Supplemental Information for:

Proteotoxic stress induces a cell cycle arrest by stimulating Lon to degrade the replication initiator DnaA

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Content:

Supplemental Figure Legends

Figure S1. Prolonged depletion of DnaK/J affects DnaA and RpoA synthesis. Western blots and ^{35}S-labeling experiments showing steady-state levels and synthesis rates of DnaA and RpoA upon DnaK/J depletion. The DnaK/J depletion strain was grown in M2G minimal medium with xylose and then shifted to M2G without xylose to deplete DnaK/J. For monitoring protein synthesis, cultures were labeled for 10 min with ^{35}S-methionine at the indicated time points followed by immunoprecipitation of DnaA or RpoA with specific antisera. Samples for Western blotting were taken from the same culture at the same time points and probed with DnaK, DnaA or RpoA antiserum, respectively. Band intensities were quantified and plotted; in the case of ^{35}S-DnaA the means from two independent experiments are shown. The results show that DnaA and RpoA synthesis decreased similarly between 10 and 14 hours. DnaA steady-state levels started to drop before 10 hours of DnaK/J depletion, while RpoA protein remained at high levels, demonstrating that the removal of DnaA protein upon DnaK/J depletion is caused mainly by DnaA degradation.

Figure S2. The effect of various protease mutations on DnaA steady-state levels in cells depleted of DnaK/J. (A) Western blots showing DnaA and RpoA protein levels in the DnaK/J depletion strain and isogenic strains with mutations in *lon*, CCNA_00108 (*lon-like*), *hslV*, *ftsH* or *clpA*. Cells were grown in PYE with xylose and shifted to PYE with glucose to deplete DnaK/J for the times indicated. (B) Western blots showing ClpP, DnaK, DnaA, and RpoA levels during simultaneous depletion of ClpP and DnaK/J. ClpP was pre-depleted for 3 hours (-IPTG) before cells were shifted to glucose to also deplete DnaK/J. (C) Western blots showing ClpX, DnaK, DnaA, and RpoA protein levels during simultaneous depletion of ClpX and DnaK/J. ClpX was depleted for 3 hours (-IPTG) before cells were shifted to glucose to also deplete DnaK/J.

Figure S3. Effects of a ClpP depletion and a *lon* deletion on DnaA and CtrA stability. (A) *In vivo* degradation assay showing the effect of a Δ*lon* mutation on CtrA stability in the

DnaK/J depletion strain. Cells were grown in xylose, or in glucose for 5.5 hrs to deplete DnaK/J, before being treated with chloramphenicol to shutdown protein synthesis. Samples were taken at the times indicated and degradation of CtrA was monitored by Western Blotting. The band intensities from two independent experiments were quantified; means with standard deviations are shown. (B) *In vivo* degradation assays showing the effect of ClpP depletion on DnaA stability in the DnaK/J depletion strain. ClpP was depleted for 8 hrs until the protein level was reduced to ~5% initial levels (see Fig. S2). DnaK/J was depleted for 5 hrs. Means with standard deviations from two independent experiments are shown. (C) *In vivo* degradation assays showing the effect of ClpP depletion on CtrA stability in the DnaK/J depletion strain. CtrA stability was measured using the same samples as in Fig. S3B.

Figure S4. The effect of allosteric activators on Lon degradation of DnaA, CtrA, SciP and CcrM. (A) *In vitro* degradation assays showing that titin-I27^{CM}-β20 does not stimulate CtrA degradation by Lon. CtrA (2 μM) was incubated with Lon_6 (0.1 μM) in the absence or presence of titin-I27^{CM}-β20 (5 μM). Creatine kinase was added to all reactions shown in this figure to facilitate ATP regeneration. Asterisks indicate the positions of the proteins analyzed. (B) *In vitro* degradation assays showing the effect of casein on DnaA degradation by Lon. DnaA (1.5 μM) was incubated with Lon_6 (0.1 μM) in the absence or presence of casein (50 μg/ml). Graphs show stimulation of Lon_6 (0.1 μM) ATPase activity (left graph) in the presence of casein, fit to a cooperative hyperbolic activation curve, and degradation rates (right graph) of DnaA (1.5 μM) by Lon_6 (0.1 μM) in the presence of increasing concentrations of casein. (C) *In vitro* degradation assays indicating that Lon degradation of SciP is not allosterically regulated. The top gel shows degradation of SciP (2 μM) by Lon_6 (0.1 μM) in the absence or presence of titin-I27^{CM}-β20 (5 μM). Note that SciP and titin-I27^{CM}-β20 run at the same position. Given that titin-I27^{CM}-β20 is rapidly degraded within less than 15 min (Fig. 5A), the data indicate that SciP degradation is not significantly

affected by titin-I27^{CM}-β20. To better assess the effect of allosteric regulators on SciP degradation we also performed degradation assays with a β20 peptide (middle gels). β20 is a 20-residue peptide containing a Lon degron derived from misfolded β-galactosidase that was found to stabilize a high efficiency state of *E. coli* Lon (Gur and Sauer, 2009). Incubation of purified SciP (2 μM) with Lon_6 (0.2 μM) in the absence or presence of β20 (50 μg/ml) shows that addition of β20 has no apparent effect on SciP degradation (upper gels). In contrast, addition of β20 (50 μg/ml) to a mixture of purified DnaA (1.5 μM) and Lon_6 (0.1 μM) clearly promoted DnaA degradation (lower gels). (D) *In vitro* degradation assays showing that Lon degradation of CcrM is only mildly stimulated by titin-I27^{CM}-β20. The gels show degradation of CcrM (1 μM) by Lon_6 (0.1 μM) in the absence or presence of titin-I27^{CM}-β20 (5 μM). The intensities of the CcrM bands from three independent experiments were quantified and plotted. (E) Increased temperature or urea denaturation do not enhance DnaA degradation in the absence of allosteric activators. Left gel: DnaA (1.5 μM) was incubated with Lon_6 (0.2 μM) and degradation was measured at 40 °C. Middle gel: Denatured DnaA (1.5 μM) (denatured by treatment with 6M urea for 6 hours) was incubated with Lon_6 (0.2 μM) at 30 °C. Right gel: DnaA was incubated for 10 min at 45 °C prior to incubation with Lon_6 (0.2 μM) at 30 °C.

Figure S5. Heat shock results in decreased DnaA levels independent of the growth medium. (A) DnaK, Lon, DnaA and RpoA levels measured by Western blots in wild type and a P_{dnaKJ}::Tn5 mutant grown in M2G medium and shifted to 40°C. (B) DnaK, Lon, DnaA and RpoA levels in wild type grown in PYE medium and shifted to 45°C.

Supplemental Experimental Procedures

Growth conditions

C. crescentus strains were grown in PYE (rich medium) or M2G medium (minimal medium), supplemented when necessary with 0.3% xylose, 0.2% glucose, 3% sucrose, 1000 μM IPTG or 500 mM vanillate. Antibiotics were added in the following concentrations as needed for solid and liquid media, respectively: oxytetracycline (2 μg ml^{-1} or 1 μg ml^{-1}), kanamycin (25 μg ml^{-1} or 5 μg ml^{-1}), chloramphenicol (1 μg ml^{-1} or 2 μg ml^{-1}) or spectinomycin (200 μg ml^{-1} or 25 μg ml^{-1}). *E. coli* strains were routinely grown in LB medium at 37 °C, supplemented with chloramphenicol (30 μg ml^{-1} or 20 μg ml^{-1}), kanamycin (50 μg ml^{-1} or 30 μg ml^{-1}), oxytetracycline (12 μg ml^{-1}), or spectinomycin (50 μg ml^{-1}) as required. Canavanine was used at a final concentration of 100 μg ml^{-1}. *Caulobacter* cultures were grown at 30 °C unless otherwise noted and diluted when necessary to maintain exponential growth. For heat shock experiments, 20 ml-cultures of *Caulobacter* were shifted from 30°C to an incubator pre-heated to 40 or 45°C, respectively, and samples were taken as indicated. Transductions were performed using ϕCr30 as described previously (Ely, 1991).

Strain construction

Strain ML2017 was constructed by transducing *dnaA*::Ω (*spec*^{r}) from GM2471 into ML76 (CB15N) containing the *dnaA* overexpression plasmid pML1716-*dnaA* (Jonas et al., 2011). ML2017 is viable in the absence of xylose because the leaky expression of P_{xyl}-*M2-dnaA* on the high copy plasmid pML1716-*dnaA* results in the accumulation of enough DnaA to allow replication initiation. Strains ML2009 and ML2008 were constructed by electroporating plasmids pCT155-*vanR*-P_{van}-*dnaA* and pCT133-P_{van}-*dnaA*, respectively, into SG400 (P_{xyl}-*dnaKJ*). To construct strain ML2002 the *lon* gene was deleted using the two-step recombination procedure (Skerker et al., 2005). First, plasmid pNPTS-*UHR*-

DHR(lon) (*kan*^{r}) was introduced into SG400 by electroporation. Clones that have integrated the vector at the *lon* locus were selected on PYE plates containing xylose, kanamycin and spectinomycin. A second recombination step was performed to select for plasmid excision. Single colonies of the first integrants were grown overnight in PYE containing xylose and spectinomycin. After overnight growth, 1 µl was plated for counter-selection on PYE containing sucrose, xylose and spectinomycin. Sucrose resistant clones, which formed colonies after 2-3 days, were restreaked to test for loss of kanamycin resistance and hence plasmid excision. The resulting clones have either regenerated the wild-type allele or generated an in-frame deletion of *lon*. To distinguish between the two outcomes, PCR was performed using primers lon_test_F and lon_test_R. The *lon* deletion in strain ML2002 was further verified by DNA sequencing. Strains ML2003, ML2005, ML2004 were constructed similarly to ML2002, except that plasmid pNPTS-UHR-DHR*(clpA)*, pNPTS-UHR-*tet*-DHR*(lon-like)* or pNPTS-UHR-*tet*-DHR*(ftsH)*, respectively, was used for the first integration. For the construction of Δ*ftsH(tet*^{r}*)* and Δ*lon-like(tet*^{r}*)* the plasmid excision step was performed in the presence of tetracycline to prevent reversion to wild type alleles. The chromosomal deletions were verified by PCR using appropriate primers (see Table 2). Strain ML2006 was constructed by moving *hslV*::Tn5 into SG400 by phage transduction and selecting for kanamycin resistance. The *hslV*::Tn5 allele was selected in a previous unpublished study as a suppressor of allele conferring DNA damage sensitivity (ML2007). Tn5 is inserted in the beginning of the *hslV* gene, thus leading to a nearly complete disruption of the gene. PCR using primers *hslV*_test_F and *hslV*_test_R was performed to verify the transduction of the mutant allele. To construct the double depletion strains ML2014 and ML2015 the P_{xyl}-*dnaKJ::spec* construct from SG400 was moved by phage transduction into strain ML2012 or ML2013 (kindly provided by C. Aakre), respectively. ML2012 contains a markerless deletion of *clpP* and a plasmid pCT133-P_{lacI}-*lacI*-P_{lac}-*clpP* allowing for the depletion of ClpP when growing cells in the absence of IPTG. Likewise, ML2013 contains a markerless deletion of *clpX* and the low-copy plasmid pCT133-P_{lacI}-

lacI-P$_{lac}$*-clpX*. Strain ML2010 was constructed by electroporating pML1716-*lon* into CB15N. Strains ML2022 and ML2023 were generated by electroporating pCT133-*vanR-P*$_{van}$*-lon* into LS2382 or ML2002, respectively.

Expression plasmids

Plasmid pCT133-*P*$_{van}$*-dnaA* was constructed by first creating pENTR-*P*$_{van}$*-dnaA* and subsequently recombining it into the destination vector pCT133, derived from the low copy vector pMR20, using the Gateway LR clonase (Invitrogen). pENTR-*P*$_{van}$*-dnaA* was constructed by amplifying *P*$_{van}$ and *dnaA* using the primer pairs Pvan_F/Pvan_R and dnaA_Pvan_F/dnaA_R, respectively from the *C. crescentus* chromosome. Both fragments were subsequently fused by PCR and cloned into pENTR using the pENTR/D-TOPO cloning system (Invitrogen). The resultant plasmid pENTR-*P*$_{van}$*-dnaA* was subsequently used to generate pCT133-*P*$_{van}$*-dnaA*. pENTR-*lon* was constructed by amplifying *lon* using primers lon_F and lon_R from the *C. crescentus* chromosome. The PCR fragment was cloned into pENTR and subsequently recombined into pML1716, yielding pML1716-*lon*. pENTR-*vanR-P*$_{van}$*-lon* was constructed by amplifying *vanR-P*$_{van}$ and *lon* using the primer pairs Pvan_F2/Pvan_R and lon_Pvan_F/lon_R, respectively from the *C. crescentus* chromosome. Both fragments were subsequently fused by PCR using primers Pvan_F2 and lon_R and cloned into pENTR. The *vanR-P*$_{van}$*-lon* construct from pENTR-*vanR-P*$_{van}$*-lon* was subsequently recombined into pCT133 using the Gateway LR clonase. For pET23b-HIS$_6$Sumo-ccDnaA, *dnaA* was amplified with dnaA_Sumo_For and dnaA_Sumo_Rev, digested with *AgeI* and *NotI* and ligated with digested pET23bHIS$_6$Sumo (Wang et al., 2007). All plasmids were sequence verified.

Integration plasmids

The integration vector pNPTS-*UHR-DHR(lon)* was constructed by amplifying an upstream and downstream homology region (*UHR* and *DHR*, respectively) of *lon* by using the primer pairs UHR_lon_F/UHR_lon_R and DHR_lon_F/DHR_lon_R, respectively. In a second

PCR step, the PCR products *UHR* and *DHR* were fused using primers UHR_lon_F and DHR_lon_R. The resultant product *UHR-DHR(lon)* was digested with *HindIII* and *EcoRI* and cloned into the *HindIII-EcoRI*-digested and dephosphorylated integration vector pNPTS138 to yield pNPTS-*UHR-DHR(lon)*. Plasmid pNPTS-*UHR-DHR(clpA)* was constructed similarly to pNPTS-*UHR-DHR(lon)*, except using the primer pairs UHR_clpA_F/UHR_clpA_R and DHR_clpA_F/DHR_clpA_R for amplifying the upstream and downstream homology regions of *clpA*, respectively. Plasmid pNPTS-*UHR-tet-DHR(ftsH)* was constructed by amplifying an *UHR* and a *DHR* of *ftsH* using the primer pairs UHR_ftsH_F/UHR_ftsH-tet_R and DHR_ftsH-tet_F/DHR_ftsH_R, respectively. In a parallel reaction the tetracycline resistance cassette (*tet*) was amplified from plasmid pKO3 using primers *tet*_ol7_F and *tet*_ol8_R. In a next PCR step, the PCR products *UHR* and *DHR* were fused with the *tet* resistance cassette yielding fragments *UHR-tet* and *tet-DHR* using primer pairs UHR_ftsH_F/*tet*_ol8_R and *tet*_ol7_F/*DHR_ftsH*_R. Finally, the resulting PCR products *UHR-tet* and *tet-DHR* were fused by using primers UHR_ftsH_F and DHR_ftsH_R. The resultant PCR fragment *UHR-tet-DHR(ftsH)* was then cloned into pNPTS using the *BamHI* and *MluI* restriction sites. Plasmid pNPTS-*UHR-tet-DHR*(*lon-like*) was constructed similarly to pNPTS-*UHR-tet-DHR*(*ftsH*) except for using primer UHR_lon-like_F, UHR_lon-like-tet_R, DHR_lon-like-tet_F and DHR_lon-like_R.

Flow cytometry

Samples from *Caulobacter* cultures grown under the appropriate conditions were fixed in 70% ethanol. Fixed cells were pelleted at 4000 rpm, resuspended in 50 mM sodium citrate buffer containing 2 μg/ml RNase and incubated for 4 hrs at 50°C to digest RNA. Samples were diluted and stained with 2.5 μM SYTOX green before being analyzed by flow cytometry using a BD Accuri C6 flow cytometer (BD biosciences). Data were collected for 50000 cells using the FL-2 detector. Data were analyzed using BD accuri software. Each experiment was repeated independently and representative results are shown.

Microscopy

Cells were fixed with 0.5% paraformaldehyde, pelleted, and resuspended in an appropriate volume of PBS. Fixed cells were mounted onto PYE 1.2% agarose pads and imaged using an Axiovert 200 microscope (Zeiss) with a 63×/1.4 NA objective (Zeiss) using software from Metamorph (Universal Imaging, PA). Cells examined were in mid-exponential phase.

Immunoblotting

Pelleted cells, normalized to the optical density of the culture, were resuspended in 1X SDS sample buffer and heated to 95°C for 10 min. Equal amounts of total protein were then subject to SDS-PAGE for 60 min at 150 V at room temperature on 10% or Tris-HCl gels (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). Proteins were detected using primary antibodies against DnaA (Taylor et al., 2011), DnaK (Baldini et al., 1998), RpoA, CtrA, *E. coli* Lon (kindly provided by R.T. Sauer), or the M2-tag, as appropriate, and a 1:5000 dilution of secondary HRP-conjugated antibody. Blots were scanned with a Typhoon scanner (GE Healthcare), images were processed with Adobe Photoshop, and the relative band intensities were quantified with ImageJ software.

^{35}S-pulse labeling

Caulobacter cultures grown to OD_{600} 0.15 were pulse-labeled with 10 μCi of [^{35}S]-methionine per ml of culture for 10 min. Cells were pelleted and snap frozen in liquid nitrogen. Cell pellets were resuspended in SDS buffer (10 mM Tris pH 8, 1% SDS, 1 mM EDTA) and boiled for 5 min before being pre-cleared in wash buffer (50 mM Tris pH 8, 150 mM NaCl, 0.5% Triton X-100) with 15 μl protein A-agarose (Roche Diagnostics). Pre-cleared protein extracts were incubated overnight with DnaA or RpoA antiserum (1:1000 dilution). Bound proteins were immunoprecipitated with 15 μl protein A-agarose, washed three times with wash buffer and resuspended in SDS loading buffer, boiled for 5 min and resolved by SDS-PAGE. Gels were dried, exposed to Phosphorimager screens and scanned with a Typhoon scanner (GE Healthcare).

Protein purification and modification

Lon protease and His_6-tagged titin^{I27}-β20 were purified as previously described (Gora et al., 2013; Gur and Sauer, 2008), with an additional ion-exchange step (MonoQ) for Lon. Purified titin^{I27}-β20 was carboxymethylated with 10 mM sodium iodoacetate, 6 M guanidine HCL and 25 mM Tris pH 8.0. After modification, the protein was exchanged in 25 mM Tris (pH 8.0) buffer and stored at 4 °C. DnaA was produced by expressing a His6 tagged SUMO-DnaA fusion, purifying this construct via Ni-NTA agarose, removal of the tagged SUMO portion with Ulp1 protease, and a second subtractive Ni-NTA passage as described (Wang et al., 2007) in buffer A (200 mM L-glutamic acid potassium salt, 10 mM magnesium acetate, 20% sucrose, 1 mM dithiothreitol) with either 10 mM or 300 mM imidazole. Cleaved DnaA was further purified using cation exchange (MonoS) chromotography. The β20 peptide (QLRSLNGEWRFAWFPAPEAV) was a gift from R. T. Sauer.

Degradation and ATPase assays

To measure protein degradation *in vivo*, cells were grown under the desired conditions. Protein synthesis was blocked by addition of 100 μg/ml chloramphenicol. Samples were taken every 10 min and snap frozen in liquid nitrogen before being analyzed by Western blotting.

ATP hydrolysis by Lon (0.1 μM Lon_6) was measured using a NADH/pyruvate kinase coupled system at 30°C with the following conditions: 0.2 mM NADH, 5 mM phosphoenol pyruvate and 0.1% pyruvate kinase/lactate dehydrogenase mix (PK/LDH Sigma P0294) in Lon reaction buffer (100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT and 25 mM Tris, pH 8.0). *In vitro* degradation assays were performed in Lon degradation buffer at 30°C with 75 μg/ml creatine kinase, 15 mM creatine phosphate and 4 mM ATP and concentrations of Lon_6, DnaA, titin-$I27^{CM}$-β20, CtrA, SciP or β20 as indicated in figure legends. Samples were taken at appropriate time points, quenched with SDS loading dye and snap frozen in liquid nitrogen. Samples were heated at 65°C for 5 minutes, separated by SDS-PAGE, and proteins detected by Coomassie Blue G-250 staining. DnaA degradation rates were calculated based on quantification of protein band intensity following scanning of stained gels by ImageJ and plotted against titin-$I27^{CM}$-β20 concentration.

Fitting of ATPase/degradation curves

In Figure 5B and S4A, the ATPase activity of Lon was fit to a hyperbolic cooperative activation equation (Eq 1);

$$\text{ATPase rate} = \frac{V_{ATPase,\max}[substrate]^n}{K_{0.5}^n + [substrate]^n} \quad (1)$$

with fitted parameters: n = 3.04 ± 0.07, $V_{ATPase,max}$ = 360.43 ± 2.89 $Lon^{-1}min^{-1}$, $K_{0.5}$ = 13.9 ± 0.14 μM for titin-$I27^{CM}$-β20 stimulation (Figure 5B) and n = 0.9 ± 0.09, $V_{ATPase,max}$ = 169 ± 10.71 $Lon^{-1}min^{-1}$, $K_{0.5}$ = 23.9 ± 4.39 μM for casein stimulation (Figure S4A).

In Figure 5C, the DnaA degradation by Lon was fit assuming a cooperative activation of DnaA degradation based on the cooperative ATPase activation from equation (1) and inhibition that stems from the amount of unoccupied Lon (amount of Lon not bound by the carboxymethylated titin substrate) at high concentrations (Eq 2):

$$\text{DnaA degradation rate} = \frac{V_{DnaA\,\deg,\max}[substrate]^n}{K_{activation}^n + [substrate]^n} * \left(1 - \frac{[substrate]^n}{K_{competition}^n + [substrate]^n}\right) \quad (2)$$

We used a Hill coefficent (n=3) derived from the ATPase stimulation experiment, assuming stimulation of ATP hydrolysis is coincident with DnaA degradation activation and fit the remaining parameters to yield, $K_{activation}$ = 2.09 ± 0.44 µM , $V_{DnaA\ deg,max}$ = 2.26 ± 0.87 $Lon^{-1}min^{-1}$ and $K_{competition}$ = 8 ± 6.03 µM.

Identification of *dnaA* overexpression suppressors

Strain ML2017 (CB15N *dnaA::Ω* + pP_{xyl}-*M2-dnaA*) was grown to late exponential phase, spread on PYE plates containing xylose, chloramphenicol and spectinomycin, incubated at 30°C, and monitored over 3 days. Small colonies were picked and restreaked on PYE plates containing chloramphenicol, spectinomycin, and either glucose or xylose. Putative suppressors were grown in liquid media overnight containing xylose, chloramphenicol and spectinomycin, and then frozen at -80°C. Plasmid DNA was isolated and transformed into wild type to test if induction of P_{xyl}-*M2-dnaA* still resulted in lethality. Plasmids that no longer led to lethality were inferred to contain mutations within the plasmid-borne *dnaA*, which were identified by sequencing the P_{xyl}-*M2-dnaA* construct using primers M13For, M13Rev and *dnaA*_For. Suppressors with plasmids that could still induce lethality were expected to contain chromosomal suppressor mutations. Genomic DNA was isolated from 10 of these candidates and prepared for Illumina whole genome re-sequencing. The

remaining suppressor mutations were identified by directly sequencing *dnaK*, *dnaJ*, and *grpE* using appropriate primers.

Identification of suppressors of DnaK/J depletion

Strain SG400 was streaked from the frozen stock on PYE plates containing glucose and spectinomycin, incubated at 30 °C, and monitored over 3 days. Small colonies were picked and restreaked on plates containing glucose or xylose. The suppressor strains were grown up in PYE with glucose overnight, frozen at -80°C, and sampled for Western blotting to verify absence of DnaK/J. Only clones in which DnaK was depleted to levels comparable to the parental strain SG400 were used for further investigation. Genomic DNA was isolated from these clones and analyzed by Illumina whole genome re-sequencing.

DNA microarrays

RNA was collected from bacteria in exponential phase that were grown under the appropriate conditions and extracted using the RNeasy mini kit (Qiagen). The generation of labeled cDNA and hybridization of custom Agilent arrays was performed as earlier described (Gora et al., 2010).

Illumina sequencing and data analysis

Genomic DNA was isolated using the Qiakit, according to the manufacturer's protocol (Qiagen). DNA concentration and quality were assessed using the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). Illumina sequencing, including sample preparation, was performed at the MIT BioMicro Center. Illumina libraries were generated from intact genomic DNA using the Nextera protocol. Up to ten samples were multiplexed in one lane in a 40 nts single read flow cell using the HiSeq 2000 platform. SNP calling was performed using SAMtools following earlier protocols (Li et al., 2009). Directed gene sequencing verified the mutations identified.

Supplemental Table 1 – Suppressor mutations

Suppressor mutant	Mutation	Genome location	Amino acid substitution
DnaA			
A_19	ATC → ACC	plasmid	DnaA(I399T)
A_50	GTG → GAG	plasmid	DnaA(V426E)
B_71	GTG → GAG	plasmid	DnaA(V426E)
F_3	GCC → ACC	plasmid	DnaA(A407T)
F_14	ATC → GTC	plasmid	DnaA(I448V)
DnaK			
B_6	ACC → GCC	A9356G	DnaK T398A
B_9	GTT → GAT	T9279A	DnaK V372D
B_21	GAA → GGA	A9255G	DnaK E364G
B_22	ACC → GCC	A9356G	DnaK T398A
B_26	FS: CAG GCC → CAG TGC CAA	9655 (+T)	DnaK 497
B_27	FS: AAG GCT GCG → AAG TGC G	9719 (-GC)	DnaK 519
B_33	FS: CAG GCC → CAG TGC CAA	9655 (+T)	DnaK 497
B_35	ACC → GCC	A9356G	DnaK T398A
B_40	ACC → GCC	A9356G	DnaK T398A
B_45	AAC → AAG	C8815G	DnaK N217K
B_56	GGC → GAC	G8376C	DnaK G74D
B_57	AAC → AGC	A9501G	DnaK N446S
B_65	GAC → TAC	G9578T	DnaK D472Y
B_68	ACC → GCC	A9356G	DnaK T398A
B_78	GAC → AAC	G8186A	DnaK D8N
B_85	CGC → GGC	C8330G	DnaK R56G
B_7	GAG → GTG	A8838T	DnaK E225V
B_20	CGC → CCC	G8385A	DnaK R71P
E_51	51 bp duplication	9046 (+51)	DnaK 294
D_8	GAC → GGC	A9618G	DnaK D485G
C_22	CAG → CGG	A9447G	DnaK Q428R
C_15	CTG → ATG	C9317A	DnaK L385M
E_9	P_{dnaK} (-199): TATCC → TAGCC	T7966G	P_{dnaK}(-199)
D_48	GGC → GCC	G8193C	DnaK G10A
D_9	ACC → TCC	A8195T	DnaK T11S
DnaJ			
B_86	FS: GGC CAG GTG → GGC CAT GGT	10848 (+T)	DnaJ 209
D_12	FS: GGC CAG GTG → GGC CAT GGT	10848 (+T)	DnaJ 209
E_45	FS: GGC CAG GTG → GGC CAT GGT	10848 (+T)	DnaJ 209
C_32	FS: CCC CAG CGC → CCC AGC GCG	10568 (-C)	DnaJ 116
GrpE			
D_42	CCC → CTC	C162899T	GrpE P164L

Supplemental Table 2 - Strains and Plasmids

Name	Genotype/description	Source
***C. crescentus* strains**		
CB15N	Synchronizable derivative of wild-type CB15	(Evinger and Agabian, 1977)
ML2017	CB15N *dnaA::Ω(spec^r)*, pML1716-*dnaA*	This study
SG400	*P_{xyl}-dnaK-dnaJ(spec^r)*	(da Silva et al., 2003)
ML2002	SG400 Δ*lon*	This study
ML2003	SG400 ΔclpA	This study
ML2004	SG400 Δ*ftsH(tet^r)*	This study
ML2005	SG400 Δ*CCNA_00108 (tet^r)*	This study
ML2006	SG400 *hslV*::Tn5	This study
ML2007	CB15N *hslV*::Tn5	Lab strain collection
ML2008	SG400 pCT133-*Pvan-dnaA*	This study
ML2009	SG400 pCT155-*vanR-Pvan-dnaA*	This study
ML2010	CB15N pML1716-*lon*	This study
ML2012	Δ*clpP*, pCT133-*P_{lacI}-lacI-P_{lac}-clpP*	Lab strain collection
ML2013	Δ*clpX*, pCT133-*P_{lacI}-lacI-P_{lac}-clpX*	Lab strain collection
ML2014	ML2012 *P_{xyl}-dnaK-dnaJ(spec^r)*	This study
ML2015	ML2013 *P_{xyl}-dnaK-dnaJ(spec^r)*	This study
ML2016	CB15N *P_{dnaK}::Tn5* (-66)	Lab strain collection
LS2382	CB15N Δ*lon (spec^r)*	(Wright et al., 1996)
ML2022	LS2382 pCT133-*vanR-P_{van}-lon*	This study
ML2023	ML2002 pCT133-*vanR-P_{van}-lon*	This study
***E. coli* strains**		
EPC517	BL21DE3 pLysS pET23b-HIS$_6$Sumo-ccDnaA	This study
EPC460	W3110 Δ*lon* pBAD-ccLon	(Gora et al., 2013)
DH5α	General cloning strain	Invitrogen
TOP10	General cloning strain	Invitrogen
General purpose vectors		
pENTR/D-TOPO	ENTRY vector for Gateway cloning system (*kan^R*)	Invitrogen
pCT155	Destination vector of pJS14, high-copy plasmid (*chlor^R*)	Lab plasmid collection
pCT133	Destination vector of pMR20, low copy plasmid (*tet^R*)	Lab plasmid collection
pML1716	Destination vector of pJS14; high-copy, *P_{xyl}*, M2 tag (*chlor^R*)	Lab plasmid collection
pNPTS138	Integration vector	Lab plasmid collection
pKO3	Template for amplifying a *tet* resistance cassette	Lab plasmid collection
Plasmids		
pENTR-*lon*	pENTR containing the *lon* open reading frame	This study
pENTR-*P_{van}-dnaA*	pENTR containing P$_{van}$ fused to *dnaA*	This study
pCT155-*vanR-P_{van}-dnaA*	pCT155 containing *dnaA* behind *vanR-P_{van}*	(Jonas et al., 2011)
pCT133-*P_{van}-dnaA*	pCT133 containing *dnaA* behind *P_{van}*	This study
pNPTS-*UHR-DHR(lon)*	for generation of a markerless in-frame deletion of *lon*	This study
pNPTS-*UHR-DHR(clpA)*	for generation of a markerless in-frame deletion of *clpA*	This study
pNPTS-*UHR-tet-DHR(ftsH)*	for generation of a *tet* marked in-frame deletion of *ftsH*	This study
pNPTS-*UHR-tet-DHR(lon-like)*	for generation of a *tet* marked in-frame deletion of *CCNA_00108*	This study
pCT133-*P_{lacI}-lacI-P_{lac}-clpP*	pCT133 containing *P_{lacI}-lacI-P_{lac}-clpP*	Lab plasmid collection
pCT133-*P_{lacI}-lacI-P_{lac}-clpX*	pCT133 containing *P_{lacI}-lacI-P_{lac}-clpX*	Lab plasmid collection
pML1716-*lon*	pML1716 containing *lon*	This study
pCT133-*vanR-P_{van}-lon*	pCT133 containing *lon* behind *vanR-P_{van}*	This study
pML1716-*dnaA*	pML1716 containing *dnaA*	(Jonas et al., 2011)
pET23b-HIS$_6$Sumo-ccDnaA	Expression vector for purification of HIS$_6$Sumo-ccDnaA	This study
pBAD-ccLon	Expression vector for purification of Lon	(Gora et al., 2013)
pSH21-HIS6-titin-I27-β20	Expression vector for purification of HIS6-titin-I27-β20	(Gur and Sauer, 2008)

Supplemental Table 3 - Primers

Primers	Sequence (5' → 3')
lon_F	CACCATGTCCGAACTACGTACGC
lon_R	TTAGTGCGTCAGCATGGC
Pvan_F	CACCGATCAAGGCGACGAACCGC
dnaA_Pvan_F	CACGATGCGAGGAAACGACGATGACCATGAAGGGCGGGGTTGC
dnaA_R	CAATCCTACGATACGGTTTCG
dnaA_Sumo_For	TATTTAACCGGTGGTATGACCATGAA
dnaA_Sumo_Rev	ATAT ATGCGGCCGCTTAGCCCCGCAG
UHR_lon_F	CAGCAGAAGCTTACGCTCTGCATCAGGTGG
UHR_lon_ml_R	GTCAGCATGGCGTCGCTGTCACGTAGTTCGGACATGTTCTCAATAC
DHR_lon_ml_F	AGAACATGTCCGAACTACGTGACAGCGACGCCATGCTG
DHR_lon_R	CAGCAGGAATTCGTGGGCAGTGAGGGGATC
lon_test_F	TCGGCCTGACCTTCACC
lon_test_R	CACTCGCTATCGATAGGGAG
UHR_clpA_F	CAGCAGAAGCTTCCTGCGCGGCAAGTTCG
UHR_clpA_ml_R	GCCATGGCCGGTTCGGTCTCCGAAAAAGAGGGCAACGGG
DHR_clpA_ml_F	CCCCGTTGCCCTCTTTTTCGGAGACCGAACCGGCCATG
DHR_clpA_R	CAGCAGGAATTCCGTTACGAGGGCGAGGCG
clpA_test_F	CCTGAAGAGCGAAACCGCC
clpA_test_R	TGCACTTCCACGTCATCCC
tet_ol7_F	CGGTATCGATAAGCTTGATATCG
tet_ol8_R	CTGCAGGAATTCAAGAAGTTCC
UHR_ftsH_F	CAGCAGGGATCCGGCCTTGCTCTGCGCCAG
UHR_ftsH_tet_R	TATCAAGCTTATCGATACCGGTTTCTGAAATTCATACGCCTTGTCTTCC
DHR_ftsH_tet_F	AACTTCTTGAATTCCTGCAGGCCAGCGTCACCGCCTGAC
DHR_ftsH_R	CAGCAGACGCGTTGCACAGGACCAGGACGCC
ftsH_test_F	GCCGGTGAGAGAGTCGCC
ftsH_test_R	CTGATAGCGACGCTCGTCGG
UHR_lon-like_F	CAGCAGGGATCCGACGCCTTCCAGGGCGC
UHR_lon-like_tet_R	TATCAAGCTTATCGATACCGGTAGGCCACCGGCATCCAAG
DHR_lon-like_tet_F	AACTTCTTGAATTCCTGCAGGATGACGAACCGACTTCGATCC
DHR_lon-like_R	CAGCAGACGCGTCCACGGCGAAGACATCG
lon-like_test_F	CCTTTGTCGACGGCCGG
lon-like_test_R	GCCGAAGTGCTGGTCGAG
dnaA_F	CACCTTGACCATGAAGGGCGGGGTTGC
dnaK_F	CACCATGAGCAAGATTATCGGTATCGACC
dnaK_R	GCATGACTCGCTTAAGCCG
dnaK_int_F	AAATCGGCGATGGCGTCTTC
dnaK_int_R	CGACTTCGTCAGGGTTCACG
dnaK_UHR_F	CACCATGACGATCGACATACCGATAGCC
dnaJ_F	CACCATGCGCGACTATTACGAAATTCTC
dnaJ_R	TGGAACGGGAAGCCCTAGC
grpE_F	CACCATGACCGACGAGCAAACG
grpE_R	GATCAGGCCTTGGCGTC
hslV_F	GCGCCCGTGATCAGCTTC
hslV_R	CTTTCCTCGGCGATCAGG
Pvan_F2	CACCGACTGGTTCACACCTAAAGCG
Lon_Pvan_for	CACGATGCGGCGAGGAAACGACGATGTCCGAACTACGTACGC

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