Supporting Information

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SI Text

SI Methods. ***Strain constructions.*** The *clpB* gene was deleted from parental strains Rosetta (DE3) (Novagen) and BTH101 (1) by P1 transduction, using a lysate from *Escherichia coli* JW2573-6: Δ*(araD-araB)567,* Δ*lacZ4787(::rrnB-3),* LAM^{-}, Δ*clpB757*::*kan, rph-1,* Δ*(rhaD-rhaB)568, hsdR514* [Keio collection, (2)]. Transductants were selected at 30 °C on LB plates supplemented with kanamycin. To eliminate the kanamycin-resistance cassette, the mutants were transformed with the FLP recombinase expression plasmid, pCP20-Amp, and subsequently cured at 42 °C (3, 4). The absence of the *clpB* gene was verified by colony PCR.

Bacterial plasmids. The chimeras were constructed by the extension of overlapping PCR products (5). *clpB* and *HSP104* genes were used as templates for PCR reactions. Internal primers that were used are shown in Table S2. The external primers (5′ to 3′) that were used to generate the full-length chimera PCR products were:

ClpB-F: (gcatcggctagcatgcgtctggatcgtcttactaa)
ClpB-R: (ggatcgtactcgagttactggacggcgacaatccggt)
104-F: (gctactgacgctagcatgaacgaccaaacgcaatttac)
104-R: (gcatgcatcctcgagttaatctaggtcatcatcaatttc)

The final PCR products were digested with *NheI* and *XhoI* and cloned into similarly digested pET24 vector (Novagen).

For plasmids used in *E. coli* cell survival assays, the above pET plasmids served as templates to amplify *SacI*/*XmaI* fragments that contain the *HSP104*, *clpB* or chimera-coding region. A Shine-Dalgarno sequence was introduced via the 5′ PCR primer. The PCR products were digested with *SacI* and *XmaI*, ligated into similarly digested pBAD18 (6) and transformed into SG22100.

For the bacterial two-hybrid assay, an *XbaI*/*XhoI* fragment of *E. coli* dnaK was generated by PCR, digested with *XbaI* and *KpnI* and ligated into similarly digested pEB354 (19). *PstI*/*XhoI* fragments containing *clpB*, *HSP104*, or chimera open reading frames were generated by PCR, digested with *PstI* and *XhoI* and ligated into similarly digested pEB355 (19).

The plasmid for the overexpression of Hsp104, pHsp104wt, was created using site-directed mutagenesis (Stratagene) to revert the pNOTAG Hsp104 N728A plasmid [Addgene, plasmid 1,244, (7)] to the wild type sequence. The resulting plasmid was sequenced and transformed into *E. coli* strain Rosetta(DE3) for overexpression and purification of Hsp104.

Plasmids for the overexpression of the ClpB M-domain mutants listed in Table S1 were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) and plasmid pET24b-clpBwt (8) as template. All constructs were verified by DNA sequencing.

The plasmid for the overexpression of human Hsp70 (HSPA1A) was a gift from Len Neckers (NIH).

Yeast plasmids. Expression in yeast of chimeras of ClpB and Hsp104 was controlled by the *HSP104* promoter (−500 bp) and terminator (+500 bp) elements on a centromeric plasmid. First, the promoter was amplified as a *Bam*HI/*Pst*I fragment using yeast genomic DNA as template and ligated into pRS314 (ATCC, Manassas, Virginia) via the same restriction sites to give p314-P_{HSP104}. The terminator sequence was then amplified as a *Xho*I/*Kpn*I fragment and ligated into p314-P_{HSP104} to give pMR116. *Hsp104*, *clpB*, or chimera open reading frames were amplified as *Pst*I/*Xho*I fragments, ligated into pMR116 and expressed in YKT52 Δ*hsp104*.

Plasmid pMR135 contains the firefly luciferase open reading frame under the control of the constitutive glyceraldehyde-3-P dehydrogenase (GPD) promoter. It was constructed by amplifying the luciferase gene as a *Spe*I/*Xho*I fragment followed by ligation into p416-GPD (9) via the same restriction sites.

Proteins. ClpB (10), ClpB M-domain mutants (10), DnaK (11), DnaJ (11), GrpE (11), Ydj1 (12), and GFP-38 (13) were prepared as described. Luciferase was purchased from Promega (Madison, WI). Hsp104 and the chimeras 44B4, 4BB4, and M-4bbb were overexpressed in Rosetta(DE3) and purified using a modified ClpB protocol (10). Briefly, cultures were grown at 25 °C to an OD_{600} of ∼0.25, shifted to 20 °C and induced overnight with 0.1 mM IPTG. Clarified cellular extracts were purified over a Q-Sepharose-FF column (GE Healthcare) in 40 mM Hepes, pH 7.5, 80 mM NaCl, 0.5 mM EDTA, 20 mM $MgCl_2$, 20% glycerol (vol/vol), 5 mM ATP and 1 mM DTT. Proteins were eluted from the column with a linear gradient of 80 to 500 mM NaCl in the same buffer. Fractions containing Hsp104 were subjected to Sephacryl S-200 chromatography (GE Healthcare) in 20 mM Hepes, pH 7.5, 100 mM KCl, 0.5 mM EDTA, 20 mM $MgCl_2$, 10% glycerol (vol/vol), 5 mM ATP and 1 mM DTT. All other chimeras were overexpressed in Rosetta(DE3) Δ*clpB* and purified as previously described for ClpB (10).

Human Hsp70 was overexpressed in BL21(DE3)pLysS cells (Promega) and purified using a modified protocol (14). Cells were grown at 30 °C to an OD_{600} ∼0.7 in LB containing carbenicillin (50 μg/mL) and chloramphenicol (30 μg/mL). Hsp70 overproduction was induced with 1 mM IPTG overnight. Clarified cellular extracts were purified over a diethylaminoethyl (DEAE)-Sepharose-FF-Sepharose-FF (GE Healthcare) column in 20 mM Tris-HCl, pH 7.5, 20 mM KCl, 0.5 mM EDTA and 1 mM DTT. Proteins were eluted with a gradient from 20 to 700 mM KCl in the same buffer. Fractions containing Hsp70 were purified further using an ATP-agarose (Sigma) column as previously described (14). Fractions containing Hsp70 were dialyzed into 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 50 mM KCl, 2 mM DTT and 10% glycerol (vol/vol).

In vivo assay for yeast thermotolerance. Cultures of *Saccharomyces cerevisiae* strain YKT52 harboring the empty vector or carrying *HSP104*, *clpB*, or chimera open reading frame under the control of the *HSP104* promoter were inoculated to $OD_{600} = 0.25$ in selective media from overnight cultures. After growth for 1 h at 30 °C, the cultures were treated for 30 min at 37 °C to induce heat-shock protein expression (15, 16). 500 μL was then transferred to preheated eppendorf tubes in a 50 °C hot block. Aliquots were removed at 0, 4, 8, and 12 min and transferred directly to ice. Fivefold serial dilutions were spread onto yeast extract, peptone, adenine, dextrose (YPAD) plates to determine the number of colony-forming units during heat-shock.

In vivo assay for E. coli survival at high temperature. SG22100 cells harboring pBAD plasmids containing *clpB*, *HSP104*, or a chimera open reading frame were grown at 30 °C in LB broth supplemented with ampicillin (100 μg/mL) to $OD_{600} = 0.2$. Protein expression was induced by the addition of arabinose (0.2%) and cultures were grown to $OD_{600} = 0.4$. The cultures were then shifted to 50 °C (17). Aliquots were removed at the indicated times, and 10-fold serial dilutions were spread on LB plates containing 100 μg/mL ampicillin. The number of viable colony-forming units was determined.

In vivo luciferase reactivation. Cultures of *S. cerevisiae* strain YKT52 harboring pMR135 in addition to the empty vector, *HSP104*, *clpB*, or chimera open reading frames under the control of the *HSP104* promoter were inoculated to $OD_{600} = 0.25$ in selective media from overnight cultures. After growth for 1 h at 30 °C, the cultures were pretreated for 30 min at 37 °C to induce heat-shock protein expression (16). After pretreatment the luciferase activity of the sample was determined, cycloheximide was added to a final concentration of 20 μg/mL to halt protein synthesis, and the cultures were shifted to 44 °C for 1 h. Cultures were then shifted to 25 °C. Luciferase activity was assayed at $t = 0$, 30, 60, 90, and 120 min after heat shock by adding 50 μL of 1 mM beetle luciferin (Promega) in 0.1 M sodium citrate, pH 5.0, to 100 μL culture and reading immediately in a Zylux Femtomaster FB15 luminometer, with a 10 s delay and 5 s read time. Percent recovery of luciferase activity was calculated using pretreated, nonheat shocked cells as reference and normalized.

In vitro luciferase reactivation. Luciferase (4 μM) was denaturated in 5 M urea for 60 min at 23 °C then diluted 80-fold in reactions containing 40 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 0.15 mg/mL BSA, 4 mM ATP, an ATP regenerating system (10 mM creatine phosphate and 30 μg/mL creatine kinase), 15 mM $MgCl_2$, ClpB, Hsp104, or chimera (0.6 μM), and either DnaK (1 μM), DnaJ (0.2 μM), GrpE (0.1 μM), or human Hsp70 (HSPA1A) (1 μM), Ydj1 (0.2 μM) (18). Reactions were incubated at 23 °C for 60 min, aliquots (5 μL) were removed at the times indicated and luciferase activity was determined by adding 15 μM luciferin (Roche) and measuring light output in a Victor^3 Multilabel Counter (Perkin Elmer). Reactivation was determined compared to a nondenatured luciferase control.

In vitro GFP-38 reactivation. Reaction mixtures (100 μL) contained 25 mM Hepes, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 5 mM DTT, 0.005% Triton X-100 (vol/vol), 4 mM ATP, an ATP regenerating system (10 mM creatine phosphate and 3 μg creatine kinase), 10 mM $MgCl_2$, 5 μL heat-aggregated GFP-38 (heated 15 min at 80 °C at 14 μM, frozen on dry ice, thawed, and used immediately), 0.5 μM ClpB, Hsp104, or chimera and either DnaK (1.3 μM), DnaJ (0.2 μM) and GrpE (0.1 μM), or human Hsp70 (HSPA1A) (1.3 μM) and Ydj1 (0.2 μM). GFP fluorescence was monitored over time at 23 °C using a Varian Cary Eclipse fluorescence spectrophotometer with a well plate reader. Excitation and emission wavelengths were 395 nm and 510 nm, respectively. Reactivation was determined compared to a nondenatured GFP-38 control.

Bacterial two-hybrid system. A bacterial two-hybrid system utilizing *Bordetella pertussis* adenylate cyclase was used as previously described (1, 19). The *clpB*, *HSP104*, or chimera-coding region was fused in frame to the 3′ end of the T18 adenylate cyclase fragment (pEB355) (19) and *dnaK* was fused to the 3′ end of the T25 adenylate cyclase fragment (pEB354) (19). Plasmids containing the T18 and T25 fusions were cotransformed into BTH101 Δ*clpB*. Cells were plated on LB plates containing ampicillin (100 μg/mL) and kanamycin (50 μg/mL) and incubated at 30 °C. Single colonies were selected and used to inoculate LB selective media and cultures grown at 30 °C. Activity of a reporter gene, β-galactosidase, was monitored on MacConkey lactose plates and in β-galactosidase assays as previously described (20).

Circular dichroism spectra. Far-UV circular dichroism spectra were collected on a Jasco J-720 spectropolarimeter at 23 °C. The spectra were recorded over the range of 200–250 nm with the protein concentration at 125 μg/mL. The step resolution was 1 nm with a speed of 50 nm/ min. A minimum of 10 scans were accumulated at a bandwidth of 2 nm and a cell path length of 5 mm. Buffer containing 25 mM Hepes, pH 7.5, 50 mM KCl, 0.1 mM EDTA and 0.1 mM Tris(2-Carboxyethyl)-Phosphine Hydrochloride (TCEP-HCl) (ThermoScientific) was used for Hsp104, the chimeras, and ClpB wild type (Fig. S6 *A–C*). Buffer containing 20 mM Tris-HCl, pH 7.5, 10% glycerol (vol/vol), 100 mM KCl, 0.1 mM EDTA with 0.1 mM TCEP-HCl was used for ClpB wild type and the M-domain point mutants (Fig. S6*D*).

Gel filtration chromatography. Protein samples (60 μL; 2 μM) were incubated with 4 mM ATP and analyzed at room temperature on a Sephacryl S-200 column (CV = 17 mL), equilibrated with 20 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol (vol/vol), 10 mM $MgCl_2$, 0.005% Triton X-100 (vol/vol) and 1 mM ATP. Fractions were analyzed for protein by the Bradford protein assay.

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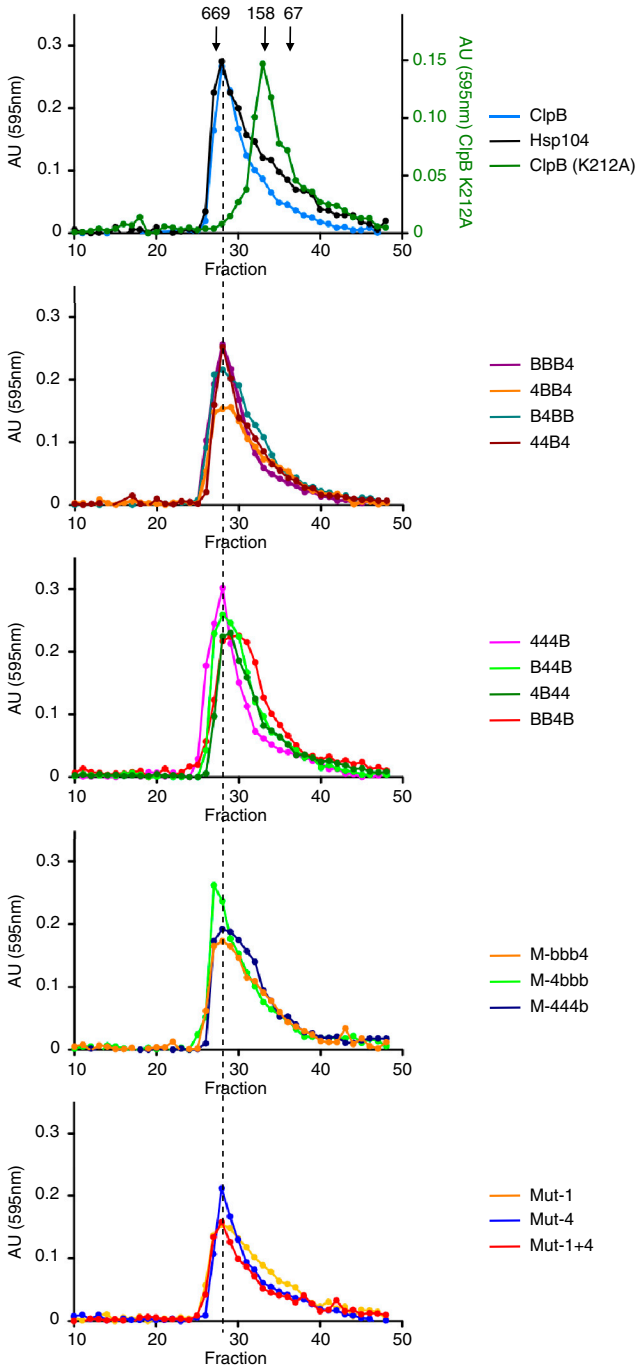

Fig. S1. Gel filtration analysis of ClpB, Hsp104, the chimeras, or the ClpB mutants. Elution profile of ClpB, Hsp104, the indicated chimera, or ClpB M-domain mutant was determined as described in *SI Methods*. Elution profile of $ClpB_{(K212A)}$ mutant that is unable to hexamerize due to a mutation in the Walker A motif (13) is shown first. Elution positions of thyroglobulin (669 kDa), aldolase (158 kDa), and BSA (67 kDa) are indicated.

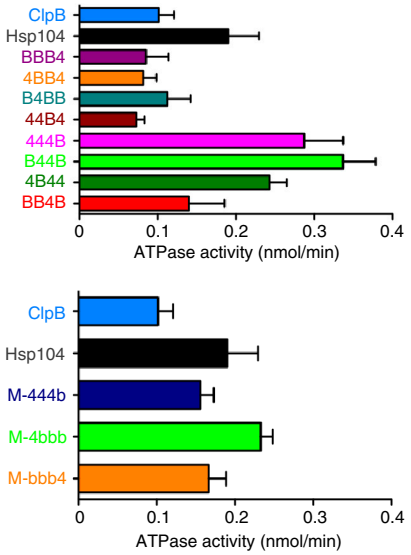

Fig. S2. ATP hydrolysis by ClpB, Hsp104, or the chimeras. ATPase activity of ClpB, Hsp104, or the indicated chimera was measured as described (8). Data are means ± SD from three experiments.

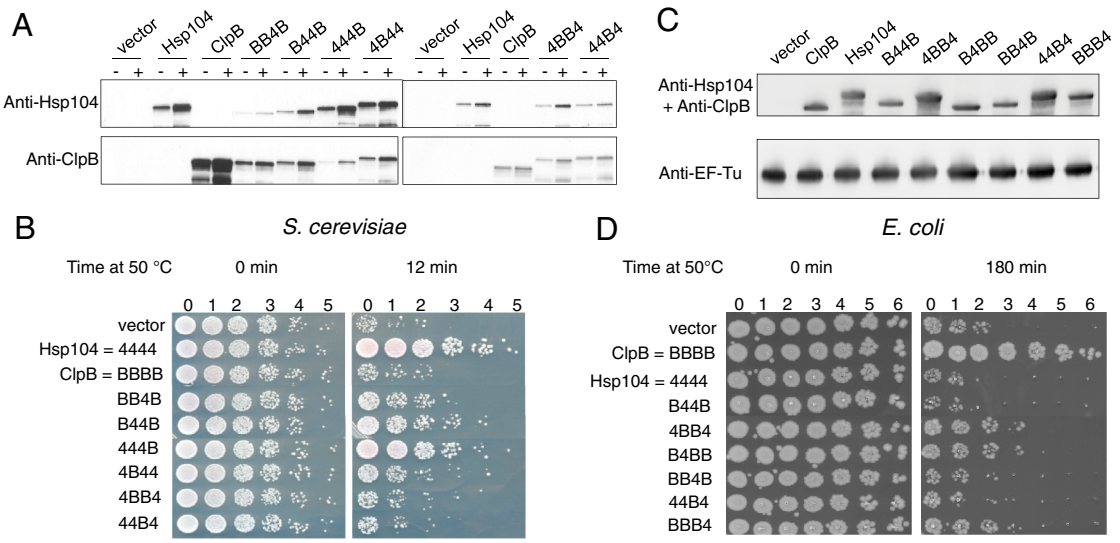

Fig. S3. Expression levels of chimeras in *S. cerevisiae* and *E. coli*. (*A*) Overnight cultures of YKT52 Δ*hsp104* cells expressing Hsp104, ClpB, or the indicated chimera under the control of the *HSP104* promoter on a centromeric plasmid were diluted to $OD_{600} = 0.5$ in selective media and incubated at either 30 °C (–) or 37 °C (+) for 30 min as described in *SI Methods*. Expression was determined by Western blot analysis using polyclonal Hsp104 (on the top) or polyclonal ClpB antisera (on the bottom). (*B*) Cells were grown as described in *SI Methods* and then fivefold serial dilutions of unheated cells (left) and heat-treated cells (right) were spotted onto YPAD plates and incubated at 30 °C for 2–3 d. (*C*) *E. coli* SG22100 cells expressing plasmid-encoded ClpB, Hsp104, or an indicated chimera under the control of the arabinose-inducible promoter was incubated at 30 °C as described in *SI Methods*. Protein expression was determined 1 h after arabinose addition by Western blot analysis using a mixture of polyclonal Hsp104 and ClpB antisera. As a control, Elongation Factor- thermal unstable (EF-Tu) was determined by Western blot using EF-Tu antiserum. (*D*) Cultures were grown as in (*C*) without heat-treatment (0 min) or after heat-treatment (180 min) and then 10-fold serial dilutions were spotted onto LB plates with ampicillin and incubated 24 h at 30 °C.

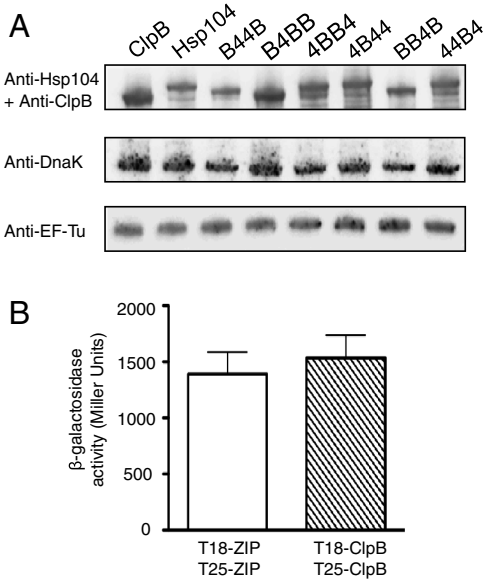

Fig. S4. Bacterial two-hybrid fusion protein expression and protein interaction positive controls. (*A*) T18 and T25 fusion proteins were coexpressed in *E. coli* MG1655 under the same conditions used for the β-galactosidase experiments and protein levels were determined by Western blot analysis using polyclonal DnaK and EF-Tu antisera and a mixture of ClpB and Hsp104 antisera. (*B*) Bacterial two-hybrid positive controls. A leucine zipper fragment (1) or *clpB* was fused to both the T18 and T25 fragments of *B. pertussis* adenylate cyclase. Plasmids were coexpressed in BTH101 Δ*clpB* and β-galactosidase activity was determined as described in *SI Methods*.

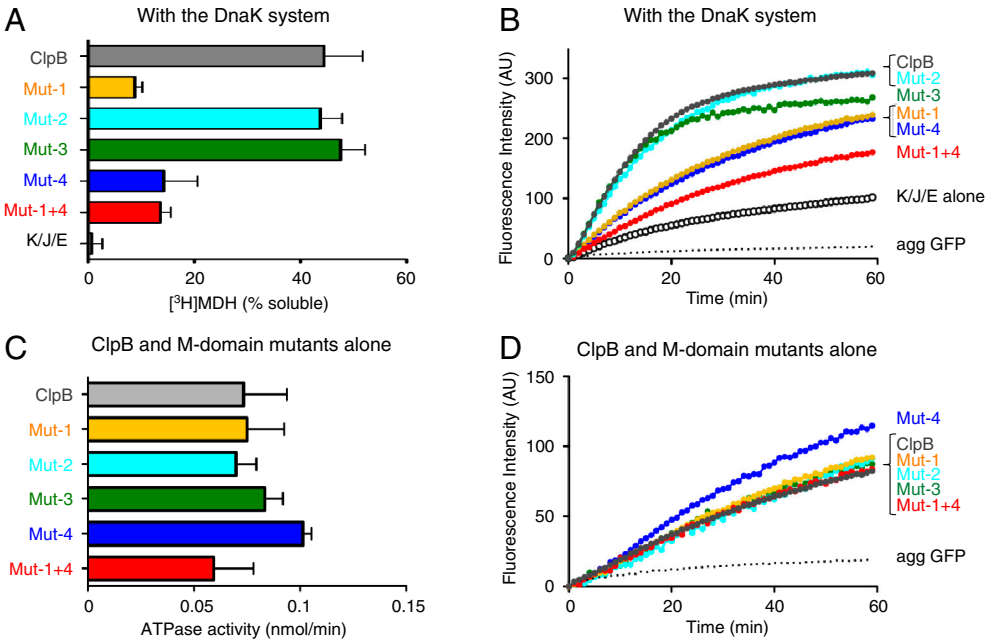

Fig. S5. Amino acid substitutions within helix 2 of the ClpB M-domain alter collaboration between ClpB and the DnaK system. (*A*) Disaggregation of heat-aggregated [^{3}H]MDH (13) and (*B*) reactivation of heat-aggregated GFP (8) by ClpB M-domain mutants in the presence of the DnaK chaperone system were determined as described. (*C*) ATPase activity of ClpB wild type and M-domain mutants was determined as described (8). (*D*) Reactivation of heat-aggregated GFP by ClpB M-domain mutants using the combination of ATP and ATPγS as the nucleotide source was measured as described (8). Data in *A* and *C* are means ± SD ($n = 3$) and a representative experiment of three independent experiments is shown in *B* and *D*.

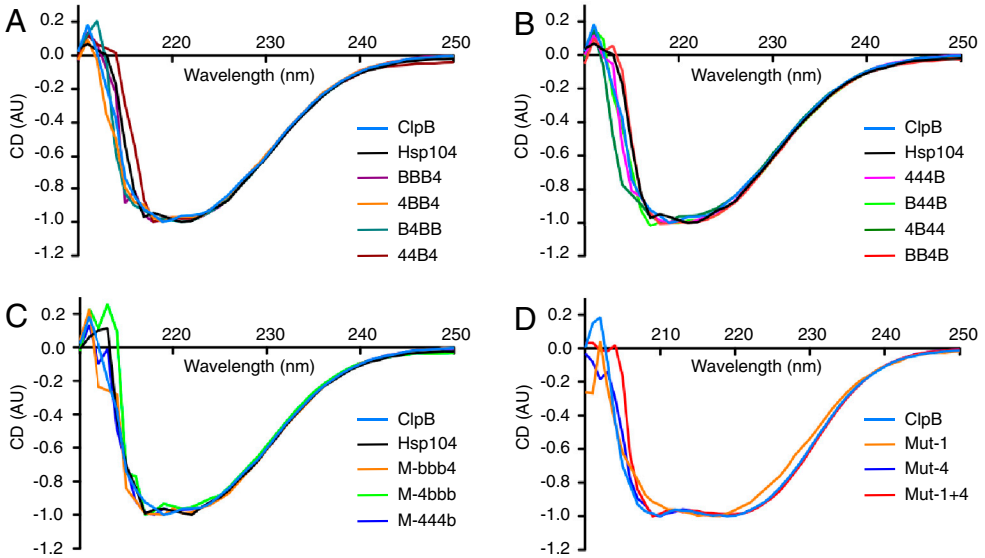

Fig. S6. CD spectra of Hsp104, ClpB, or chimeras. CD spectra were determined as described in *SI Methods* for: (*A*) ClpB, Hsp104, and chimeras that collaborate with the DnaK system, (*B*) ClpB, Hsp104, and chimeras that collaborate with the Hsp70 system, (*C*) ClpB, Hsp104, and M-domain chimeras, and, (*D*) ClpB wild type and ClpB M-domain mutants.

Table S1. Luciferase reactivation by ClpB wild type or mutants with the DnaK system

ClpB or ClpB mutant	Type of change to ClpB	Rate of luciferase reactivation (% min^{-1}) *	Relative rate of luciferase reactivation
ClpB wild type		5.02 ± 0.25	1
Mut-1(K438E, K439E, R440E)	charge change in helix 2	1.78 ± 0.20	0.35 ± 0.04
Mut-2 (L444A)	substitution to Hsp104 amino acids in helix 2	4.89 ± 0.10	0.98 ± 0.02
Mut-3 (R453A, Q454A)	charge change in helix 2	4.97 ± 0.40	0.99 ± 0.08
Mut-4 (A467H, S470E, G471E)	substitution to Hsp104 amino acids in helix 2	1.56 ± 0.05	0.31 ± 0.01
Mut-1+4 (K438E, K439E, R440E +A467H, S470E, G471E)	double mutant	1.62 ± 0.18	0.32 ± 0.04
DnaK system alone		0.045 ± 0.018	0.01± > 0.01

*% of initial luciferase activity without urea treatment

Table S2. Primers used for the construction of ClpB-Hsp104 chimeras

Primer name	Primer sequence (5′ to 3′)	Chimera name
clpB-N1-R	gtcaattctagtgttaccacgaagttgttcaatcgcttgagtaatgttg	B44B, B4BB
hsp104-N1-F	caacattactcaagcgattgaacaacttcgtggtaacactagaattgac	B44B, B4BB
hsp104-M2-R	agaaaccggaatccccgtccatcttgcagctgtttcagaaatgg	B44B, 444B
clpB-M2-F	ccatttctgaaacagctgcaagatggacggggattccggtttct	B44B, 444B
hsp104-dM-R	aatcgcaatggtatcttcaacagatggttcagcgacttcaattttctg	B4BB, 44B4
clpB-dM-F	cagaaaattgaagtcgctgaaccatctgttgaagataccattgcgatt	B4BB, 44B4
clpB-1M-R	atccaattcttctggcttagaatcaatctgcatacgaatgctggatg	BB4B, M-4bbb, M-444b
hsp104-1M-F	catccagcattcgtatgcagattgattctaagccagaagaattggat	BB4B, M-4bbb, M-444b
hsp104-Md-R	cttcagcaatttcggcgtcggtgaccacattttggatcatggag	BB4B
clpB-Md-F	ctccatgatccaaaatgtggtcaccgacgccgaaattgctgaag	BB4B
clpB-M2-R	cttcttaacagggataccagtcaaacgcgccagcacttcagcaat	BBB4, 4BB4
hsp104-M2-F	attgctgaagtgctggcgcgtttgactggtatccctgttaagaag	BBB4, 4BB4
hsp104-N1-R	ggtatatttttttcaaagcctgcaaaggtgtgttcgtatcagc	4BB4, 4B44
clpB-N1-F	gctgatacgaacacacctttgcaggctttgaaaaaatatacc	4BB4, 4B44
clpB-1M-R	cgagttcttctggttttgagtctcttgcgacggcgacaccag	44B4
hsp104-1M-F	ctggtgtcgccgtcgcaagagactcaaaaccagaagaactcg	44B4
clpB-Md-R	gatcatggagttggcaccagcttcgagctgcgttgcggcttc	44B4
hsp104-Md-F	gaagccgcaacgcagctcgaagctggtgccaactccatgatc	44B4
clpB-m3m4-R	cgatttgctttttgatatctgggattttgccgtattgcagttcag	M-bbb4
hsp104-m3m4-F	ctgaactgcaatacggcaaaatcccagatatcaaaaagcaaatcg	M-bbb4
hsp104-m1m2-R	gtttttttactggcttcatcagactcatcttcatctctctctagagc	M-4bbb
clpB-m1m2-F	gctctagagagagatgaagatgagtctgatgaagccagtaaaaaac	M-4bbb
hsp104-m3m4-R	cttccagttgctttttccagttccgggatggcgaagtaccttaaatc	M-444b, M-b44b
clpB-m3m4-F	gatttaaggtacttcgccatcccggaactggaaaagcaactggaag	M-444b, M-b44b
clpB-m1m2-R	ctatctttagtggtggagtcggctttcattaacgcctgttgttcc	M-b4bb, M-b44b
hsp104-m1m2-F	ggaacaacaggcgttaatgaaagccgactccaccactaaagatag	M-b4bb, M-b44b
hsp104-m2m3-R	cagttcagacatccgcgccagatcatatctacgttcagcatc	M-b4bb
clpB-m2m3-F	gatgctgaacgtagatatgatctggcgcggatgtctgaactg	M-b4bb

clpB and hsp104 indicate the PCR template from *clpB* or *hsp104*, respectively; *F* = forward primer; *R* = reverse primer.