHE21; p *dnaK*, p *clpG_{GI}* = respective genes cloned in pUHE21 (C–E).

Ade2 reporter protein required for adenine biosynthesis depends on the respective prion state. We expressed ClpG_{GI} in the yeast reporter cells and concurrently inhibited the endogenous Hsp104 disaggregase by low concentrations of guanidine hydrochloride (GuHCl), a specific inhibitor of Hsp104 activity (37). We confirmed that ClpG_{GI} disaggregation activity is only mildly affected by 5 mM GuHCl, in contrast to Hsp104, which is strongly inhibited (SI Appendix, Fig. S124). On adenine-limiting medium, the yeast reporters form white colonies if the prion state is maintained but turn red upon prion loss because of the accumulation of a metabolite of the Ade2 substrate. Hsp104 inhibition led to formation of red reporter colonies, indicating prion loss, while the presence of ClpG_{GI} changed colony color to pink (SI Appendix, Fig. S12B). This suggests a weak [PSI⁺] and [URE3] phenotype and thus partial ClpG_{GI} activity in prion maintenance. On medium lacking adenine, loss of the prion state abolishes growth of the reporter strains. On such a medium, ClpG_{GI} allowed for growth of the 1075, but not the 779-6A strain, again suggesting that ClpG_{GI} can partially rescue Hsp104 functionality in yeast prion propagation. Notably, a comparable, partial activity in yeast prion propagation had been determined for *E. coli* ClpB, when coexpressed with cooperating *E. coli* DnaK in yeast prion reporter cells (38).

ClpG Overcomes Mechanistic Restrictions of ClpB, Rationalizing Its Factor-Independent Disaggregation Activity. ClpG/ClpG_{GI} show high stand-alone disaggregation activity, whereas ClpB strictly re-

quires cooperation with its Hsp70 (DnaK) partner chaperone. ClpB has low basal ATPase activity and does not bind protein aggregates efficiently, while DnaK targets protein aggregates to ClpB to concurrently stimulate ClpB ATPase activity (39–42). We speculated that the partner-independent ClpG/ClpG_{GI} and DnaK-dependent ClpB disaggregation activities must be reflected in different biochemical properties of the AAA+ chaperones. We therefore tested whether ClpG/ClpG_{GI} and ClpB differ in (i) ATPase activities and (ii) their abilities to bind model protein aggregates.

ClpG_{GI} and ClpG show a 12-fold higher basal ATPase activity compared with *Ec*ClpB (Fig. 5 and SI Appendix, Fig. S13). ClpG/ClpG_{GI} therefore do not require stimulation by a partner protein to reach increased ATPase activities. The ATPase activity is specific, as a double Walker B mutant in ClpG_{GI} (ClpG_{GI} E383A E723A) did not display ATPase activity (SI Appendix, Fig. S13). We next assessed substrate-stimulated ATPase activities by using casein and peptide B1 (AHAWQHOGKTLFISRKTYRIC) as these model substrates are directly recognized by ClpB without DnaK assistance. Casein binds to the N-terminal domain and the substrate processing pore site of ClpB, whereas peptide B1 exclusively interacts with the central ClpB pore site (43). Casein and peptide B1 stimulated ClpB ATPase activity by 8- and 12-fold, respectively (Fig. 5). ClpG_{GI} ATPase activity was also increased by peptide B1 (1.8-fold) while casein showed no effect, indicating that ClpG_{GI} and ClpB differ in substrate recognition in parts.

Difference in substrate binding can be likely attributed to differences between the N-terminal domains of ClpG/ClpG_{GI} and ClpB. Its unique N1 domain might hinder ClpG/ClpG_{GI} to interact with casein while enabling the binding to protein aggregates at the same time. To assess the role of the ClpG/ClpG_{GI} N-terminal domains we either deleted the entire N-terminal region (Δ N1) or only N2, the very N-terminal, ClpG/ClpG_{GI}-specific extension harboring a putative Zn²⁺-binding motif (Δ N2) (Fig. 6A). ClpG/ClpG_{GI} wild-type and N-terminal deletion constructs were expressed at increased levels largely comparable for all constructs in *P. aeruginosa* Δ clpB Δ clpG Δ clpG_{GI} mutant cells and analyzed for their ability to restore heat tolerance upon temperature upshift to 50 °C (Fig. 6B and *SI Appendix*, Fig. S14). All ClpG/ClpG_{GI} N-terminal deletion variants failed to restore heat tolerance, indicating that the unique N-terminal extensions are essential for functionality (Fig. 6B and *SI Appendix*, Fig. S2). In contrast, deletion of the N-terminal domain of *P. aeruginosa* ClpB (Δ N-ClpB) still conferred heat tolerance, in agreement with former studies showing that the N-terminal domain of *Ec*ClpB is not essential for protein disaggregation (Fig. 6B) (44–47).

To further characterize the role of the unique N2 N-terminal extension, we purified and characterized Δ N2-ClpG/ Δ N2-ClpG_{GI} deletion mutants. The deletions did not affect global ClpG/ClpG_{GI} protein structure as revealed by CD spectroscopy (*SI Appendix*, Fig. S15). Also ATP-dependent oligomerization of Δ N2-ClpG_{GI} was similar to ClpG_{GI} and ClpG (*SI Appendix*, Fig. S7). Consistent with the *in vivo* results, reactivation of aggregated luciferase by ClpG/ClpG_{GI} *in vitro* was strongly reduced (ClpG_{GI}) or entirely abrogated (ClpG) upon deletion of the N2 N-terminal extensions in contrast to ClpB (Fig. 6C and *SI Appendix*, Fig. S16).

The crucial role of the ClpG/ClpG_{GI} N2 N-terminal extension for heat tolerance and protein disaggregation could be explained by the N-terminal domain to control ClpG/ClpG_{GI} ATPase function or to target ClpG/ClpG_{GI} to protein aggregates. Whereas

basal ATPase activity of Δ N2-ClpG was comparable to ClpG, Δ N2-ClpG_{GI} exhibited a 5.8-fold increased ATPase rate compared with ClpG_{GI}, indicating that the N2 part of the N-terminal extension down-regulates ATPase activity (Fig. 6D). We next performed an *in vitro* binding assay with ClpG/ClpG_{GI} wild-type and the N2 deletion variants using aggregated luciferase as substrate to test for N2 subdomain-mediated aggregate targeting. The binding of ClpG/ClpG_{GI} to aggregated luciferase was monitored in the presence of nonhydrolyzable ATP γ S, which stabilizes Hsp100-substrate interaction. As luciferase aggregates are insoluble, binding of ClpG/ClpG_{GI} was monitored by separating soluble and insoluble protein fractions by centrifugation (Fig. 6E). We ensured that only very minor amounts of ClpG/ClpG_{GI} and their N-terminal deletion variants are found in the pellet fraction in the absence of protein aggregates (*SI Appendix*, Fig. S17). We observed substantial binding of full-length ClpG/ClpG_{GI} to both aggregated substrates and stronger binding compared with ClpB (Fig. 6E), confirming that ClpB requires DnaK as crucial accessory factor for aggregate binding (48). Binding to ClpG was entirely dependent on its unique N-terminal extension as Δ N2-ClpG no longer bound aggregated proteins (Fig. 6E). In contrast, Δ N2-ClpG_{GI} binding to protein aggregates was not affected, unraveling an unexpected difference between ClpG and ClpG_{GI}. Notably, Δ N2-ClpG_{GI} still retained minor disaggregation activity in contrast to Δ N2-ClpG (Fig. 6C), consistent with its ability to still bind aggregated luciferase (Fig. 6E).

Together these findings provide a mechanistic rationale for why ClpG/ClpG_{GI} act as potent, stand-alone disaggregases and unravel distinct functions of the unique N-terminal extensions in ATPase control in ClpG_{GI} and aggregate targeting in ClpG rationalizing their crucial roles in protein disaggregation.

Discussion

In this work, we characterized the AAA+ chaperones ClpG/ClpG_{GI} as stand-alone disaggregases, which confer superior heat tolerance to bacteria (Fig. 7). So far protein disaggregation and heat tolerance in bacteria have been predominantly linked to the AAA+ disaggregase ClpB, which forms a bichaperone system with the DnaK (Hsp70) partner chaperone. ClpB activity is crucial for thermotolerance in Gram-negative (4, 49, 50), but also Gram-positive bacteria (6, 51). The lack of ClpB in some Gram-positive bacteria is compensated by the presence of an alternative disaggregation system composed of ClpC and its adaptor proteins for aggregate targeting and ATPase activation (12, 30, 52, 53). While other bacterial AAA+ proteins (ClpE and ClpL) have also been implicated in protein disaggregation, a disaggregation activity has either not been documented or is very limited *in vitro* (51, 54–56). Here, we demonstrate that ClpG/ClpG_{GI} overcome both mechanistic restrictions of ClpB and ClpC by exhibiting a high basal ATPase activity and directly binding to protein aggregates. These features allow for efficient ATP-dependent threading of aggregated proteins explaining why ClpG/ClpG_{GI} function in protein disaggregation without assistance of accessory factors.

The unique N2 N-terminal extensions are characterized by distinct amino acid sequences, while ClpG_{GI} harbors an additional insertion (*SI Appendix*, Fig. S2). This sequence diversity is reflected by functional diversity, as the N2 subdomain is essential for aggregate targeting of ClpG, while its counterpart regulates ClpG_{GI} ATPase activity. Furthermore, the unique N2 N-terminal extensions likely interplay with the C-terminal “classical” Hsp100 N domains in ClpG/ClpG_{GI}-mediated disaggregation. Hsp100 N domains can directly bind substrates (57) and coordination of both ClpG/ClpG_{GI} N-terminal domains might be crucial for activity. Dissecting the interplay and specific roles of ClpG/ClpG_{GI} N-terminal domains will be essential to understand the mechanistic basis of these stand-alone disaggregases. Such studies will also unravel whether substrate specificity of ClpG/ClpG_{GI} is restricted to thermally aggregated proteins or extends to, e.g.,

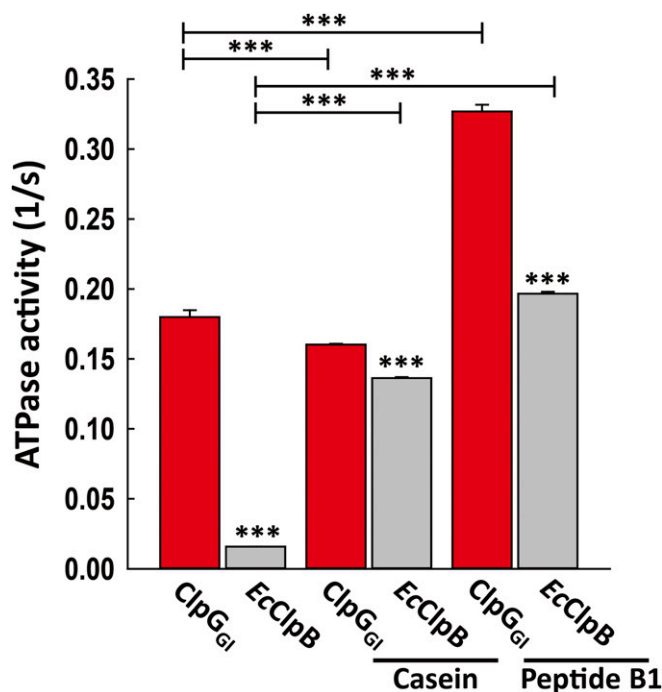


Fig. 5. ClpG_{GI} exhibits high basal ATPase activity and differs in substrate specificity. Basal and substrate-stimulated ATPase activities of EcClpB and ClpG_{GI} were determined. The substrates, casein and peptide B1, enhanced the ATPase activity of ClpB, while ClpG_{GI} activity was only further enhanced by peptide B1 (***) ($P < 0.001$; EcClpB compared with ClpG_{GI} and ClpG/ClpG_{GI} \pm substrate stimulation).

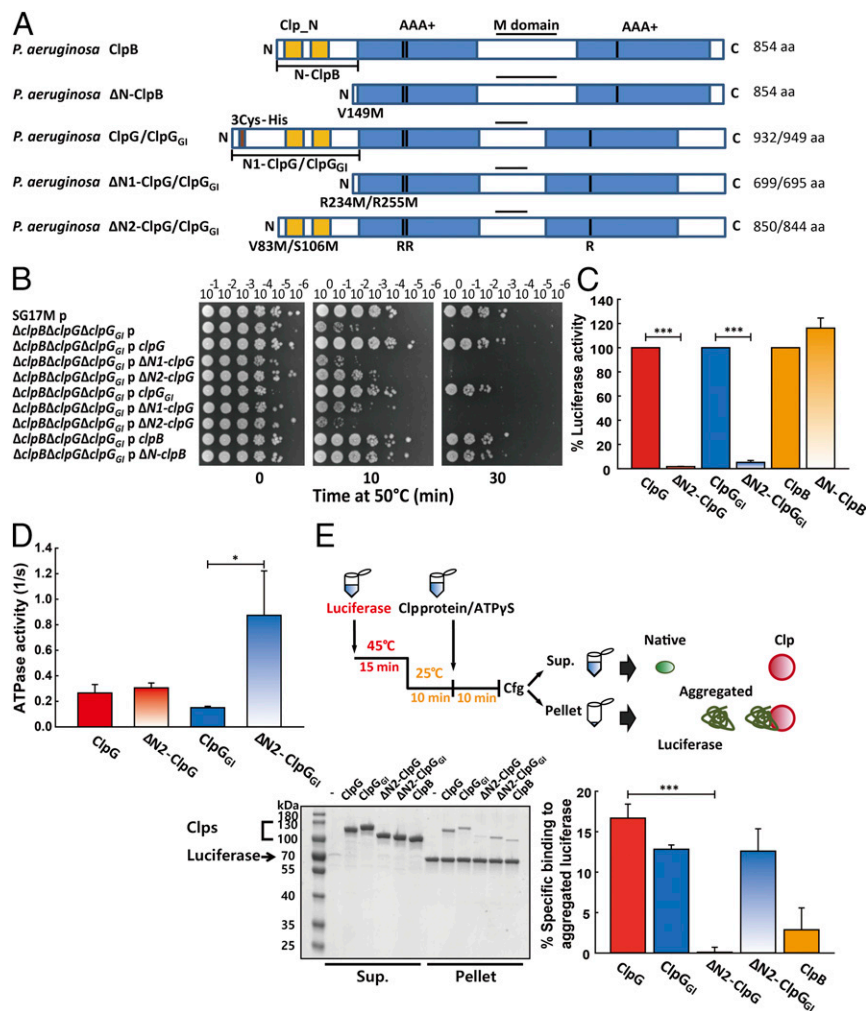


Fig. 6. The unique N-domain extensions of ClpG/ClpG_{GI} are essential for disaggregation activity. (A) Domain organization of ClpG/ClpG_{GI} and ClpB. The generated N-terminal deletion variants of ClpG/ClpG_{GI} (ΔN1 and ΔN2) and ClpB (ΔN) are indicated (B) *P. aeruginosa* SG17M $\Delta clpB \Delta clpG \Delta clpG_{GI}$ expressing full-length, N-terminal truncated ClpG/ClpG_{GI} or ClpB proteins from the vector pJN105 were subjected to heat shock at 50 °C for 10 min and 30 min. Cellular viabilities were determined by spotting serial dilutions (10^0 – 10^{-6}) of cells on LB plates. $p = pJN105$. (C) Refolding of heat-aggregated luciferase was monitored in the presence of indicated chaperones. The activity of native luciferase in the presence of wild-type chaperones ClpG, ClpG_{GI}, and ClpB after 90 min of refolding was set as 100%. (D) Basal ATPase activities of indicated ClpG/ClpG_{GI} variants were determined. (E) Experimental scheme for monitoring binding of ClpG/ClpG_{GI} and ClpB proteins to heat-aggregated luciferase. (Left) Heat-aggregated luciferase was incubated with the indicated chaperones in the presence of 2 mM ATPγS. Soluble (Sup.) and insoluble (pellet) fractions were analyzed by SDS-PAGE. (Right) Intensities of protein bands were quantified by ImageJ with the fraction (%) of chaperone present in the pellet fraction versus chaperone without luciferase aggregates calculated. Binding experiments were performed three times with a representative SDS-PAGE shown. Quantifications are based on three independent experiments (* $P < 0.05$; *** $P < 0.001$).

irreversible oxidized proteins as it is the case for ClpB of *Mycobacterium tuberculosis* (14).

ClpG/ClpG_{GI} are as potent as the DnaK-ClpB system in protein disaggregation, can complement the thermotolerance defect of *E. coli* $\Delta clpB$ and $dnaK103$ mutant cells, and together increase thermotolerance in *P. aeruginosa* SG17M clone C cells substantially. We even noticed higher disaggregation activity of the horizontally transferred ClpG_{GI} toward aggregated luciferase in vitro and in vivo, potentially explaining why ClpG_{GI} is promiscuous and functional in many bacteria, including *E. coli*, even compared with ClpG (Fig. 4) (23, 35).

As the well-established disaggregase ClpB, ClpG/ClpG_{GI}-mediated protein disaggregation is linked to refolding of aggregated substrates, independent from the peptidase ClpP. This underlines the concept that thermotolerance largely relies on reactivation of essential proteins aggregated upon heat induction (3, 58).

What is the relationship between the coexisting disaggregation systems, DnaK-ClpB and ClpG/ClpG_{GI}? We show here that both systems work independently from one another and exert largely overlapping and compensatory activities. Constitutive expression of either ClpB or ClpG/ClpG_{GI} to higher levels largely restores heat tolerance of *P. aeruginosa* cells lacking the chromosomal copies of all disaggregases (Fig. 6B and SI Appendix, Fig. S4). The contribution of the three disaggregase systems to *P. aeruginosa* heat tolerance differ in logarithmic and stationary phase. Strong increase of ClpG/ClpG_{GI} levels in *P. aeruginosa* clone C SG17M cells during stationary phase, likely enables ClpG/

ClpG_{GI} to outcompete DnaK, rationalizing their growth phase-specific contributions to heat tolerance (Fig. 7).

Despite superior biochemical performance, why is ClpG/ClpG_{GI} not more widespread? No detrimental effect was observed upon overproduction of ClpG/ClpG_{GI}, but expression of $shsp20_{GI}$ encoding the small heat shock protein in the $dna-shsp20_{GI-clpG_{GI}}$ operon has been detrimental in *E. coli*. Furthermore, we can only speculate that, for example, the high intrinsic ATPase activity of ClpG/ClpG_{GI} is hard to control. Alternatively, to covalently link the substrate binding domain to disaggregate functionality in the same protein may provide reduced regulatory flexibility. ClpG/ClpG_{GI} might have developed in bacteria indwelling a demanding ecological niche in parallel to the Hsp70-ClpB system, or ClpG/ClpG_{GI} is an evolutionary younger protein. Recent acquisition of ClpG_{GI} homologs, often on plasmids and in more than one copy, by unconventional pathogens associated with industrial and clinical settings (see also below) suggests that certain man-made environments provide a strong selection pressure aiding predominantly the spread of the $dna-shsp20_{GI-clpG_{GI}}$ core unit (59, 60).

Bioinformatic analysis indicates that both copies of the ClpG disaggregase, $clpG$ and $clpG_{GI}$, have been acquired by horizontal gene transfer. Growth benefits provided by mobile genetic elements have been so far largely linked to acquisition of antibiotic resistance, specific virulence factors, or the utilization of alternative carbon sources (e.g., degradation of aromatic compounds) (61, 62). Recently, a growth advantage provided by transmissible loci is becoming apparent: increased bacterial fitness during stress conditions (23, 25, 63). Exposure to elevated temperature

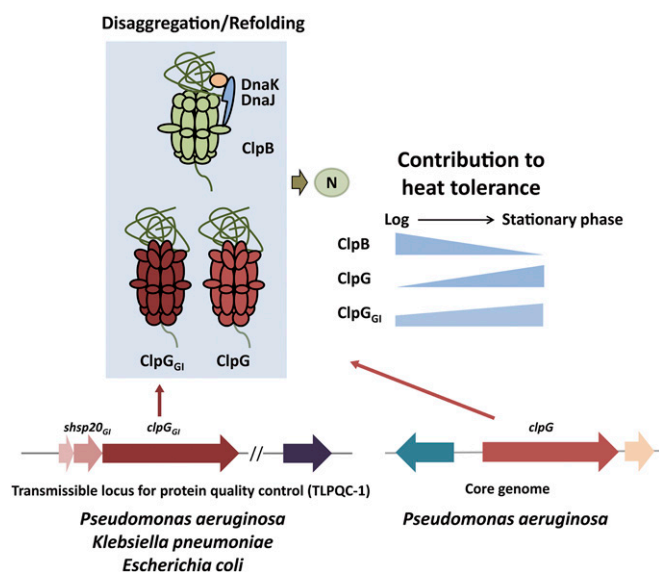


Fig. 7. The ClpG/ClpG_{GI} disaggregases increase disaggregation potential and heat resistance of *P. aeruginosa* SG17M. *P. aeruginosa* clone C strain harbors the classical disaggregating DnaK–ClpB bichaperone system and the ClpG and ClpG_{GI} disaggregases (23). ClpG is located on the core genome, while ClpG_{GI} is located on the TLPQC-1 locus as part of PACGI-1 genomic island. The contributions of the different disaggregating systems to heat tolerance differ at logarithmic and stationary growth. ClpB/ClpG_{GI} are most important for heat resistance during logarithmic growth, while ClpG/ClpG_{GI} dominate heat tolerance during stationary phase. Compared with ClpB, ClpG/ClpG_{GI} have unique and extended N-terminal N1 domains to bind to substrates. TLPQC-1 encoding ClpG_{GI} is also present in outbreak strains of *K. pneumoniae* and *E. coli*.

represents one of the most adverse conditions for environmental bacteria, but is also relevant for pathogens in interaction with the host. Acquisition of the ClpG/ClpG_{GI} disaggregases boosts protein quality control and increases viability of *P. aeruginosa* clone C during severe heat stress. The identification of NuoCD, subunits of the NAD quinolone oxidoreductase, as potential ClpG_{GI} substrates (Fig. 2) suggests a role of ClpG_{GI} in energy production through respiration. The ClpB disaggregase plays a role in virulence and host persistence of pathogenic and commensal bacteria (6, 7, 13–16). It remains to be tested whether a $\Delta clpB \Delta clpG_{GI}$ mutant exhibits phenotypes beyond heat sensitivity such as in microbial–host interactions. A potential function in virulence is emphasized as ClpG is uniquely encoded on the core genome of *P. aeruginosa*, the most important human nosocomial pathogen of the *Pseudomonas* genus. Remarkably, more clones of *P. aeruginosa*, such as clone J strains successful in acute and chronic infections and the aquatic habitat, additionally harbor ClpG_{GI} on PACGI-1/TLPQC-1 (25), while the PA14 strain, which is lacking PACGI-1/TLPQC-1, is predominantly found in acute infections (22). The occurrence of ClpG_{GI} extends beyond *P. aeruginosa* as ClpG_{GI} is found in extremely heat-tolerant *E. coli* from food factories (2% of all strains), where ClpG_{GI} and TLPQC-1 additively contribute to heat tolerance (35). Also 2/3 of *Klebsiella pneumoniae* strains from the clinical environment harbor ClpG_{GI} (called ClpK in this species) suggesting that *K. pneumoniae* requires the extended heat tolerance phenotype to successfully colonize the clinical habitat (60). Infections caused by *K. pneumoniae* harboring *clpG_{GI}* are, however, not characterized by increased virulence (60). This indicates heat tolerance to become an increasingly important virulence, persistence, or resistance factor, probably promoted by modern food production and medical

sterilization procedures that involve heat treatment. Since ClpG/ClpG_{GI} are AAA+ chaperones in *P. aeruginosa* clone C and other common clonal strains of different genera, they might represent a potential target to develop novel antimicrobial or antivirulence compounds and approaches.

Materials and Methods

Detailed information of all experimental procedures is provided in *SI Appendix*.

Strains, Plasmids, and Growth Condition. The aquatic isolate *P. aeruginosa* SG17M was selected as the representative clone C strain. All strains, plasmids, and primers used in this study are listed in *SI Appendix*, Tables S2–S4. Growth conditions and construction of mutants and plasmids are described in *SI Appendix*.

Bioinformatic Analysis. Protein sequences of ClpG (acc. no. EWH25562), ClpG_{GI} (acc. no. EWH27925), and ClpB (acc. no. EWH24017) of *P. aeruginosa* SG17M (25) were used as queries to search for ClpG, ClpG_{GI}, and ClpB homologs as described in *SI Appendix*.

Heat Shock Tolerance Assay. *P. aeruginosa* cells incubated in LB broth with shaking at 20 °C were harvested from logarithmic (OD₆₀₀ = 0.7) and stationary (OD₆₀₀ = 2.5) phase. The cells were exposed to 50 °C for 10, 30, and 60 min and subsequently cell viability was determined by the spotting assay as described in *SI Appendix*.

In Vitro Disaggregating Activity Assay. Disaggregating activity was determined with minor modification (64) as described in *SI Appendix*. MDH and luciferase (both from Roche) were used as model substrates.

In Vivo Chaperone Assay. An *E. coli* MC4100 $\Delta clpB$ strain expressing YFP-luciferase and the respective chaperone was cultured in LB medium at 30 °C. To heat shock, cells were incubated at a nonlethal 45 °C for 20 min. Afterward, the cells were incubated at 30 °C for recovery and luciferase activity was measured at certain time points as described in *SI Appendix*.

ATPase Assay. ATPase activity was determined by a coupled ADP monitoring/recycling reaction of pyruvate kinase (PK) and lactate dehydrogenase (LDH) as described in *SI Appendix*.

Prion Propagation Test. Propagation of [PSI⁺] and [URE3] was monitored by their ability to promote expression of Ade2p using yeast strains, 779-6A (*MAT α* , *kar1-1*, *SUQ5*, *ade2-1*, *his3 Δ 202*, *leu2 Δ 1*, *trp1 Δ 63*, and *ura3-52*) and 1075 (*MAT α* , *kar1-1*, *P_{DALS}::ADE2*, *his3 Δ 202*, *leu2 Δ 1*, *trp1 Δ 63*, and *ura3-52*) (38) as described in *SI Appendix*.

Disaggregation of Proteins in Heat-Denatured Crude Extracts. Disaggregation of proteins in heat-denatured crude extracts was performed with minor modifications (32) as described in *SI Appendix*.

Isolation of in Vivo Protein Aggregates. In vivo protein aggregates were examined with minor modifications (65) as described in *SI Appendix*.

In Vitro Substrate Binding Assay. The interaction between denatured luciferase and Clp protein variants was conducted as described in *SI Appendix*.

ACKNOWLEDGMENTS. We thank Daniel C. Masison (National Institute of Diabetes and Digestive and Kidney Diseases, NIH) for providing materials and valuable discussion for the prion propagation test, Johannes Gescher (Karlsruhe Institut für Technology) for providing the material for yeast cloning, Matthias P. Mayer [Center for Molecular Biology of the University of Heidelberg (ZMBH)] for help with CD measurements, and Regina Zahn (ZMBH) for technical assistance. Changhan Lee received a personal scholarship through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (NRF-2014R1A6A3A03057742), and has been provided a travel grant from the Karolinska Institutet for a research visit to the University of Heidelberg. The project was funded by the Swedish Research Council for Medicine and Health (K2012-56X-22034-01-3), the Karolinska Institutet (U.R.), and by grants from the Deutsche Forschungsgemeinschaft (BB617/17-2 and MO 970/4-2) (to B.B. and A.M.).

1. Kim YE, Hipp MS, Bracher A, Hayer-Hartl M, Hartl FU (2013) Molecular chaperone functions in protein folding and proteostasis. *Annu Rev Biochem* 82:323–355.

2. Saibil H (2013) Chaperone machines for protein folding, unfolding and disaggregation. *Nat Rev Mol Cell Biol* 14:630–642.

3. Weibezahn J, et al. (2004) Thermotolerance requires refolding of aggregated proteins by substrate translocation through the central pore of ClpB. *Cell* 119:653–665.
4. Squires CL, Pedersen S, Ross BM, Squires C (1991) ClpB is the *Escherichia coli* heat shock protein F84.1. *J Bacteriol* 173:4254–4262.
5. Sanchez Y, Lindquist SL (1990) HSP104 required for induced thermotolerance. *Science* 248:1112–1115.
6. Chastanet A, Derre I, Nair S, Msadek T (2004) *clpB*, a novel member of the *Listeria monocytogenes* CtsR regulon, is involved in virulence but not in general stress tolerance. *J Bacteriol* 186:1165–1174.
7. de Oliveira NE, et al. (2011) *clpB*, a class III heat-shock gene regulated by CtsR, is involved in thermotolerance and virulence of *Enterococcus faecalis*. *Microbiology* 157:656–665.
8. Queitsch C, Hong SW, Vierling E, Lindquist S (2000) Heat shock protein 101 plays a crucial role in thermotolerance in Arabidopsis. *Plant Cell* 12:479–492.
9. Krüger E, Völker U, Hecker M (1994) Stress induction of *clpC* in *Bacillus subtilis* and its involvement in stress tolerance. *J Bacteriol* 176:3360–3367.
10. Krüger E, Witt E, Ohlmeier S, Hanschke R, Hecker M (2000) The *clp* proteases of *Bacillus subtilis* are directly involved in degradation of misfolded proteins. *J Bacteriol* 182:3259–3265.
11. Kirstein J, Dougan DA, Gerth U, Hecker M, Turgay K (2007) The tyrosine kinase MscB is a regulated adaptor protein for ClpCP. *EMBO J* 26:2061–2070.
12. Schlothauer T, Mogk A, Dougan DA, Bukau B, Turgay K (2003) MecA, an adaptor protein necessary for ClpC chaperone activity. *Proc Natl Acad Sci USA* 100:2306–2311.
13. Meibom KL, et al. (2008) 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* 67:1384–1401.
14. Vaubourgeix J, et al. (2015) Stressed mycobacteria use the chaperone ClpB to sequester irreversibly oxidized proteins asymmetrically within and between cells. *Cell Host Microbe* 17:178–190.
15. Yuan L, Rodrigues PH, Bèlanger M, Dunn W, Jr, Progulske-Fox A (2007) The *Porphyromonas gingivalis* *clpB* gene is involved in cellular invasion in vitro and virulence in vivo. *FEMS Immunol Med Microbiol* 51:388–398.
16. Brodmann M, Dreier RF, Broz P, Basler M (2017) *Francisella* requires dynamic type VI secretion system and ClpB to deliver effectors for phagosomal escape. *Nat Commun* 8:15853.
17. Kerr KG, Snelling AM (2009) *Pseudomonas aeruginosa*: A formidable and ever-present adversary. *J Hosp Infect* 73:338–344.
18. Rakhimova E, et al. (2009) *Pseudomonas aeruginosa* population biology in chronic obstructive pulmonary disease. *J Infect Dis* 200:1928–1935.
19. Tielen P, et al. (2011) Genotypic and phenotypic characterization of *Pseudomonas aeruginosa* isolates from urinary tract infections. *Int J Med Microbiol* 301:282–292.
20. Römling U, Wingender J, Müller H, Tümmler B (1994) A major *Pseudomonas aeruginosa* clone common to patients and aquatic habitats. *Appl Environ Microbiol* 60:1734–1738.
21. Fischer S, et al. (2016) Intracolon genome diversity of the major *Pseudomonas aeruginosa* clones C and PA14. *Environ Microbiol Rep* 8:227–234.
22. De Soya A, et al.; EU FP7 funded COST Action BM1003 “Cell surface virulence determinants of cystic fibrosis pathogens” (2013) Developing an international *Pseudomonas aeruginosa* reference panel. *MicrobiologyOpen* 2:1010–1023.
23. Lee C, Wigren E, Lünsdorf H, Römling U (2016) Protein homeostasis: More than resisting a hot bath. *Curr Opin Microbiol* 30:147–154.
24. Bojer MS, Struve C, Ingmer H, Hansen DS, Krogfelt KA (2010) Heat resistance mediated by a new plasmid encoded Clp ATPase, ClpK, as a possible novel mechanism for nosocomial persistence of *Klebsiella pneumoniae*. *PLoS One* 5:e15467.
25. Lee C, et al. (2015) A novel protein quality control mechanism contributes to heat shock resistance of worldwide-distributed *Pseudomonas aeruginosa* clone C strains. *Environ Microbiol* 17:4511–4526.
26. Lee S, et al. (2003) The structure of ClpB: A molecular chaperone that rescues proteins from an aggregated state. *Cell* 115:229–240.
27. Wang F, et al. (2011) Structure and mechanism of the hexameric MecA-ClpC molecular machine. *Nature* 471:331–335.
28. Kim YI, et al. (2001) Molecular determinants of complex formation between Clp/Hsp100 ATPases and the ClpP peptidase. *Nat Struct Biol* 8:230–233.
29. Mogk A, Kummer E, Bukau B (2015) Cooperation of Hsp70 and Hsp100 chaperone machines in protein disaggregation. *Front Mol Biosci* 2:22.
30. Trentini DB, et al. (2016) Arginine phosphorylation marks proteins for degradation by a Clp protease. *Nature* 539:48–53.
31. Parsell DA, Kowal AS, Singer MA, Lindquist S (1994) Protein disaggregation mediated by heat-shock protein Hsp104. *Nature* 372:475–478.
32. Mogk A, et al. (1999) Identification of thermolabile *Escherichia coli* proteins: Prevention and reversion of aggregation by DnaK and ClpB. *EMBO J* 18:6934–6949.
33. Carroni M, et al. (2014) Head-to-tail interactions of the coiled-coil domains regulate ClpB activity and cooperation with Hsp70 in protein disaggregation. *Elife* 3:e02481.
34. Schlieker C, Tews I, Bukau B, Mogk A (2004) Solubilization of aggregated proteins by ClpB/DnaK relies on the continuous extraction of unfolded polypeptides. *FEBS Lett* 578:351–356.
35. Li H, Gänzle M (2016) Some like it hot: Heat resistance of *Escherichia coli* in food. *Front Microbiol* 7:1763.
36. Thomas JG, Baneyx F (1996) Protein folding in the cytoplasm of *Escherichia coli*: Requirements for the DnaK-DnaJ-GrpE and GroEL-GroES molecular chaperone machines. *Mol Microbiol* 21:1185–1196.
37. Jung G, Jones G, Masison DC (2002) Amino acid residue 184 of yeast Hsp104 chaperone is critical for prion-curing by guanidine, prion propagation, and thermotolerance. *Proc Natl Acad Sci USA* 99:9936–9941.
38. Reidy M, Miot M, Masison DC (2012) Prokaryotic chaperones support yeast prions and thermotolerance and define disaggregation machinery interactions. *Genetics* 192:185–193.
39. Oguchi Y, et al. (2012) A tightly regulated molecular toggle controls AAA+ disaggregase. *Nat Struct Mol Biol* 19:1338–1346.
40. Winkler J, Tyedmers J, Bukau B, Mogk A (2012) Hsp70 targets Hsp100 chaperones to substrates for protein disaggregation and prion fragmentation. *J Cell Biol* 198:387–404.
41. Rosenzweig R, Moradi S, Zarrine-Afsar A, Glover JR, Kay LE (2013) Unraveling the mechanism of protein disaggregation through a ClpB-DnaK interaction. *Science* 339:1080–1083.
42. Lee HW, et al. (2013) Heat shock protein 70 (HSP70) expression is associated with poor prognosis in intestinal type gastric cancer. *Virchows Arch* 463:489–495.
43. Schlieker C, et al. (2004) Substrate recognition by the AAA+ chaperone ClpB. *Nat Struct Mol Biol* 11:607–615.
44. Beinker P, Schlee S, Groemping Y, Seidel R, Reinstein J (2002) The N terminus of ClpB from *Thermus thermophilus* is not essential for the chaperone activity. *J Biol Chem* 277:47160–47166.
45. Liu Z, Tek V, Akoev V, Zolkiewski M (2002) Conserved amino acid residues within the amino-terminal domain of ClpB are essential for the chaperone activity. *J Mol Biol* 321:111–120.
46. Mogk A, et al. (2003) Roles of individual domains and conserved motifs of the AAA+ chaperone ClpB in oligomerization, ATP hydrolysis, and chaperone activity. *J Biol Chem* 278:17615–17624.
47. Clarke AK, Eriksson MJ (2000) The truncated form of the bacterial heat shock protein ClpB/Hsp100 contributes to development of thermotolerance in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J Bacteriol* 182:7092–7096.
48. Acebrón SP, Martín I, del Castillo U, Moro F, Muga A (2009) DnaK-mediated association of ClpB to protein aggregates. A bichaperone network at the aggregate surface. *FEBS Lett* 583:2991–2996.
49. Eriksson MJ, Clarke AK (1996) The heat shock protein ClpB mediates the development of thermotolerance in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J Bacteriol* 178:4839–4846.
50. Simão RC, Susin MF, Alvarez-Martinez CE, Gomes SL (2005) Cells lacking ClpB display a prolonged shutoff phase of the heat shock response in *Caulobacter crescentus*. *Mol Microbiol* 57:592–603.
51. Frees D, et al. (2004) Clp ATPases are required for stress tolerance, intracellular replication and biofilm formation in *Staphylococcus aureus*. *Mol Microbiol* 54:1445–1462.
52. Kummer E, et al. (2016) Bacterial and yeast AAA+ disaggregases ClpB and Hsp104 operate through conserved mechanism involving cooperation with Hsp70. *J Mol Biol* 428:4378–4391.
53. Kirstein J, Molière N, Dougan DA, Turgay K (2009) Adapting the machine: Adaptor proteins for Hsp100/Clp and AAA+ proteases. *Nat Rev Microbiol* 7:589–599.
54. Suokko A, Poutanen M, Savijoki K, Kalkkinen N, Varmanen P (2008) ClpL is essential for induction of thermotolerance and is potentially part of the HrcA regulon in *Lactobacillus gasseri*. *Proteomics* 8:1029–1041.
55. Miethke M, Hecker M, Gerth U (2006) Involvement of *Bacillus subtilis* ClpE in CtsR degradation and protein quality control. *J Bacteriol* 188:4610–4619.
56. Park SS, et al. (2015) ClpL is a chaperone without auxiliary factors. *FEBS J* 282:1352–1367.
57. Rosenzweig R, et al. (2015) ClpB N-terminal domain plays a regulatory role in protein disaggregation. *Proc Natl Acad Sci USA* 112:E6872–E6881.
58. Tessarz P, Mogk A, Bukau B (2008) Substrate threading through the central pore of the Hsp104 chaperone as a common mechanism for protein disaggregation and prion propagation. *Mol Microbiol* 68:87–97.
59. Nguyen SV, Harhay GP, Bono JL, Smith TP, Harhay DM (2017) Genome sequence of the thermotolerant foodborne pathogen *Salmonella enterica* serovar Senftenberg ATCC 43845 and phylogenetic analysis of loci encoding increased protein quality control mechanisms. *mSystems* 2:e00190-16.
60. Jørgensen SB, et al. (2016) Heat-resistant, extended-spectrum β -lactamase-producing *Klebsiella pneumoniae* in endoscope-mediated outbreak. *J Hosp Infect* 93:57–62.
61. Johnson CM, Grossman AD (2015) Integrative and conjugative elements (ICEs): What they do and how they work. *Annu Rev Genet* 49:577–601.
62. Dobrindt U, Hochhut B, Hentschel U, Hacker J (2004) Genomic islands in pathogenic and environmental microorganisms. *Nat Rev Microbiol* 2:414–424.
63. Krajewski SS, Joswig M, Nagel M, Narberhaus F (2014) A tricistronic heat shock operon is important for stress tolerance of *Pseudomonas putida* and conserved in many environmental bacteria. *Environ Microbiol* 16:1835–1853.
64. Mogk A, Deuerling E, Vordervölbecke S, Vierling E, Bukau B (2003) Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. *Mol Microbiol* 50:585–595.
65. Tomoyasu T, Mogk A, Langen H, Goloubinoff P, Bukau B (2001) Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the *Escherichia coli* cytosol. *Mol Microbiol* 40:397–413.