Supporting Information Lee et al. 10.1073/pnas.1712051115

Supporting Figures and Figure legends

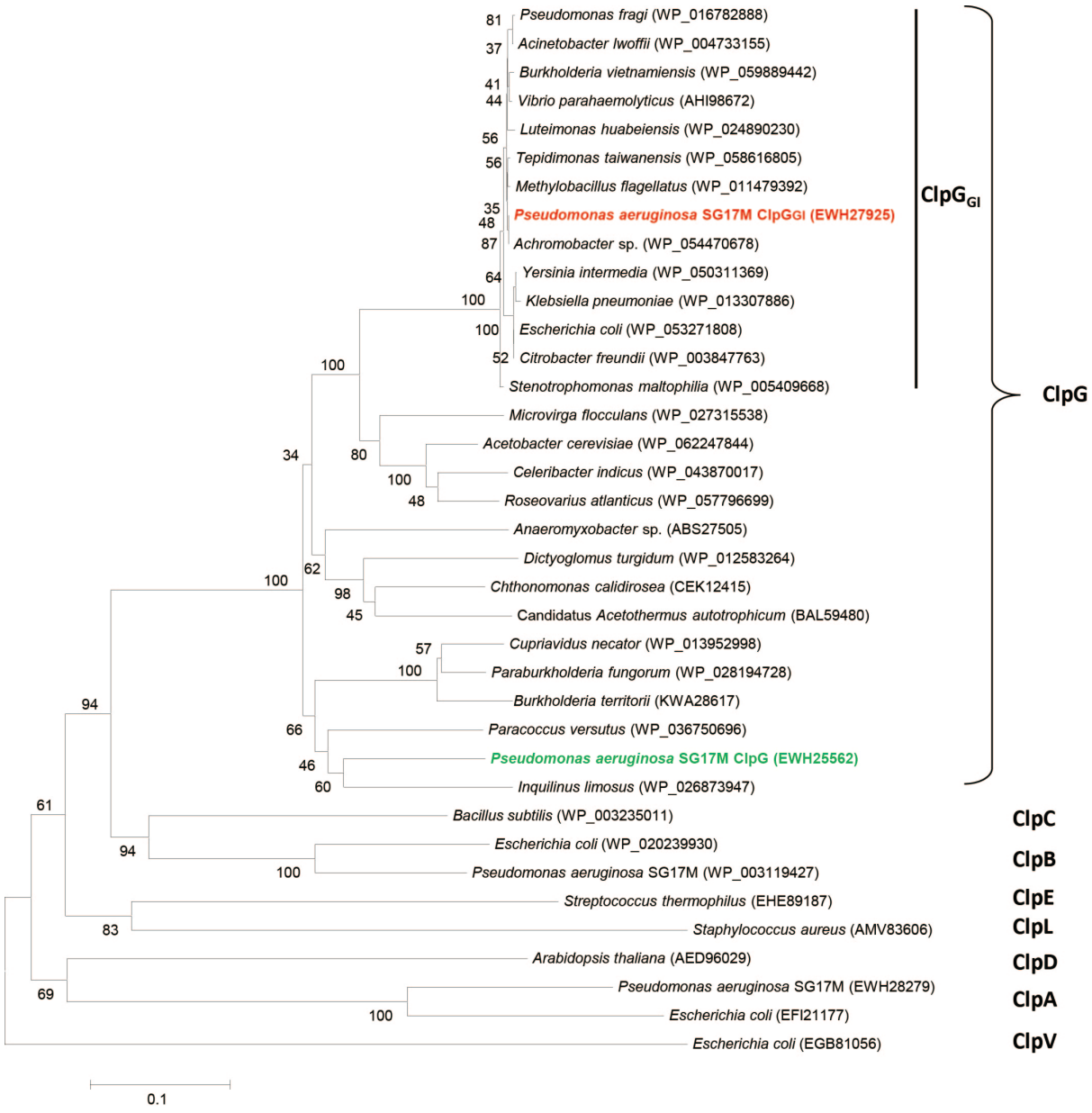
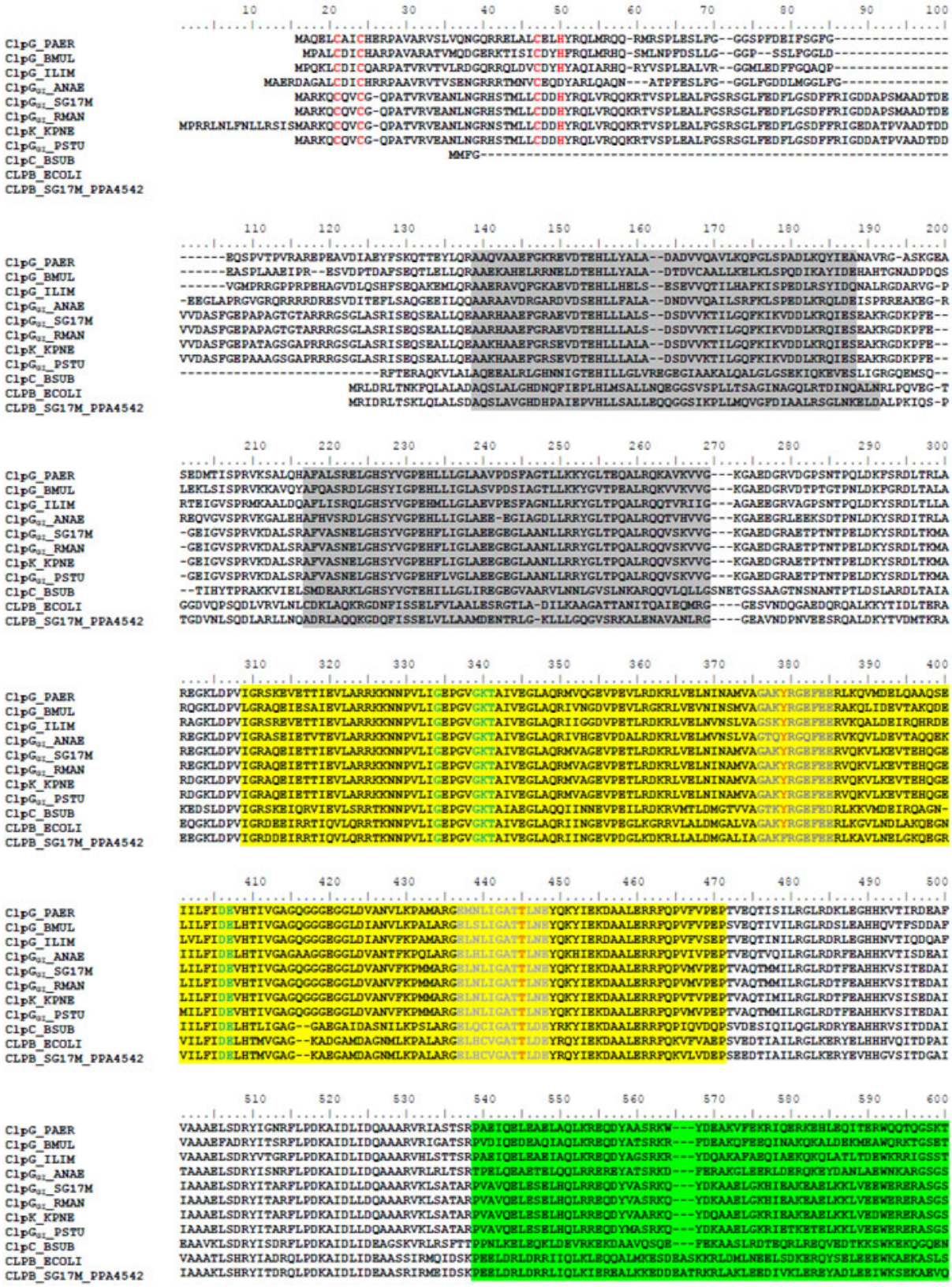


Fig. S1 Phylogenetic analysis of ClpG/ClpG$_{GI}$ proteins. ClpG/ClpG$_{GI}$ proteins form a distinct group within the Clp protein family distant from ClpB, ClpC and other class I Clp family members (ClpA, B, C, D, E and V). The ClpG$_{GI}$ group members located on the TLPQC-1 like genomic islands form a specific sub-group. Protein sequences were aligned with ClustalX2 using standard parameters and a neighbor-joining tree was built using MEGA 7.0. Branch lengths correspond to substitutions per site and branch support values are indicated in %.


ClpG_PAER
ClpG_BMUL
ClpG_ILIM
ClpG$_{ANAE}$
ClpG$_{SG17M}$
ClpG$_{RMAN}$
ClpK_KPNE
ClpG$_{PSTU}$
ClpC_BSUB
CLPB_ECOLI
CLPB_SG17M_PPA4542

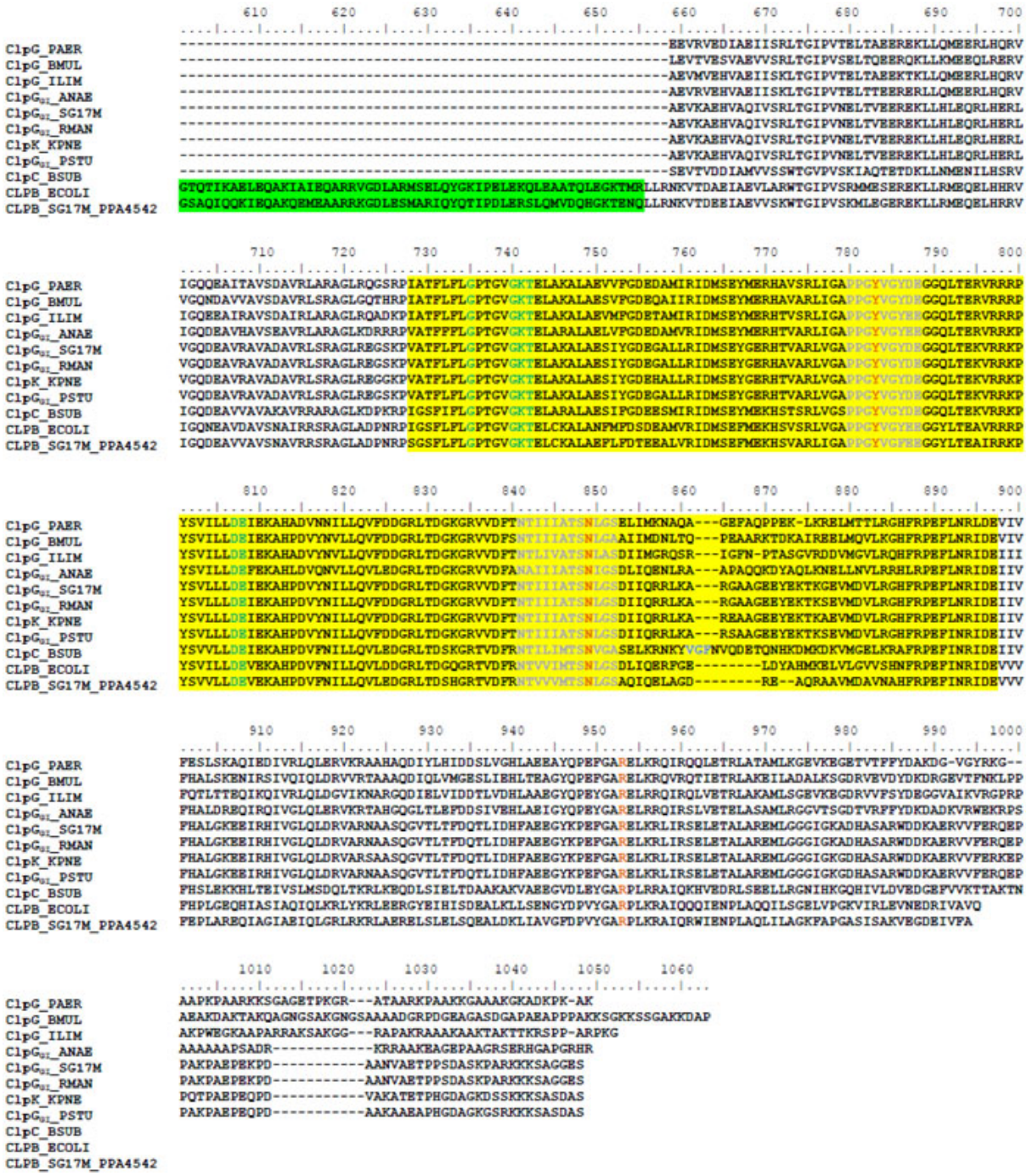


Fig. S2 Sequence alignment of selected AAA+ Hsp100 family proteins. Alignment of selected ClpG/ClpG$_{GI}$ homolog in comparison with *B. subtilis* ClpC and ClpB of *E. coli* and *P. aeruginosa* SG17M. ClpG and ClpG$_{GI}$ homolog contain an N-terminal extension with a conserved 3Cys-His motif (red letters). Conserved domains present in all proteins were boxed: Clp_N domain (pfam02861, gray background), AAA+ ATPase domains (AAA-1, AAA-2) (cd00009, yellow background) and M-domains (green background). WalkerA/B motifs are in green letters; the pore loop motifs in AAA-1 and AAA-2 are in gray letters with the conserved tyrosine residues (Y) in brown, sensor 1 motifs in AAA-1 and AAA-2 are in gray letters with the conserved threonine (T) and asparagine (N) in AAA-1 and AAA-2, respectively, in orange and sensor 2 motif at C-terminal region after AAA-2 is in orange

letter. The VGF ClpP interaction loop of ClpC is in turquoise. ClpC, P37571.1 (*Bacillus subtilis*); ClpG_BMUL, KGC07149.1 (*Burkholderia multivorans*), ClpG_ILIM, WP_026873947.1 (*Inquilinus limosus*); ClpG_ANAE, ABS27505.1 (*Anaeromyxobacter* sp. Fw109-5); $ClpG_{GI}$_RMAN, WP_045786171.1, (*Ralstonia mannitolilytica*); ClpK_KPNE, AFV70479.1 (*Klebsiella pneumoniae*); $ClpG_{GI}$_PSTU, F2MZ57 (*Pseudomonas stutzeri*).

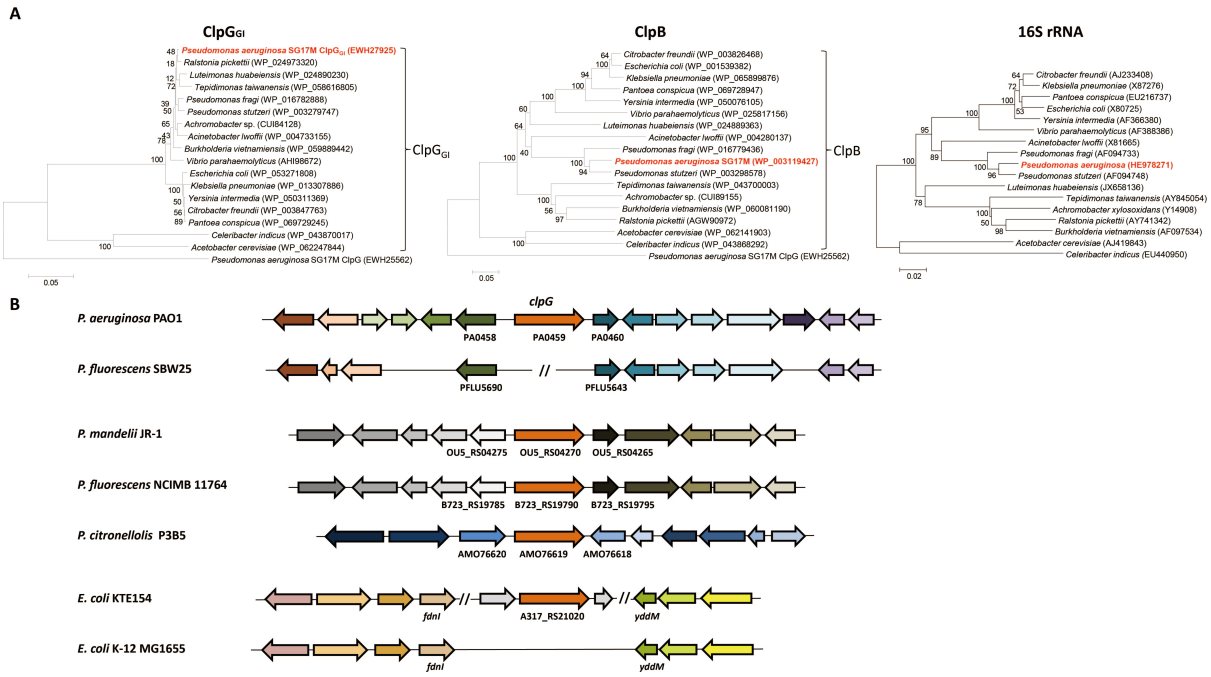
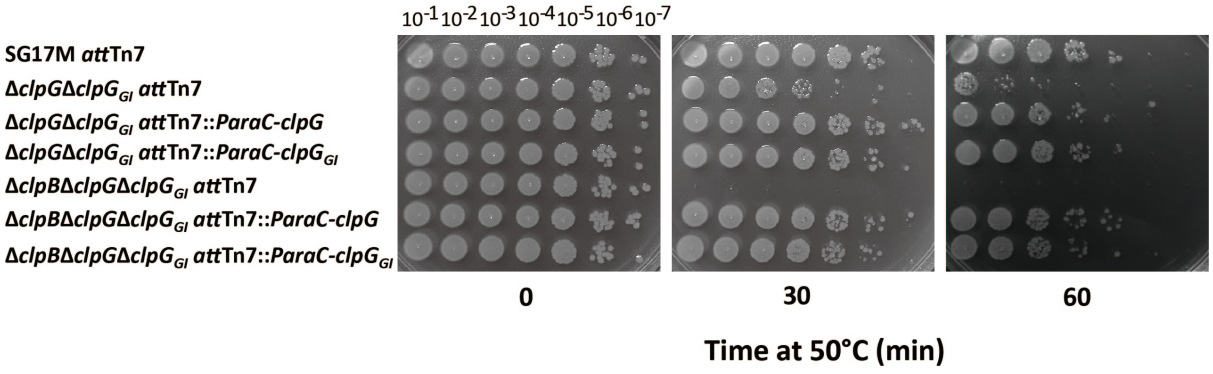


Fig. S3 Comparative phylogenetic analysis of ClpG, $ClpG_{GI}$ and ClpB proteins. (A) Phylogenetic neighbor-joining tree of $ClpG_{GI}$ homologs compared to the ClpB and 16S RNA phylogenetic tree. (B) Unique position of ClpG between the major facilitator superfamily (MFS) transporter PA0458 and a DUF2780 superfamily protein (PA0460) in *P. aeruginosa*. The corresponding genomic region of *Pseudomonas fluorescens* SBW25, which is lacking ClpG, is given. Genomic organizations of ClpG homologs from *P. fluorescens* NCIMB 11764, *Pseudomonas mandelii* JR-1 and *Pseudonomas citronellolis* P3B5 are different and shown for comparison. Also in *E. coli* KTE154, the ClpG homolog is found on a putative genomic island as compared with *E. coli* K-12 MG1655.



41

42 **Fig. S4 Complementation of *P. aeruginosa* SG17M Δ*clpG* Δ*clpG*$_{GI}$-double and Δ*clpB***
43 **Δ*clpG* Δ*clpG*$_{GI}$-triple disaggregase mutants by arabinose-induced expression of ClpG**
44 **and ClpG$_{GI}$**. *P. aeruginosa* SG17M wild type and indicated mutant cells expressing *clpG* or
45 *clpG*$_{GI}$ from the *araC* promoter (P*araC*) at the chromosomal Tn*7* site (*att*Tn*7*) were grown to
46 stationary phase at 20°C and heat shocked at 50°C for 30 and 60 min. Cellular viabilities
47 were determined by spotting serial dilutions (10^{-1} – 10^{-7}) of cells on LB plates followed by
48 incubation at 37°C for 18 h.

49

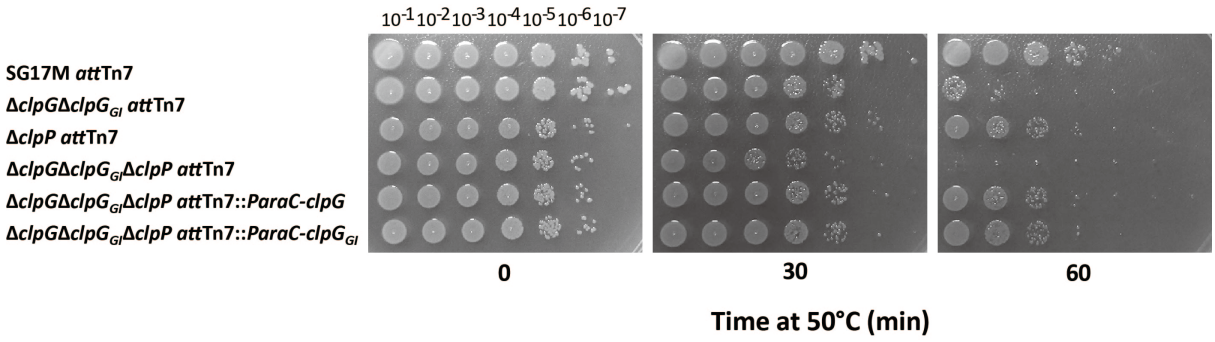


Fig. S5 ClpG/ClpG$_{GI}$ function independent from ClpP in heat tolerance. *P. aeruginosa* SG17M wild type and indicated mutant cells expressing *clpG* or *clpG$_{GI}$* from the *araC* promoter (P*araC*) at the chromosomal Tn7 site (*att*Tn*7*) were grown to stationary phase at 20°C and heat shocked at 50°C for 30 and 60 min. Cellular viabilities were determined by spotting serial dilutions (10^{-1} – 10^{-7}) of cells on LB plates followed by incubation at 37°C for 18 h.

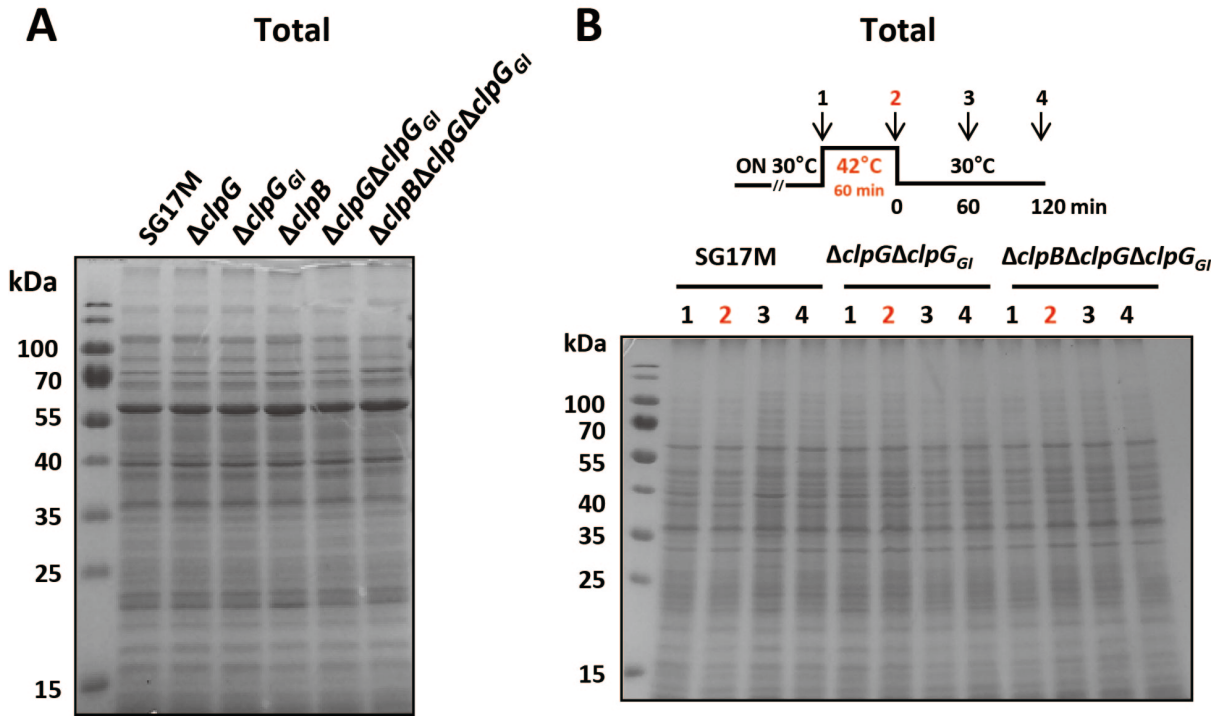


Fig. S6 Total protein of *P. aeruginosa* SG17M cells lacking ClpB and ClpG/ClpG$_{GI}$. Total protein content loading controls for estimation of isolated protein aggregates in *P. aeruginosa* strains (Fig. 2) analyzed by SDS-PAGE. (A) *P. aeruginosa* SG17M and mutants were cultured at 42°C for 24 h. (B) *P. aeruginosa* SG17M and mutants were grown at 30°C overnight (1), incubated at 42°C for 60 min (2), and then shifted to 30°C for 60 (3) and 120 (4) min for recovery.

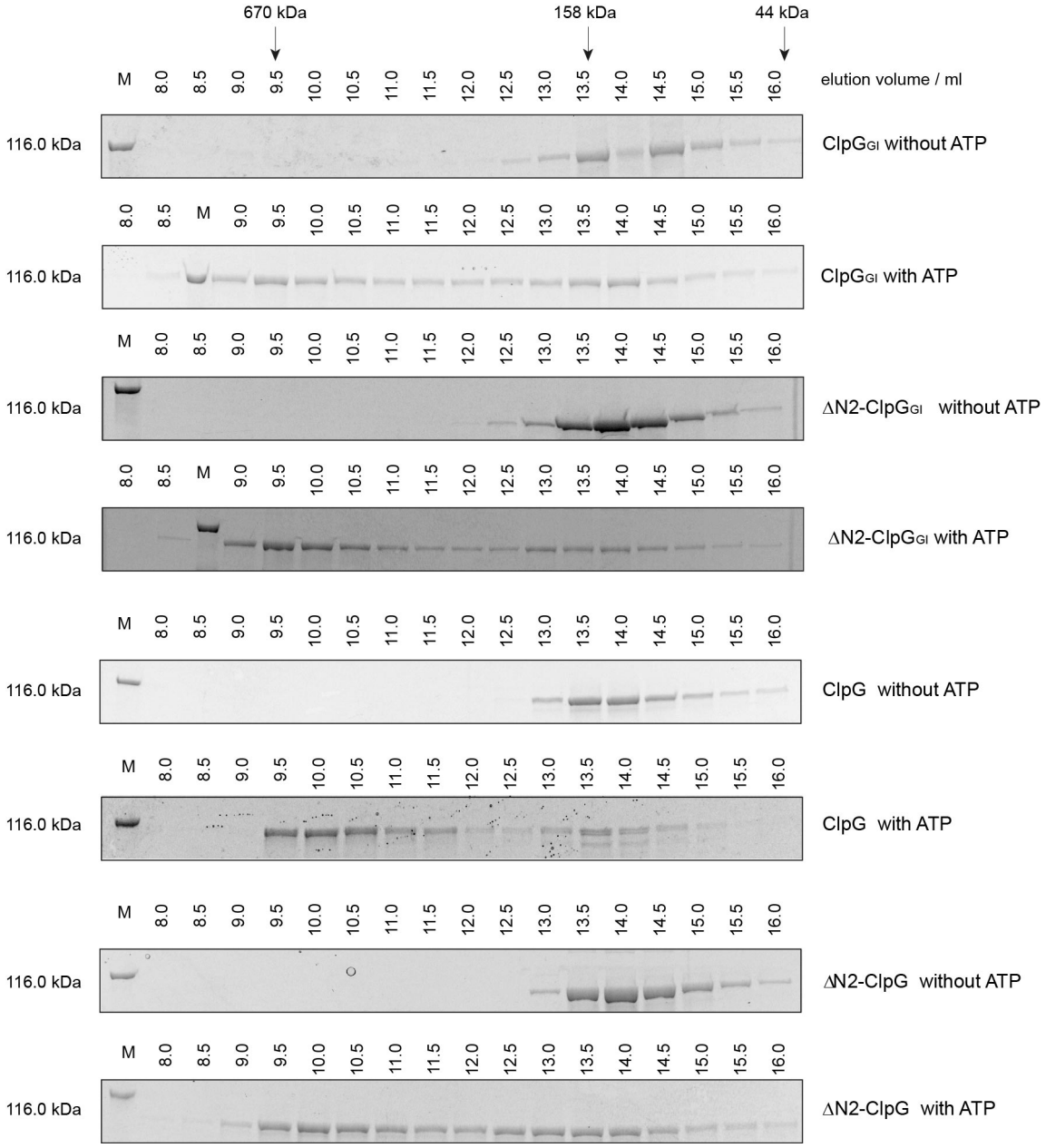


Fig. S7 Oligomerization of ClpG, $ClpG_{GI}$ and respective ΔN2 deletion mutants. Oligomerization studies of 10 µM ClpG and $ClpG_{GI}$ wild type and indicated deletion mutants were conducted using a Superdex 200 10/300 GL size exclusion column. Individual runs were performed in the absence or presence of nucleotide (2 mM ATP) in assay buffer (50 mM Tris pH 7.5, 150 mM KCl, 20 mM $MgCl_2$, 2 mM DTT) at room temperature. For visualization the fractions were collected and analyzed by SDS-PAGE and subsequent SYPRO Red staining. A molecular weight marker is shown and the elution volumes of standard proteins are indicated.

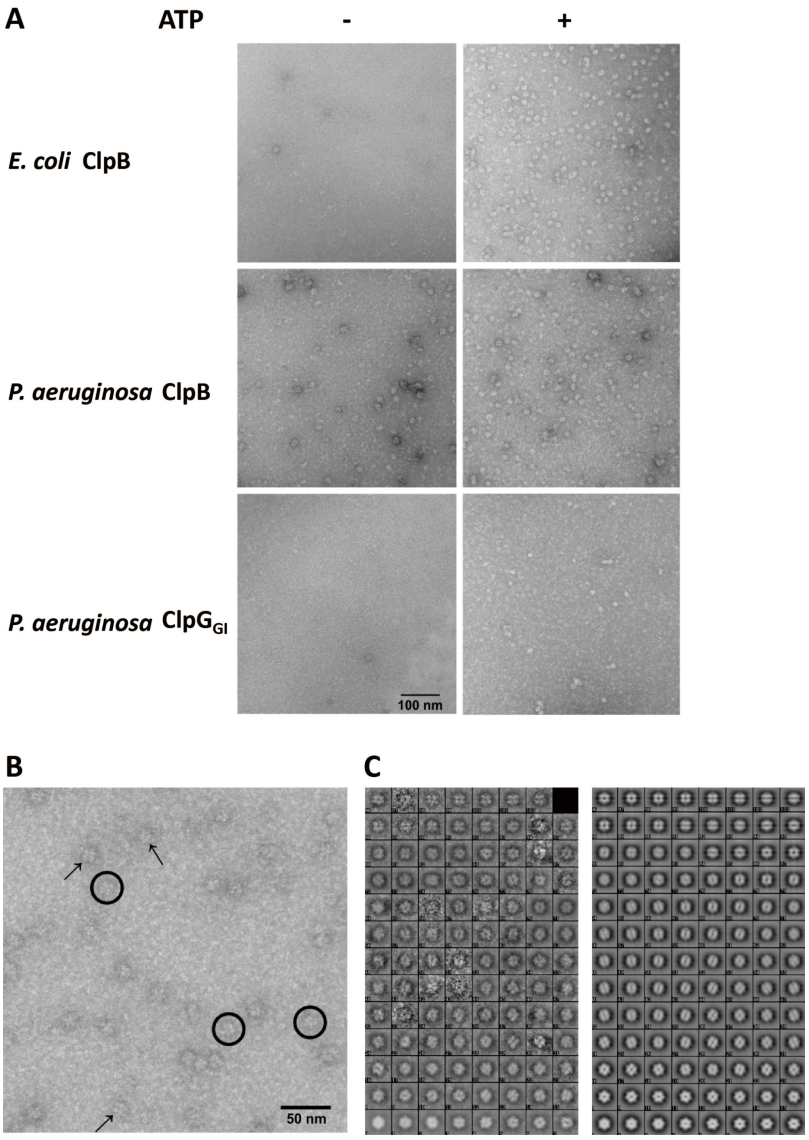


Fig. S8 Analysis of ClpG$_{GI}$ particles by transmission electron microscopy (TEM). (A) Oligomerization of *E. coli* K-12 ClpB and *P. aeruginosa* SG17M ClpB and ClpG$_{GI}$. An oligomeric protein structure for ClpB and ClpG$_{GI}$ was observed by TEM. *E. coli* ClpB showed a shift from monomer to apparent hexamer formation in the presence of 2 mM ATP. Formation of hexamers by *P. aeruginosa* ClpB is induced by ATP with hexamers already present before the addition of ATP. Oligomerization of ClpG$_{GI}$ is hardly induced upon addition of ATP under the same condition. Samples were incubated with 2 mM ATP at room temperature for 10 min and subsequently subjected to TEM. (B) Upon incubation with ATPγS, monodispersed molecules with roughly hexagonal top-views (circles) and bipartite side-views (arrows) can be recognized in the negatively stained sample by TEM. However, a significant background of low-mass proteins exists. The diameter of top-view molecules ranges from 12.2 nm to 18.6 nm with a mean of 14.6 nm ± 1.4 nm (N=56). The height, as measured from side-view projections reaches a mean of 9.75 nm ± 0.86 nm (N=89) within a range from 7.03 nm to 11.82 nm. (C) The appearance of class averages (left) and views of their back projections (right) sufficiently coincide at the base of the resolution reached. For

3D-reconstruction of $ClpG_{GI}$ molecules 1897 particles have been chosen and a first reference-free class average was calculated.

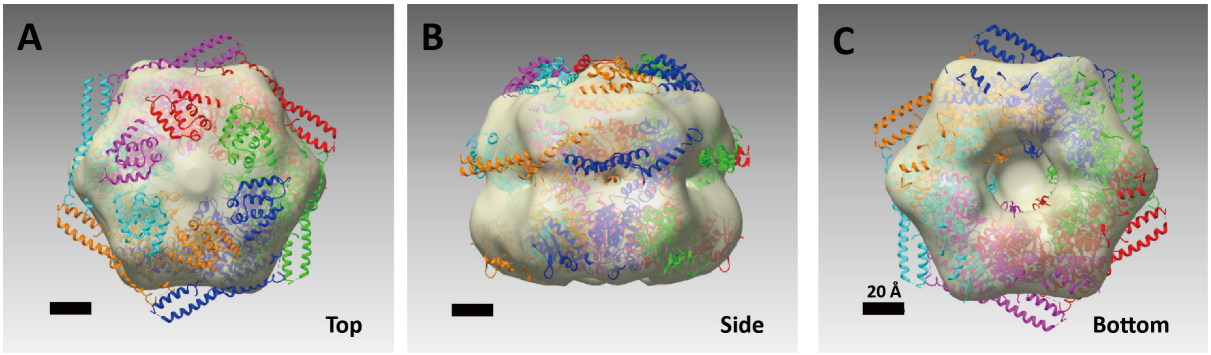


Fig. S9 Modeling of the $ClpG_{GI}$ hexameric structure. (A/B/C) Top, side and bottom view of the electron density model of $ClpG_{GI}$. The top-view shows a closed surface, while the bottom-view with its C-termini shows an open molecule. The model diameter is 13.6 nm versus 10.3 nm as model height, given a height/diameter ratio of 0.76 and thus appears smaller relative to direct measurements on negatively stained molecules (see Fig. S8). Assessment of the $ClpG_{GI}$ density model by semi-automatically fitting of X-ray data of homologous *E. coli* ClpB (PDB code: 4D2Q) into the model.

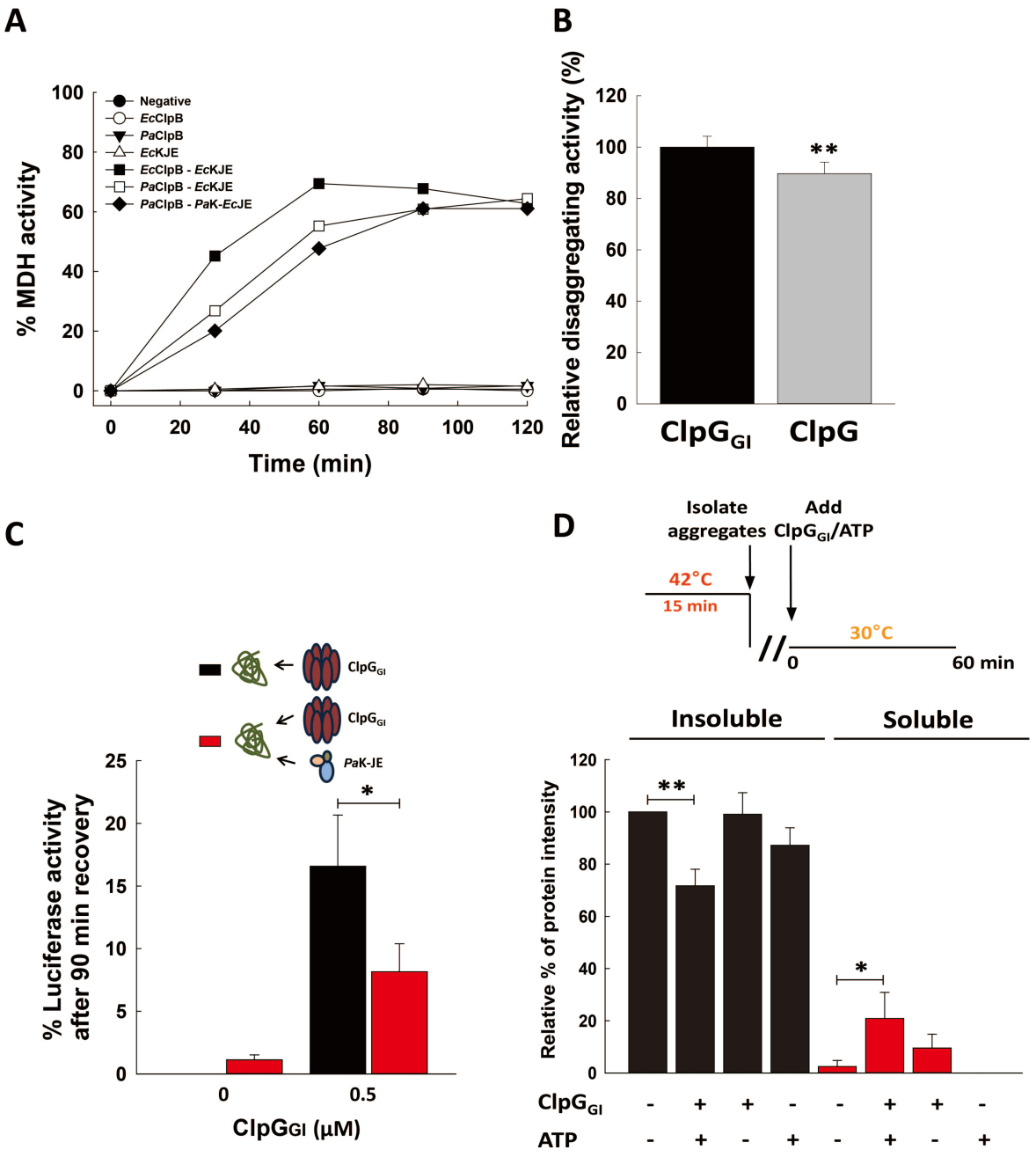


Fig. S10 KJE-dependent disaggregating activities of *P. aeruginosa* and *E. coli* ClpB and stand-alone disaggregating activity of *P. aeruginosa* ClpG. (A) ClpB of *P. aeruginosa* SG17M and *E. coli* K-12 show KJE-dependent disaggregation activity. *E. coli* DnaK (*Ec*KJE) or *P. aeruginosa* DnaK (*Pa*K-*Ec*JE) together with *E. coli* DnaJ and GrpE cooperate with *E. coli* ClpB (*Ec*ClpB) and *P. aeruginosa* ClpB (*Pa*ClpB) in the refolding of heat-aggregated Malate Dehydrogenase (MDH). Negative: no addition of disaggregating chaperones. All reactions were performed in the presence of GroES/EL. (B) ClpG exhibits comparable disaggregating activity to $ClpG_{GI}$. Refolding of heat-aggregated luciferase was monitored in presence of ClpG or $ClpG_{GI}$. After 90 min of luciferase disaggregation ClpG activity corresponds to 90% of the activity of $ClpG_{GI}$. (C) Refolding of aggregated luciferase was monitored in absence or presence of *Pa*DnaK-*Ec*DnaJE(*Pa*K-JE) at 0 and 0.5 μM of $ClpG_{GI}$.

(D) Total intensities of protein bands below $ClpG_{GI}$ were quantified by ImageJ for each lane of the SDS-PAGE (see Fig. 3D). *, $p<0.05$; **, $p<0.01$.

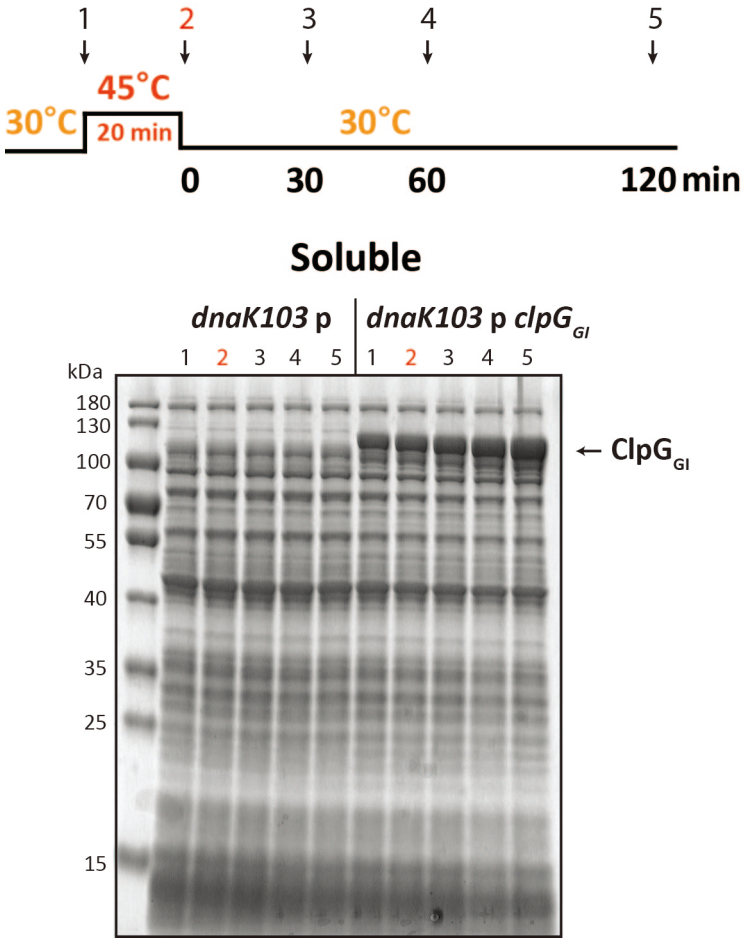


Fig. S11 Soluble fraction of *E. coli dnaK103* mutant cells containing an empty vector (p) or expressing *clpG*$_{GI}$ (p*clpG*$_{GI}$). Cells were grown at 30°C in the presence of 250 μM IPTG and shifted to 45°C for 20 min followed by a recovery period at 30°C. Soluble protein fractions (Fig. 4E) were isolated at the indicated time points and analyzed by SDS-PAGE. p=pUHE21; p *clpG*$_{GI}$=*clpG*$_{GI}$ cloned in pUHE21.

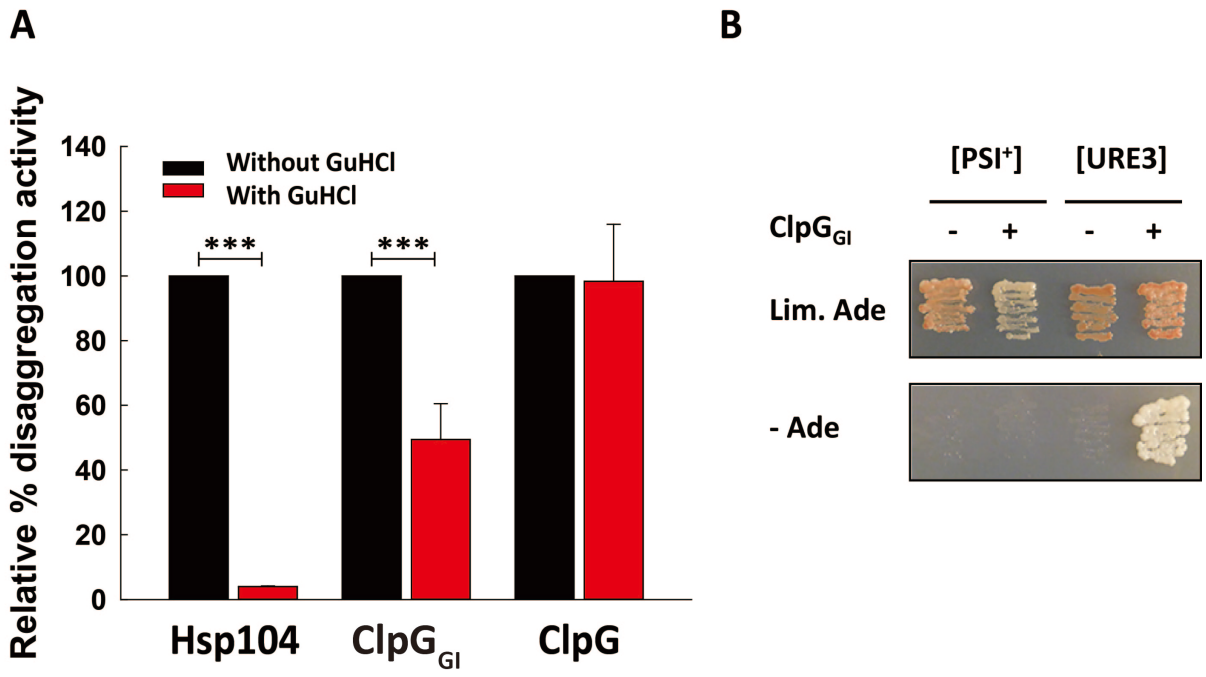


Fig. S12 Effect of guanidine hydrochloride (GuHCl) on Hsp104, ClpG and ClpG$_{GI}$ and the prion phenotype in *Saccharomyces cerevisiae* upon expression of ClpG$_{GI}$. (A) Disaggregation and refolding of urea-denatured luciferase was determined for Hsp104 (in presence of cooperating yeast Hsp70 system: Ssa1/Ydj1/Sse1) and ClpG/ClpG$_{GI}$ in absence and presence of 5 mM GuHCl. Disaggregation activities of Hsp104, ClpG and ClpG$_{GI}$ determined in absence of GuHCl were set at 100% (***, p<0.001). (B) *S. cerevisiae* 779-6A and 1075 cells (without or with a ClpG$_{GI}$-expression plasmid), reporting on [PSI+] and [URE3] prion phenotypes, respectively, were grown in 3 mM guanidine hydrochloride to inhibit Hsp104 activity. The prion states after Hsp104 inhibition were monitored on plates with limiting adenine medium (Lim. Ade) and on adenine depletion medium (-Ade).

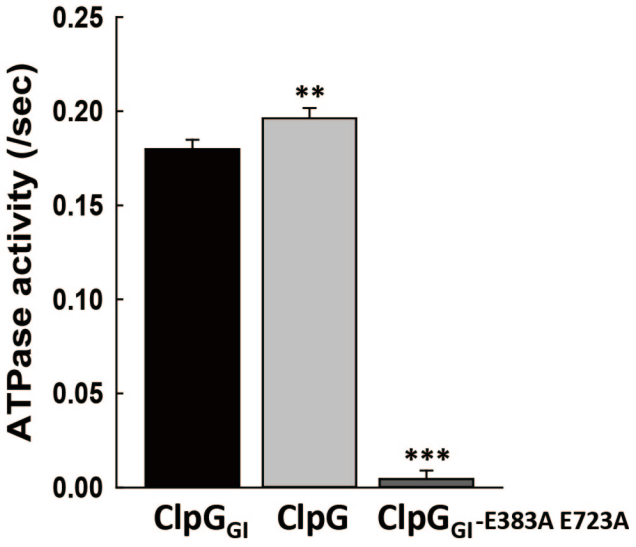


Fig. S13 Basal ATPase activities of *P. aeruginosa* SG17M ClpG$_{GI}$ and its double Walker B mutant. ClpG$_{GI}$ exhibits similar basal ATPase activity as ClpG, while the double Walker B mutant ClpG$_{GI\ E383A\ E723A}$ has lost the ATPase activity (**, p<0.01; ***, p<0.001; compared to ClpG$_{GI}$).

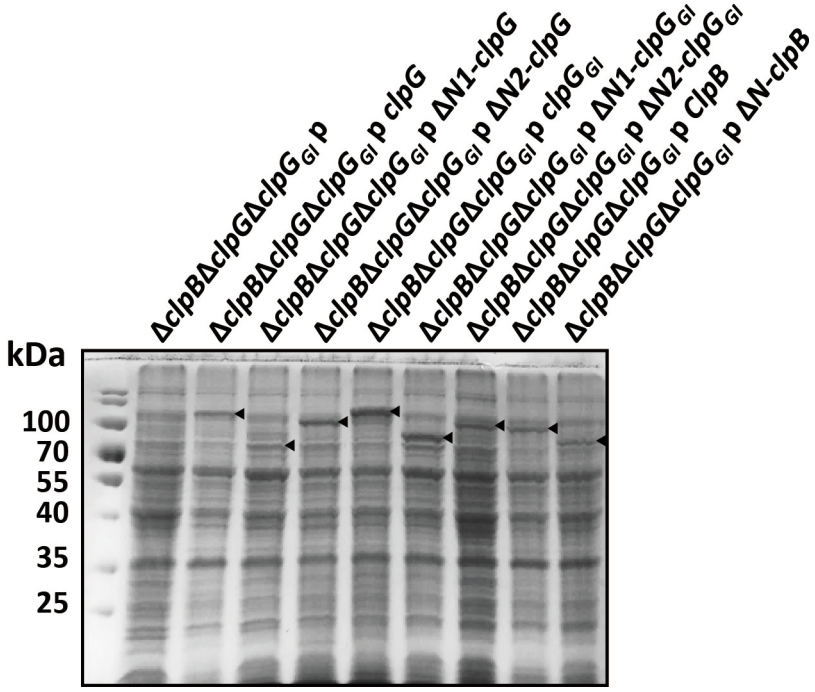
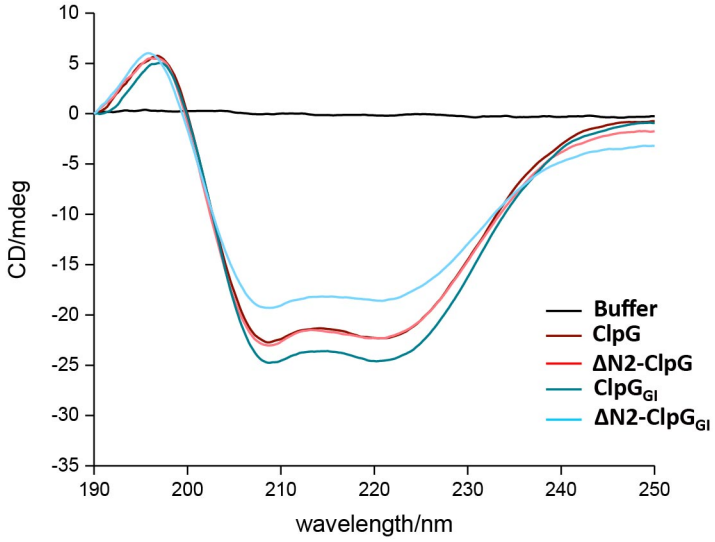


Fig. S14 Expression levels of ClpB, ClpG and ClpG$_{GI}$ and respective N-terminal deletion variants. *P. aeruginosa* SG17M Δ*clpB* Δ*clpG* Δ*clpG$_{GI}$* mutant cells containing pJN105 plasmid derivatives expressing full-length or N-terminally shortened ClpB, ClpG, ClpG$_{GI}$ were subjected to SDS-PAGE analysis. Full-length and truncated ClpB, ClpG and ClpG$_{GI}$ are marked by arrows. p=pJN105.



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159 **Fig. S15 Secondary structure analysis of ClpG, ΔN2-ClpG, ClpG$_{GI}$ and ΔN2-ClpG$_{GI}$.**
160 Circular Dichroism Spectra were recorded from 250-190 nm at a protein concentration of 2.5
161 µM in sodium phosphate buffer (50 mM, pH 7.5). The buffer control spectrum is also given
162 and shown in black. ClpG/ΔN2-ClpG are shown in brown/red and ClpG$_{GI}$/ΔN2-ClpG$_{GI}$ are
163 shown in green/blue.

164

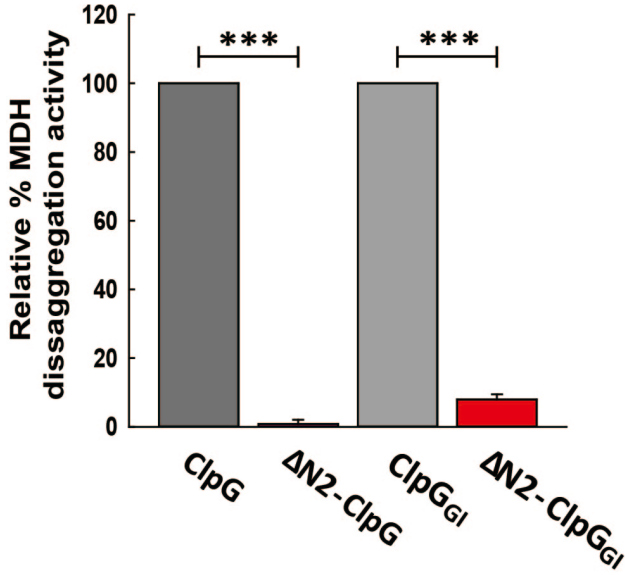

Fig. S16 MDH disaggregation activities of ClpG/ClpG$_{GI}$ and respective ΔN2 deletion mutants. Refolding of heat-aggregated MDH was monitored in the presence of indicated chaperones. The activity of respective full-length proteins was set as 100% (***, p<0.001).

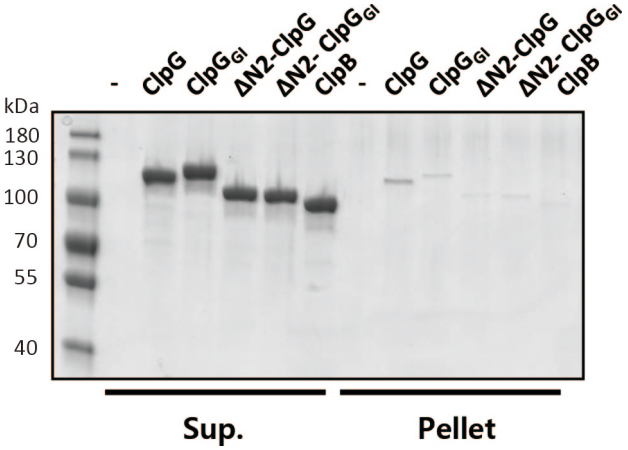

Fig. S17 Solubilities of ClpG, ΔN2-ClpG, ClpG$_{GI}$ and ΔN2-ClpG$_{GI}$ and ClpB. Indicated Hsp100 proteins were incubated with 2 mM ATPγS in the absence of heat-aggregated luciferase and soluble (Sup) and insoluble (Pellet) fractions were isolated and analyzed by SDS-PAGE.

Supporting Tables

Table S1. Identification of *in vivo* aggregation-enhanced proteins in *P. aeruginosa* SG17M

Name	Size (kDa)	Genbank accession number	Function	Strain background where the aggregate was observed
HtpG	90	WP_004349034.1	Hsp90 family chaperone	Δ*clpB* Δ*clpG* Δ$clpG_{GI}$
NuoCD	70	WP_003090458.1	NADH-ubiquinone oxidoreductase, subunit CD	Δ$clpG_{GI}$
IbpA	15	WP_003091405.1	Small heat shock protein 20	Δ*clpB* Δ*clpG* Δ$clpG_{GI}$
LasI	23	WP_024082598.1	Quorum sensing protein	Δ*clpB*

Table S2. Bacterial strains used in this study

Strain	Genotype	Source or reference
P. aeruginosa **SG17M and derivatives**		
SG17M	Wild type, Environment, river water	(1, 2)
SG17M002	SG17M Δ*dna-shsp20*$_{GI}$-*clpG*$_{GI}$ (previously SG17M Δ*dna-shsp20c-clpBc*)	(3)
SG17M008	SG17M Δ*clpG*	This study
SG17M009	SG17M Δ*clpG*$_{GI}$	This study
SG17M010	SG17M Δ*clpB*	This study
SG17M011	SG17M Δ*clpB* Δ*clpG*	This study
SG17M012	SG17M Δ*clpB* Δ*clpG*$_{GI}$	This study
SG17M013	SG17M Δ*clpB* Δ*clpG* Δ*clpG*$_{GI}$	This study
SG17M014	SG17M *attTn7*	This study
SG17M015	SG17M Δ*clpG* Δ*clpG*$_{GI}$ *attTn7*::*araC-clpG*	This study
SG17M016	SG17M Δ*clpG* Δ*clpG*$_{GI}$ *attTn7*::*araC-clpG*$_{GI}$	This study
SG17M017	SG17M Δ*clpB* Δ*clpG* Δ*clpG*$_{GI}$ *attTn7*::*araC-clpG*	This study
SG17M018	SG17M Δ*clpB* Δ*clpG* Δ*clpG*$_{GI}$ *attTn7*::*araC-clpG*$_{GI}$	This study
SG17M019	SG17M Δ*clpP*	This study
SG17M020	SG17M Δ*clpG* Δ*clpG*$_{GI}$ Δ*clpP*	This study
SG17M021	SG17M Δ*clpP attTn7*	This study
SG17M022	SG17M Δ*clpG* Δ*clpG*$_{GI}$ Δ*clpP attTn7*::*araC-clpG*	This study
SG17M023	SG17M Δ*clpG* Δ*clpG*$_{GI}$ Δ*clpP attTn7*::*araC-clpG*$_{GI}$	This study
Other ***P. aeruginosa*** **strains**		
PAO1	Wild type, genetic reference strain, burn wound isolate, Australia, 1953	(4)
PAO1-001	PAO1 Δ*clpB*	This study
PAO1-002	PAO1 Δ*clpG*	This study
PAO1-003	PAO1 Δ*clpB* Δ*clpG*	This study
E. coli **K-12 derivatives**		
TOP10	Strain for cloning and harboring plasmids	Invitrogen
HB101 pRK2013	Helper strain for conjugation, Km^R	(5)
DH5α λpir	Cloning and harboring plasmids of R6K origin of replication	Lab collection
DH5α λpir pTNS2	Helper strain for chromosomal integration of mini-*Tn7* element, Ap^R	(6)
MC4100	*In vivo* heat shock and chaperone assay, MC4100 placIq pHSG-YFP-Luciferase	(7)
MC4100 Δ*clpB*	*In vivo* heat shock and chaperone assay, MC4100 △*clpB*::Km placIq pHSG-YFP-Luciferase	(7)
MC4100 *dnaK103*	*In vivo* heat shock, temperature-sensitive *dnaK* mutant	(8)
Yeast		
779-6A	*MATα*, *kar1-1*, *SUQ5*, *ade2-1*, *his3Δ202*, *leu2Δ1*, *trp1Δ63*, *ura3-52*	(9)
1075	*MATα*, *kar1-1*, *P*$_{DAL5}$*::ADE2*, *his3Δ202*, *leu2Δ1*, *trp1Δ63*, *ura3-52*	(9)

JG26	*Saccharomyces cerevisiae* InvSc1strain for yeast cloning	Invitrogen

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183

184 **Table S3. Plasmids used in this study**

Plasmid	Description	Source or reference
pEX18Tc	Gene replacement vector; ColE1 origin of replication, Tc^R, *oriT*$^+$, *sacB*$^+$	(10)
pSG001	pEX18Tc containing FRT-Gm^R-FRT cassette cloned in *Bam*HI and *Sal*I site	(3)
pSG010	pSG001-Δ*clpB*, construct for *clpB* deletion with fragment up and downstream of *clpB* cloned in *Sac*I/*Bam*HI and *Pst*I/*Hin*dIII	This study
pSG011	pSG001-Δ$clpG_{GI}$, construct for $clpG_{GI}$ deletion with fragment up- and downstream of $clpG_{GI}$ cloned in *Sac*I/*Bam*HI and *Sal*I/*Hind*III	This study
pMQ150	Vector for yeast cloning and gene replacement; Km^R (selection in *E. coli*), URA3 (selection in yeast)	(11)
pSG013	pMQ150-Δ*clpP*, construct for *clpP* deletion, Gm cassette flanked by fragment up and downstream of *clpP*	This study
pSG014	pMQ150-Δ*clpG*, construct for *clpG* deletion, Gm cassette flanked by fragment up and downstream of *clpG*	This study
pMR116	Expression vector in yeast, Ap^R, TRP1 (selection in yeast)	(12)
pSG015	pMR116 with C-terminal 6xHis tag fusion to $clpG_{GI}$ cloned in *Nhe*I and *Xba*I sites	This study
pUC18T-mini-Tn7T-Gm*Tn7*	Genome insertion vector; ColE1 origin of replication, Ap^R, Gm^R	(6)
pTNS2	Helper plasmid for chromosomal integration of mini-Tn*7* element; Ap^R	(6)
pSG016	pUC18T-mini-Tn7T-Gm with *araC-clpG* cloned in *Eco*RI and *Kpn*I sites	This study
pSG017	pUC18T-mini-Tn7T-Gm with *araC*-$clpG_{GI}$ cloned in *Spe*I and *Kpn*I sites	This study
pJN105	A broad-host range vector with arabinose inducible *araBAD* promoter; pBBR1origin of replication; Gm^R	(13)
pSG018	*ClpB* -6xHis cloned in pJN105 in *Nhe*I and *Xba*I sites	This study
pSG019	*ClpG* -6xHis cloned in pJN105 *Nhe*I and *Xba*I sites	This study
pSG020	$ClpG_{GI}$-6xHis cloned in pJN105 in *Nhe*I and *Xba*I sites	This study
pSG021	*P. aeruginosa dnaK* -6xHis cloned in pJN105 in *Nhe*I and *Xba*I sites	This study
pSG022	*P. aeruginosa* 6xHis-*ibpA* cloned in pJN105 in *Nhe*I and *Xba*I sites	This study
pSG007	6xHis-$shsp20_{GI}$ cloned in pJN105 in *Nhe*I and *Xba*I sites	(3)
pSG023	$shsp20_{GI}$ $clpG_{GI}$-6xHis cloned in pJN105 in *Nhe*I and *Xba*I sites	This study
pSG027	Δ*N(V149M) clpB*-6xHis cloned in *Nhe*I and *Xba*I sites	This study
pSG028	Δ*N(V83M) clpG*-6xHis cloned in pJN105 in *Nhe*I and *Xba*I sites	This study
pSG029	Δ*N(R234M) clpG*-6xHis cloned in pJN105 in *Nhe*I and *Xba*I sites	This study
pSG030	Δ*N(S106M)* $clpG_{GI}$-6xHis cloned in pJN105 in *Nhe*I and *Xba*I sites	This study
pSG031	Δ*N(R255M)* $clpG_{GI}$-6xHis cloned in pJN105 in *Nhe*I and *Xba*I sites	This study
pSG032	pJN105 with C-terminal 6xHis fusion to N-terminal region of *clpB* (M1 to V149) cloned in *Nhe*I and *Xba*I sites	This study
pSG033	pJN105 with C-terminal 6xHis fusion N-terminal region of *clpG* (M1 to A82) cloned in *Nhe*I and *Xba*I sites	This study
pSG034	pJN105 with C-terminal 6xHis fusion N-terminal region of *clpG* (M1 to R234) cloned in *Nhe*I and *Xba*I sites	This study
pSG035	pJN105 with C-terminal 6xHis fusion N-terminal region of $clpG_{GI}$ (M1 to G105) cloned in *Nhe*I and *Xba*I sites	This study
pSG036	pJN105 with C-terminal 6xHis fusion N-terminal region of $clpG_{GI}$ (M1 to R255) cloned in *Nhe*I and *Xba*I sites	This study
pSG037	pJN105 $clpG_{GI\ E383A\ E723A}$ 6xHis cloned in *Nhe*I and *Xba*I sites	This study
pFLP2	FRT cassette excision vector, Tc^R	(14)
pRK2013	Helper plasmid for mobilization of non-self-transmissible plasmids, Km^R	(5)
pUHE21	Vector for IPTG-inducible gene expression in *E. coli*	(8)
pUHE21-*dnaK*	Vector for IPTG-inducible expression of *dnaK* in *E. coli*	(8)
pUHE21-$clpG_{GI}$	Vector for IPTG-inducible expression of $clpG_{GI}$ in *E. coli*, cloned into *BamH*I and *Xba*I sites	This study
pET24a-clpG_{GI}	Vector for IPTG-inducible expression of $clpG_{GI}$ in *E. coli* BL21 cloned into *Nde*I and *Xho*I sites	This study
pET24a-c*lpG*	Vector for IPTG-inducible expression of *clpG* in *E. coli* BL21 cloned into *Nde*I and *Xho*I sites	This study
pET24a-*ΔN2*-clpG_{GI}	Vector for IPTG-inducible expression of Δ*N2*-clpG_{GI} in *E. coli* BL21 cloned into *Nde*I and *Xho*I sites	This study

pET24a-*ΔN2-clpG*	Vector for IPTG-inducible expression of Δ*N2*-c*lpG* in *E. coli* BL21 cloned into *Nde*I and *Xho*I sites	This study

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187 **Table S4. Primers used in this study**

Primer	Sequence[1]	Purpose	Source or reference
clpB Up F SacI	5’ GGAGCTCGACTGAGCTAACGACCCAAG 3’	Construction of a *clpB*-deletion mutant (upstream region)	This study
clpB Up R BamHI	5’ CGCGGATCCGTTCGGCACAGATCCAGGAGCTG 3’	Construction of a *clpB*-deletion mutant (upstream region)	This study
clpB Dn F PstI	5’ GCGCTGCAGAGCTTGCTGGTCAAACGGTCTATTC 3’	Construction of a *clpB*-deletion mutant (downstream region)	This study
clpB Dn R HindIII	5’ CGCAAGCTTCTGCTCAGCCTGTTGCGCCAGG 3’	Construction of a *clpB*-deletion mutant (downstream region)	This study
$clpG_{GI}$ Up F SacI	5’ GGAGCTCCTTGCAAGAACTTCCTCAGG 3’	Construction of a $clpG_{GI}$-deletion mutant (upstream region)	This study
$clpG_{GI}$ Up R BamHI	5’ CGCGGATCCAGACTTGGCATTGTTTTCTGGC 3’	Construction of a $clpG_{GI}$-deletion mutant (upstream region)	This study
$clpG_{GI}$ Dn F SalI	5’ GCGGTCGACAAGTCAGCGGGCGGCGAATCT 3’	Construction of a $clpG_{GI}$-deletion mutant (downstream region)	This study
$clpG_{GI}$ Dn R HindIII	5’ CGCAAGCTTCACGAAGTAAGGTGTCCAGATG 3’	Construction of a $clpG_{GI}$-deletion mutant (downstream region)	This study
ClpP Up F	5’ GTTGTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGGCTGGAAGACATCAAGGTCGAG 3’	Construction of a *clpP*-deletion mutant (yeast cloning)	This study
ClpP Up R	5’ AAGAGTTGCGAGACATGTCTTG 3’	Construction of a *clpP*-deletion mutant (yeast cloning)	This study
ClpP Gm F	5’ TCTGTTAGGG AGTGATCGCA AGACATGTCT CGCAACTCTTCGAATTAGCTTCAAAAGCGCTCTGA 3’	Construction of a *clpP*-deletion mutant (yeast cloning)	This study
ClpP Gm R	5’ CTTGGTCGGGTCACTTGGCCTTCGGCTTGTCGGCCTTGCCCGAATTGGGGATCTTGAAGTTCCT 3’	Construction of a *clpP*-deletion mutant (yeast cloning)	This study
ClpP Dn F	5’ GACCTGGCCGTCTAAGGCTC 3’	Construction of a *clpP*-deletion mutant (yeast cloning)	This study
ClpP Dn R	5’ CAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCGGGCACTTCGGCATTGAAACCGATG 3’	Construction of a *clpP*-deletion mutant (yeast cloning)	This study
ClpG Up F	5’ GTTGTAAAACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGGCTTCCAGGGCGATGGAGATCAG 3’	Construction of a *clpG*-deletion mutant (yeast cloning)	This study
ClpG Up R	5’ AGATAGCGCAAAGTTCCTGGGC 3’	Construction of a *clpG*-deletion mutant (yeast cloning)	This study
ClpG Gm F	5’ GA AACAGGAGTCCTCCCATGGCCCAGGAACTTTGCGCTATCTCGAATTAGCTTCAAAAGCGCTCTGA 3’	Construction of a *clpG*-deletion mutant (yeast cloning)	This study
ClpG Gm R	5’ CTTGGTCGGGTCACTTGGCCTTCGGCTTGTCGGCCTTGCCCGAATTGGGGATCTTGAAGTTCCT 3’	Construction of a *clpG*-deletion mutant (yeast cloning)	This study
ClpG Dn F	5’ GGCAAGGCCGACAAGCCGAAG 3’	Construction of a *clpG*-deletion mutant (yeast cloning)	This study
ClpG Dn R	5’ CAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCGGGGTGATCGTCGACATCCAGAC 3’	Construction of a *clpG*-deletion mutant (yeast cloning)	This study
pMQ150 F	5’ CTGGCGAAAGGGGGATGTG 3’	Confirmation of positive clones of pMQ105	This study
pMQ150 R	5’ GTTGTGTGGAATTGTGAGCGG 3’	Confirmation of positive clones of pMQ105	This study
ΔClpB confirm F	5’ GCCAACTGCTCTACCGACTG 3’	Confirmation of the *clpB*-deletion mutant	This study
ΔClpB confirm R	5’ CACATGAGCCATATCGGCTATC 3’	Confirmation of the *clpB*-deletion mutant	This study
ΔClpG confirm F	5’ GAACAGGTTGCCGAGGATGC 3’	Confirmation of the *clpG*-deletion mutant	This study
ΔClpG confirm R	5’ CAGTCGCCCTATCGCCACCTG 3’	Confirmation of the *clpG*-deletion mutant	This study
Δ$clpG_{GI}$ confirm F	5’ GACTTTCGGCATGTTAGGCG 3’	Confirmation of the $clpG_{GI}$-deletion mutant	This study
Δ$clpG_{GI}$ confirm R	5’ CGAAGTAAGGTGTCCAGATG 3’	Confirmation of the $clpG_{GI}$-deletion mutant	This study

ΔClpP confirm F	5’ CGATGAGTGTCATTCGTCAG 3’	Confirmation of the *clpP*-deletion mutant	This study
ΔClpP confirm R	5’ TCCGGACGGAATTCGAGATC 3’	Confirmation of the *clpP*-deletion mutant	This study
PglmS-up	5’ CTGTGCGACTGCTGGAGCTGA 3’	Confirmation of the genome integration	(15)
PglmS-down	5’ GCACATCGGCGACGTGCTCTC 3’	Confirmation of the genome integration	(15)
ClpB pJN F NheI	5’ GCAGCTAGCCTATAGAAGGAAGGACGACC 3’	Cloning *clpB*-6xHis for expression	This study
ClpB-His pJN R XbaI	5’ CGCTCTAGATCAGTGATGATGATGATGATGGGCGAAGACGATCTCGTCGC 3’	Cloning *clpB*-6xHis for expression	This study
ClpG pJN F NheI	5’ GCAGCTAGCCCTTCCGTGGAAACAGGAGTC 3’	Cloning *clpG*-6xHis for expression	This study
ClpG-His pJN R XbaI	5’ CGCTCTAGATCAGTGATGATGATGATGATGCTTGGCCTTCGGCTTGTCG 3’	Cloning *clpG*-6xHis for expression	This study
$sHsp20_{GI}$ pJN F NheI	5’ GCAGCTAGCCTTCTGGAGGTGTGACATGGA 3’	Cloning $shsp20_{GI}$-$clpG_{GI}$-6xHis for expression	This study
$ClpG_{GI}$ pJN F NheI	5’ CGCGGCTAGCCTTTACAGGAGCATCAGCATGGCCA 3’	Cloning $clpG_{GI}$-6xHis for expression	This study
$ClpG_{GI}$-His pJN R XbaI	5’ GCATCTAGATCAGTGATGATGATGATGATGAGATTCGCCGCCCGCTGACTTC 3’	Cloning $clpG_{GI}$-His/$shsp20_{GI}$-$clpG_{GI}$-6xHis for expression	This study
DnaK pJN F NheI	5’ GCAGCTAGCCAAAGTTTCTGGAGAGTGAAT 3’	Cloning *dnaK*-6xHis for expression	This study
DnaK-His pJN R XbaI	5’ CGCTCTAGATTAGTGATGATGATGATGATGCTTGTTGTCCTTGACCTCTTC 3	Cloning *dnaK*-6xHis for expression	This study
ClpB V149M pJN F NheI	5’ GCAGCTAGCCTATAGAAGGAAGGACGACCCATGAACGACCCGAACGTCGAGGAGTC 3’	Cloning ΔN-*clpB* for expression	This study
ClpG R234M pJN F NheI	5’ GCAGCTAGCCCTTCCGTGGAAACAGGAGTCCTCCCATGGTGGACGGCCCGAGCAACACTC 3’	Cloning ΔN1-*clpG* for expression	This study
ClpG V83M pJN F NheI	5’ GCAGCTAGCCCTTCCGTGGAAACAGGAGTCCTCCCATGGACATCGCCGAGTACTTCAG 3’	Cloning ΔN2-*clpG* for expression	This study
$ClpG_{GI}$ R255M pJN F NheI	5’ CGCGGCTAGCCTTTACAGGAGCATCAGCATGGCCGAGACGCCGACCAACAC 3’	Cloning ΔN1-$clpG_{GI}$ for expression	This study
$ClpG_{GI}$ S106M pJN F NheI	5’ CGCGGCTAGCCTTTACAGGAGCATCAGCATGGGGCTCGCCAGCCGTATCAG 3’	Cloning ΔN2-$clpG_{GI}$ for expression	This study
ClpB V149-His pJN R XbaI	5’ CGCTCTAGATCAGTGATGATGATGATGATGCACCGCTTCGCCGCCACGCAG 3’	Cloning N-term of *clpB* for expression	This study
ClpG R234-His pJN R XbaI	5’ CGCTCTAGATCAGTGATGATGATGATGATGGCGGCCGTCCTCGGCGCCCT 3’	Cloning N-term of *clpG* for expression	This study
$ClpG_{GI}$ R255-His pJN R XbaI	5’ CGCTCTAGATCAGTGATGATGATGATGATGGCGGCCATCCTCGGCCCCTT 3’	Cloning N-term of $clpG_{GI}$ for expression	This study
araC pJN F EcoRI	5’ GCGAAGCTTGATTCGTTACCAATTATGAC 3’	Amplifying *araC* and *araBAD* promoter	This study
araC pJN F SpeI	5’ GCGCACTAGTGATTCGTTACCAATTATGAC 3’	Amplifying *araC* and *araBAD* promoter	This study
ClpG-His mini R KpnI	5’ CGCGGTACCTCAGTGATGATGATGATGCTTGGCCTTCGGCTTGTCG 3’	Cloning *araC*-*clpG*-6xHis for single copy complementation	This study
$ClpG_{GI}$-His mini R KpnI	5’ CGCGGTACCTTAGTGATGATGATGATGAGATTCGCCGCCCGCTGACTTC 3’	Cloning *araC*-$clpG_{GI}$-6xHis for single copy complementation	This study
$ClpG_{GI}$ pMR F EcoRI	5’ TATATTGAATTCATGGCCAGAAAACAATGCCAAG 3’	Cloning $clpG_{GI}$-6xHis into pMR116 vector for yeast	This study
$ClpG_{GI}$-His pMR R XhoI	5’ GCGCCTCGAGTTAGTGATGATGATGATGATGAGATTCGCCGCCCGCTGACTTC 3’	Cloning $clpG_{GI}$-6xHis into pMR116 vector for yeast	This study
$clpG_{GI}$-BamHI-A	5’ GGCCATGGATCCATGGCCAGAAAACAATGCCAAGTCTGC 3’	Cloning $clpG_{GI}$ into pUHE21 for expression in *E. coli*	This study
$clpG_{GI}$-XbaI-B	5’ GGCCATTCTAGATCAAGATTCGCCGCCCGCTGACTTCTTC 3’	Cloning $clpG_{GI}$ into pUHE21 for expression in *E. coli*	This study
$ClpG_{GI}$ E383A F	5’ GATTCTCTTCATCGACGCCGTGCACACCATCGTCG 3’	Amino acid replacement E383A in $ClpG_{GI}$	This study

$ClpG_{GI}$ E383A R	5’ CGACGATGGTGTGCACGGCGTCGATGAAGAGAATC 3’	Amino acid replacement E383A in $ClpG_{GI}$	This study
$ClpG_{GI}$ E723A F	5’ CGTGTTGCTGCTGGACGCCATCGAGAAGGCTCACC 3’	Amino acid replacement E723A in $ClpG_{GI}$	This study
$ClpG_{GI}$ E723A R	5’ GGTGAGCCTTCTCGATGGCGTCCAGCAGCAACACG 3’	Amino acid replacement E723A in $ClpG_{GI}$	This study

[1] Restriction sites are underlined

Supporting experimental procedures

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 tables S2–S4. If not otherwise specified, cells were grown in LB medium at 37°C containing appropriate antibiotics with agitating speed 200 rpm. For *P. aeruginosa* SG17M, gentamicin (Gm) was used at 30 μg ml^{-1} and tetracycline (Tc) at 70 μg ml^{-1}. *Escherichia coli* TOP10 was used for cloning and retaining of plasmids requiring Gm at 30 μg ml^{-1}, Tc at 20 μg ml^{-1}, kanamycin (Km) at 25 μg ml^{-1} and ampicillin (Ap) at 100 μg ml^{-1} for plasmid propagation.

Mutant construction

All primers are described in supplemental table S4. Briefly, in-frame-deletion mutants were constructed in *E. coli* TOP10 by cloning flanking regions of the target genes into pSG001 (Table S3), a derivative of pEX18Tc harboring a FLP-excisable Gm cassette (3). Constructed vectors were introduced into SG17M by triparental mating aided by *E. coli* HB101 pRK2013. Selection was on citrated-based Vogel-Bonner minimal medium (VBMM) containing Gm (16).

The gene replacement vectors to construct *clpP* and *clpG* in-frame-deletion mutants were assembled by yeast cloning. The flanking regions of target genes and the Gm cassette were amplified by specific primers (Table S4) which overlap with vector pMQ150 and each other for subsequent recombination in yeast strain JG26 (Table S2) (10, 11). The suicide vector pMQ150 was digested by *Bam*HI and *Sal*I. Restricted vector and all three amplified fragments were transformed into JG26 using the lithium acetate method (17). Positive colonies were screened by colony PCR with primers pMQ150 F and pMQ150 R (Table S4). Recombinant plasmids from positive colonies were isolated by Zymoprep™ Yeast Plasmid Miniprep II kit (Zymo Research), transformed into DH5α λpir, which was subsequently used as the conjugal donor strain. The final constructs were verified by sequencing.

To identify double crossover events, Tc^S and Gm^R conjugants were selected and streaked for single colony. The pFLP3 plasmid was used for Flp-mediated excision of the Gm marker following established procedures (16). Deletion mutants were confirmed by PCR using primers outside of the region of potential homologous recombination.

Bioinformatic analysis

Protein sequences of ClpG (acc. no.: EWH25562), $ClpG_{GI}$ (acc. no.: EWH27925) and ClpB (acc. no.: EWH24017) of *P. aeruginosa* SG17M (3) were used as queries to search ClpG, $ClpG_{GI}$ and ClpB homologs in NCBI databases by BLASTP using standard parameters. *Cupriavidus necator* (ClpG, WP_013952998; ClpB, WP_013957113; 16s rRNA, AF191737), *Ralstonia* sp. (ClpG, WP_010809302; ClpB, SDO57215; 16s rRNA, AY741342), *Paraburkholderia fungorum* (ClpG, WP_028194728; ClpB, WP_074763873; 16s rRNA, AF215705), *Burkholderia territorii* (ClpG, KWA28617; ClpB, WP_060105492; 16s rRNA, LK023503), *P. citronellolis* (ClpG, AMO76619; ClpB, WP_061560717; 16s rRNA, Z76659), *P. fluorescens* (ClpG, WP_031318856; ClpB, KTC62816; 16s rRNA, D84013), *P. mandelii*

(ClpG, WP_042932128, ClpB, SDU43686; 16s rRNA, AF058286), *E. coli* (ClpG, WP_063856138; ClpB, ESE24594; 16s rRNA, X80725), *Serratia* sp. (ClpG, WP_006327187; ClpB, WP_006323719; 16s rRNA, AJ233431), *P. aeruginosa* (16s rRNA, HE978271), *I. limosus* (ClpG, WP_026873947; ClpB, WP_026872601; 16s rRNA, AY043374), *Paracoccus versutus* (ClpG, WP_036750696; ClpB, WP_036753552; 16s rRNA, AY014174), *Chthonomonas calidirosea* (ClpG, CEK12415; ClpB, WP_016481690; 16s rRNA, AM749780), *Candidatus Acetothermus autotrophicum* (ClpG, BAL59480; ClpB, BAL58994; 16s rRNA, AP011801), *Dictyoglomus turgidum* (ClpG, WP_012583264; ClpB, WP_012583832; 16s rRNA, NR_074885), *Anaeromyxobacter* sp. (ClpG, ABS27505; ClpB, WP_041448603; 16s rRNA, NR_074927) are the Acc. No. of protein sequences used in Fig. 1C. The CDART database was searched for proteins with identical domain structure. Proteins homologous over the entire length of the sequence (>93%) were considered. These database searches retrieved ClpG homologs (including $ClpG_{GI}$ members) with an identity/homology down to 63/80%. There was a clear distinction to the next class of proteins, which showed identity of 53 % or less over <90% of protein length. Within their subgroup, $ClpG_{GI}$ proteins were as low as 92/94% identical/homologous. On most circumstances, not more than one protein homolog from each genus was selected and most distant members were considered. Proteins were aligned using ClustalX2 using standard parameters (18). Representative protein sequences of experimentally characterized class I Clp proteins, ClpV, ClpA, ClpL, ClpD, ClpE and ClpC were included. The aligned sequences were subjected to phylogenetic analysis by applying neighbor-joining, maximum likelihood and maximum parsimony method in MEGA7.0 (19) with essentially the same results. The protein distance matrices were calculated by using the PROTDIST program from the PHYLIP package, with the Dayhoff PAM matrices/Poisson as amino acid replacement models. The tree was drawn with the Treeview program. The robustness of the phylogenetic tree topologies was evaluated by bootstrap analysis with 1000 replications in MEGA7.0.

Plasmid construction and single-copy complementation

Genes were cloned into pJN105 with a 6xHis tag at the C terminal end (Table S3, 4) (13). Subsequently, *clpG* open reading frames including *araC* and *araC*-promoter were amplified (primers described in Table S4) and cloned into the *Bam*HI and *Kpn*I site of the multiple cloning site of the pUC18T-mini-Tn*7*T-Gm vector for single copy complementation on the chromosome. The vector and pTNS2 helper plasmid encoding the TnsABCD transposase subunits were co-conjugated into SG17M aided by *E. coli* HB101 containing pRK2013 (Table S2). Gm^R conjugants were selected and chromosomal integration was confirmed by PCR with primers PglmS-up and PglmS-down (Table S4). Excision of the Gm marker was obtained with the pLFP3 plasmid (16). The double Walker B mutant $ClpG_{GI\ E383A\ E723A}$ was constructed by site-directed mutagenesis (Table S2, S4). The double Walker B mutant of the ClpB protein binds to ATP and forms hexamers but does not hydrolyze ATP (20).

Heat shock tolerance assay

P. aeruginosa cells incubated in LB broth with shaking at 200 rpm at 20°C and 37°C were harvested from logarithmic (OD_{600} = 0.7) and stationary (OD_{600} = 2.5) phase. For

examination of the impact of the N-terminal region of ClpB and ClpG proteins, cells harboring the cloned gene on pJN105 were grown at 37°C for 24 h in LB broth and 0.1% L-arabinose was added at 6 h. *E. coli* K-12 strains were grown at 30°C and 0.1% L-arabinose or 100 μM IPTG was added at OD_{600} = 0.2 with heat treatment at OD_{600} = 0.6. A cell suspension of 500 μl was exposed to 50°C for 10, 30 and 60 min and a control cell suspension was kept on ice. Cell viability after exposure to the lethal heat shock was determined by the spotting assay. 5 μl of 10-fold dilutions were spotted onto a LB plate. Growth was assessed after 12–14 h of incubation at 37°C or 24 h at 30°C.

Production of antisera

Polyclonal antibodies were raised against purified *P. aeruginosa* ClpB, ClpG and $ClpG_{GI}$ in rabbits by Davids Biotechnologie GmbH.

Western blot analysis

As for heat shock analysis, *P. aeruginosa* cells incubated in LB broth with shaking at 20°C were harvested from logarithmic (OD_{600} = 0.7) and stationary (OD_{600} = 2.5) phase. Cell lysates with equal protein content, as assessed by Coomassie brilliant blue staining of protein gels, were subjected to SDS-PAGE (4% stacking and 10% separating gel) and subsequently blotted onto polyvinylidene difluoride membrane (Millipore). As loading control, the membrane was stained with Ponceau S and then incubated in blocking solution (1 × Tris-buffered saline (TBS) pH 7.4, 0.01% Tween 20, 5% skim milk powder) overnight at 4°C. After washing, ClpG, $ClpG_{GI}$ and ClpB were detected with anti-ClpG, anti-$ClpG_{GI}$ and anti-ClpB antiserum at 1:500 000, 1:1 000 000 and 1:2 000 000 dilution, respectively, and with 1:4 000 goat anti-rabbit secondary antibody (Jackson Immuno Research, UK). Bound antibody was visualized with enhanced chemiluminescence (ECL) detection reagent (Roche). Purified proteins were used as a concentration control. Detection of protein production was performed at least twice in independent experiments.

Protein purification

Full length C-terminal 6xHis-tagged ClpB, DnaK, ClpG, $ClpG_{GI}$ and deletion variants of ClpB, ClpG and $ClpG_{GI}$ were expressed from pJN105 (Table S3) (13) in *P. aeruginosa* SG17M derivatives or in *E. coli* BL21 cells. SG17M derivatives or *E. coli* BL21 were grown to mid-exponential phase (OD_{600} = 0.5) in LB broth at 37°C (exceptional, ClpG harboring strain was grown at 42°C). Protein expression was induced by adding 0.1% L-arabinose or 500 μM IPTG (in case of *E. coli* BL21). Cells were grown for an additional 4 h, harvested and resuspended in binding buffer (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl). After disruption by sonication or French press (BL21), cell debris was removed by centrifugation at 15 000 g for 30 min and the protein was purified according to standard procedures using Ni-NTA columns (Qiagen) or Ni-IDA (Macherey-Nagel). ClpG proteins purified from *E. coli* were additionally subjected to Superdex S200 size exclusion chromotography in assay buffer (50 mM Tris-Cl pH 7.5, 150 mM KCl, 20 mM $MgCl_2$, 2 mM DTT). The protein concentration was determined by using Bradford reagent, and purity was assessed by SDS-PAGE. *E. coli* ClpB, DnaK, DnaJ, GrpE, GroEL and GroES were purified

as described (21, 22).

Transmission electron microscopy (TEM)

Protein samples of ClpB and $ClpG_{GI}$ were prepared for energy-filtered transmission electron microscopic (EF-TEM) analysis (Libra 120 plus, Zeiss, Oberkochen, Germany) and 3D-reconstruction was performed (23). 20 µl (0.5 mg/ml) of *E. coli*/*P. aeruginosa* ClpB proteins and $ClpG_{GI}$ was incubated with 2 mM ATP/ATPγS at ambient temperature for 10 min. 180 µl of assay buffer were added to result in 50 µg/ml final protein concentration and the sample was immediately assessed (Fig. S8 A). $ClpG_{GI}$ in the assay buffer with $MnCl_2$ replacing $MgCl_2$ was incubated on ice for 24 h (Fig. S8 B,C). Electron micrographs were recorded with a bottom-mount, cooled 2048x2048 CCD frame transfer camera (SharpEye; Tröndle, Wiesenmoor, Germany) at close to low dose conditions in the elastic bright field mode as Zero-loss images (nominal magnification: 40,000x; slit width: 10 eV; 0.5 mrad illumination aperture, 60 µm objective aperture, beam current: 1 µA), close to the Gaussian focus, i.e. about 0 to 100 nm underfocus.

3D-reconstruction of $ClpG_{GI}$ molecules

For $ClpG_{GI}$ 3D-reconstruction a data set of 1897 particles was extracted from raw data, using 'boxer' of the EMAN2 program (24), setting the box-size to 100 pixel, which leads to a final sampling size of 0.29 nm at the probe level. Reference-free classification of the data set, based on the box size and the molecular protein mass, was done and refinement calculations based on C1 and finally C6-symmetries were performed, running into conversion within 12 cycles, which led to the final 3D-density model of $ClpG_{GI}$. With the aid of Chimera-1.8 (25), X-ray model data, i.e. a highly analogous *E. coli* ClpB mutant E432A (PDB code: 4D2Q; (26)), were semi-automatically fitted into the electron microscopic density map, which was median-filtered with two iterations and was set to a level of 0.0283 within a range of -0.044 to 0.0446, using voxel size 0.33 nm. Further modeling and analytical work were done accordingly.

Size exclusion chromatography

To estimate the molecular mass and oligomeric state, $ClpG_{GI}$, ClpG and N-terminal deletion variants were subjected to size exclusion chromatography (SEC) using a Superdex 200 10/300 GL (GE Healthcare) equilibrated with assay buffer. 10 µM of proteins was preincubated +/- 2 mM ATP for 5 min at room temperature and column-applied in assay buffer +/- 2 mM ATP at room temperature, respectively, as a running buffer. Since ATP in the running buffer interferes with protein detection, eluted fractions were analyzed by SDS-PAGE. Blue-dextran, thyroglobulin, ferritin, aldolase, conalbumin and ovalbumin were used as molecular mass standards.

Circular Dichroism (CD)

CD measurements were performed in 50 mM Na phosphate (pH 7.5) at 20°C by using a Jasco J-175 Spectropolarimeter using 2.5 µM ClpG/$ClpG_{GI}$ and N-terminal deletion mutants.

In vitro disaggregating activity assay

Disaggregating activity was determined as described with minor modification (27). Malate dehydrogenase (MDH; Roche) and luciferase (Roche) were used as model substrates. 1 µM of MDH and 0.1 µM of luciferase in assay buffer were heat-denatured at 47°C for 30 min and at 45°C for 15 min, respectively, and incubated at room temperature for 5 min. 50 µl of denatured substrate was mixed with 50 µl assay buffer containing 1 µM disaggregating chaperone and the ATP-generating system (2 mM ATP, 3 mM phosphoenolpyruvate and 20 ng/µl pyruvatekinase final concentration in 100 µl of reaction volume) in common and, optionally, accessory proteins, 1 µM DnaK-0.2 µM DnaJ-0.1 µM GrpE (KJE) (for ClpB) and 1 µM each GroEL/ES (for refolding of MDH by ClpB and ClpG/$ClpG_{GI}$). Samples were incubated at 30°C for disaggregation and refolding and samples were removed at certain time points after incubation, To detect recovered MDH activity, consumption of NADH was measured by monitoring decrease of absorbance at 340 nm using UV spectrophotometer (Novaspec Plus, Amersham). 10 µl of sample was added into 690 µl of MDH assay buffer (150 mM potassium phosphate pH 7.6, 1 mM DTT, 0.5 mM oxaloacetate, 0.28 mM NADH). To assess luciferase activity, 2 µl of sample was mixed with 125 µl 2X luciferase assay buffer (25 mM glycylglycin, 12.5 mM $MgSO_4$, 5 mM ATP) and 125 µl 25 mM luciferin (Gold Biotech.) solution and activity measured by Berthold Lumat LB 9507. 100% activity corresponds to the activity before heat denaturation.

In vivo chaperone assay

A single colony of the *E. coli* MC4100 Δ*clpB* strain expressing YFP-luciferase and the respective chaperone (Table S2) was cultured in LB-medium at 30°C overnight, re-inoculated into fresh LB medium containing 0.1% L-arabinose and 0.25 mM isopropyl-β-d-thiogalactopyranoside (IPTG). Cells were grown to OD_{600} = 0.6 and 800 µg/ml of erythromycin was added to cease further translation. To heat shock, cells were incubated at non-lethal 45°C for 20 min in the water bath. Afterwards, the cells were incubated at 30°C for recovery and 200 µl cell suspension at 30, 60 and 90 min was used to measure luciferase activity. 200 µl of 25 mM luciferin was added to monitor luciferase activity in a Berthold Lumat LB 9507. 100% of luciferase activity corresponds to the activity before heat shock.

ATPase assay

ATPase activity was determined by a coupled ADP monitoring/recycling reaction of pyruvate kinase (PK) and lactate dehydrogenase (LDH). The targeted ATPase converts the substrate ATP to produce ADP. PK converts phosphoenolpyruvate (PEP) into pyruvate with ADP to yield ATP. LDH converts pyruvate into lactate by oxidizing NADH to NAD^+ which is monitored by decrease of absorbance at 340 nm. ATPase activity was calculated by assuming that one oxidized NAD^+ corresponds to the production of one molecule of ADP. 1 µM ATPase was examined in 100 µl assay buffer with 5 mM NADH, 10 mM PEP (Sigma) and PK/LDH (Sigma) in a 96-well plate (TPP) format. 50 µM B1 peptide (28) or 0.1 mg/ml casein was added to assess stimulation of ATPase activity. 100 µl of 4 mM ATP was added to each sample to start the reaction. Decrease of absorbance at 340 nm was measured by FLUOstar-Omega (BMG-Labtech).

Prion propagation test

Propagation of [PSI$^+$] and [URE3] was monitored by their ability to promote expression of Ade2p using yeast strains 779-6A (*MATα*, *kar1-1*, *SUQ5*, *ade2-1*, *his3Δ202*, *leu2Δ1*, *trp1Δ63*, *ura3-52*) and 1075 (*MATα*, *kar1-1*, P_{DAL5}*::ADE2*, *his3Δ202*, *leu2Δ1*, *trp1Δ63*, *ura3-52*) (29). Cells deficient in Ade2p (Ade$^-$) display red on adenine-limited medium due to accumulation of a metabolite of adenine biosynthesis and cannot grow on medium without adenine. Sup35p catalyzes translation termination at stop codons, thus preventing translation of *ade2-1* mRNA containing amber mutation in 779-6A strain. Integration of Sup35p into [PSI+] prion aggregates depletes Sup35p to cause transcription of full-length *ade2-1* mRNA and restoration of adenine production. Ure2p is a transcriptional repressor of *DAL5* gene. In 1075 strain, the *ADE2* gene is fused to the *DAL5* promoter. Integration of soluble Ure2p into [URE3] prion aggregates relieves Ure2p-mediated transcriptional repression and cells express Ade2p to restore adenine-related phenotypes. Yeast strains containing pMR116 empty vector and pMR116 $clpG_{GI}$ were cured from prions in the presence of 3 mM guanidine hydrochloride on Synthetic Dextrose (SD)-Trp (Sunrise Science Products) containing limited concentration of adenine (8 mg/l) (29) (Table S3). Subsequently, the cured colonies were streaked on SD-TRP-limited adenine and SD-TRP-ADE plates and incubated for 3-4 days at 30°C.

Refolding of urea-denatured luciferase

Urea-denatured luciferase was used as substrate when determining the impact of guanidine hydrochloride (GuHCl) on Hsp104, $ClpG_{GI}$ and ClpG disaggregation activities. 4 M luciferase was denatured in 6 M urea at 30°C for 30 min and 80-fold diluted in buffer (50 mM HEPES pH 7.5, 150 mM KCl, 20 mM $MgCl_2$, 2 mM DTT) containing chaperones (1 M Hsp104/2 M Ssa1/1 M Ydj1/0.1 M Sse1; 1 M $ClpG_{GI}$; 1 M ClpG) and an ATP regenerating system in the absence or presence of 5 mM GuHCl. Luciferase activities were determined as described before.

Disaggregation of proteins in heat-denatured crude extracts

Disaggregation of proteins in heat denatured crude extracts was performed with minor modifications (30). Bacterial cells were cultured at 30°C for 24 h, 10 ml cell suspension collected and resuspended in 1 ml breakage buffer (50 mM HEPES pH 7.6, 150 mM KCl, 20 mM $MgCl_2$, 10 mM DTT). Cells were disrupted by sonication and the cell lysate centrifuged at 15,000 g for 15 min at 4°C to acquire soluble proteins. Proteins (approx. 3 µg/µl) were incubated at 42°C for 15 min in a water bath and formed aggregates isolated by centrifugation at 15,000 g for 15 min at 4°C. 100 µl of aggregates were supplemented with 5 µM $ClpG_{GI}$ and 20 mM ATP in the breakage buffer at a final volume of 200 µl. The samples were incubated at 30°C for 60 min for disaggregation and refolding. After incubation, soluble and insoluble proteins were separated by centrifugation at 15,000 g for 15 min at 4°C and visualized with Coomassie staining after SDS-PAGE separation.

Isolation of *in vivo* protein aggregates

To assess protein aggregation *in vivo* (31), cells were cultured at 30°C for 24 h in LB medium.

Subsequently, cells were heat shocked at 42°C for 1 h and recovered at 30°C for 1 and 2 h. Cells were collected before, during and after heat shock at various time points.

To identify substrates of disaggregating chaperones, cells were grown at 42°C for 24 h and the aggregated protein content was analyzed. 2 ml cell culture was harvested and collected cell pellet was resuspended in 40 µl buffer A (10 mM potassium phosphate buffer pH 6.5, 1 mM EDTA, 20% sucrose, 1 mg/ml lysozyme) and incubated on ice for 30 min. 360 µl buffer B (10 mM potassium phosphate buffer pH 6.5, 1 mM EDTA) was added and cells disrupted by sonication. Intact cells were separated by mild centrifugation at 2,000 g for 15 min at 4°C. Subsequently, soluble protein was separated by centrifugation at 15,000 g for 30 min at 4°C and aggregated protein in the pellet was separated from the membrane associated proteins by washing twice with 400 µl buffer B containing 2% NP40. Lastly, aggregated proteins were washed with 400 µl buffer B and resuspended in 50 µl of buffer B. Total crude extract, soluble and insoluble aggregated proteins were analyzed by Coomassie staining after SDS-PAGE.

***In vitro* substrate binding assay**

To examine the interaction between aggregated luciferase or aggregated MDH and Clp protein variants, luciferase and MDH were heat-denatured at 45°C for 15 min or 47°C for 30 min in assay buffer. The samples were cooled down to 25°C for 10 min. 2 µM of aggregated luciferase or 4 µM of aggregated MDH were mixed with 1.5 µM ClpB/ClpG$_{GI}$/ClpG protein or N2 subdomain deletion variants with 2 mM of ATPγS in 100 µl assay buffer. Samples were incubated at 25°C for 10 min. Soluble and insoluble fractions were separated by centrifugation at 13,000 rpm for 25 min at 4°C. The pellet fraction was washed once with 150 µl assay buffer and centrifuged again at 13,000 rpm for 10 min at 4°C. Binding assays were performed in Low binding micro tubes (Sarstedt). Supernatant and pellet fractions were mixed with protein sample buffer and analyzed by Coomassie staining after SDS-PAGE (8-16% gradient gels). Each assay was repeated three times independently. As control purified Clp proteins without aggregated proteins were subjected to the same protocol. Band intensities were analyzed using ImageJ.

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