Supplementary Materials

Part 1. Experimental Procedures

Reagents. ACS grade or molecular biology grade reagents were purchased from the following manufacturers: Sigma-Aldrich, Invitrogen, Gibco/BRL, Mallinckrodt, J. T. Baker, Burdick and Jackson, and BioRad. Molecular biology kits and enzymes and other specialized reagents were used according to manufacturer's recommendations and were obtained from the following: PfuTurbo DNA polymerase (Stratagene), Qiaprep Spin Mini-prep Kits, Qiaquick PCR Purification Kit, and Qiaquick Gel Extraction Kit (QIAgen), Bis-Tris NuPAGE gels and MES Running Buffer (Invitrogen), AminoLink Plus Immobilization Kit (Pierce).

Strains, plasmids, and primers. Strain names and relevant genotypes are listed in Table S1. Kanamycin insertion mutations in targeted genes in ML13, ML14, ML19, ML33, ML35, and ML36 were constructed by linear transformation of strain NM200, as detailed below. Mutated genes were moved into MG1655 by P1 transduction with selection on Luria broth (LB) plates containing 25 μg/ml kanamycin. Primers used in this study are listed in Table S2 with other supplementary material. To amplify the open reading frames for GDH and SspB from *E. coli* genomic DNA we used the same PCR reagent conditions and cycling parameters described for the amplification of antibiotic resistance cassettes. The forward primer for GDH (see Table S2) contains restriction sites for NheI and NdeI (underlined) and an RBS (bold) upstream of the start codon for *gdhA* (ATG in the NdeI site). The reverse primer encodes the C-terminus of GdhA and its stop codon (bold), which overlaps with a unique HindIII site (underlined). The ~1.4 kb PCR product was gel purified, digested with NheI and HindIII, and ligated to pBAD24 that had been similarly digested, dephosphorylated with calf-intestinal alkaline phosphatase (New England Biolabs), and gel purified. After transformation into electrocompetent MG1655, an ampicillin

resistant clone expressing high levels of GDH upon arabinose induction was identified and the plasmid, pGDH, was purified and used for the studies reported here. For SspB the forward and reverse primers (Table S2) created unique NdeI and HindIII sites (underlined) at the 5' and 3' end of the *sspB* ORF, respectively. The PCR conditions were the same except that the 72 °C incubation during cycling was 60 seconds, and the annealing temperature during cycling was 63 °C. The ~500 bp PCR product was gel purified, digested with NdeI and HindIII, and purified again. For a vector, we digested pGDH with NdeI and HindIII to remove the *gdh* ORF and gel purified the 5 kb vector DNA band. The dephosphorylated vector was ligated to the digested *sspB* PCR product and transformants were obtained as above and screened for SspB production upon arabinose induction. The identity of the SspB was confirmed by western blotting with anti-SspB antibody (supplied by R.T. Sauer). The plasmid expressing SspB, pSspB, was purified and used for the studies reported here.

P1 transductions. Mutant alleles containing inserted or linked antibiotic resistance genes were moved using standard P1 transduction protocols (1). Transductants were purified once on LB agar plates containing the antibiotic for the selective marker and twice on LB agar, generally at 32° C. *lon* mutants were selected and purified at 37° C to avoid complications due to excess capsular polysaccharide production. To transduce the *ftsH3::kan* mutation, the suppressor, *sfhC21* (2), was first introduced into our strains taking advantage of its linkage to the *glnD* gene. We constructed a *glnD::kan* mutation in the recombination-proficient strain, NM200, by linear transformation with a mutagenic double stranded oligonucleotide (see below) selecting for kanamycin resistance. The *glnD::kan* was moved into YN594 creating ML30078, which cannot grow in the absence of glutamine. ML30078 was transduced with a lysate of P1 grown on CAG39499, carrying the *sfhC21* allele tightly linked to a wild type *glnD* gene. Glutamine

prototrophs were screened for loss of kanamycin resistance and designated ML30094 (MG1655 *clpP::cat sfhC21*). The *ftsH3::kan* mutation was brought into ML30094 by transduction with a lysate of P1 grown on CAG39502. A purified transductant that was both kanamycin and chloramphenicol resistant was designated ML30102 (MG1655 *clpP::cat sfhC21* Δ*ftsH3::kan*). ML30102 forms small colonies on LB agar after 2-3 days at 37 °C and grows slowly in liquid LB, reaching significantly lower cell densities than our other MG1655 derivatives.

To construct ML30110 (MG1655 *clpP::cat clpQ::cat argE::Tn10*), we co-transduced *clpQ::cat* linked to *argE::Tn10* (3). The *argE::Tn10* mutation was moved into by ML30009 by P1 transduction, selecting for tetracycline resistance and screening for chloramphenicol resistance and inability to grow on minimal medium without arginine. After purification, one such transductant was designated ML30076 (MG1655 *clpQ::cat argE::Tn10*). A lysate of P1 grown on ML30076 was used to transduce YN594 (MG1655 *clpP::cat*), and tetracycline-resistant transductants were screened for arginine auxotrophy and chloramphenicol resistance. The strain was designated ML30110 (MG1655 *clpP::cat clpQ::cat argE::Tn10*). The absence of ClpP and ClpQ was confirmed by Western blotting using anti-ClpP and anti-ClpQ polyclonal antibodies.

Construction of deletion mutants. A kanamycin cassette flanked by recognition sites for the FLP recombinase (FRT sites) (4) was amplified from plasmid pKD13 using primers containing overhanging regions of homology to sites upstream and downstream of the genetic regions of interest, usually the open reading frame and stop codon. The oligonucleotides used as primers are provided in Table S2 in the Supplementary Material accompanying this article. The following components, listed at their final concentrations, were combined in a final volume of 50 μl: PfuTurbo Buffer (1X), deoxynucleotide triphosphates (0.2 mM each), pKD-13 (0.2 ng/μl),

forward primer (0.2 pmol/μl), reverse primer (0.2 pmol/μl), PfuTurbo DNA polymerase (0.05 U/μl). Reaction components were mixed on ice, and Pfu DNA polymerase was added last. The cycling parameters used for amplification were: 95 °C for 2 minutes, 30 cycles consisting of steps of 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 90 sec, followed by one cycle at 72 °C for 10 min before the temperature was lowered to 4 °C. DpnI (Stratagene) was added to digest the parental vector, and the PCR product was separated on an agarose gel, excised, and purified using a QIAgen Gel Purification Kit. The PCR product was used for linear transformation of the recombination-proficient strain NM200 (5) selecting for kanamycin resistant recombinants. Colonies were purified on kanamycin plates and screened by Western blotting to confirm that the deleted gene product was absent. To excise the kanamycin resistance gene, cells were transformed with the plasmid, pCP20, encoding the flip recombinase, which catalyzes site-specific excision at the FRT sites. Cells were subsequently cured of pCP20 (4).

Production of rabbit anti-SsrA antiserum and purification of IgG. N-terminal cysteinyl-SsrA (CAANDENYALAA) was synthesized as described (6). The peptide was conjugated to keyhole limpet hemocyanin using the kit provided by Pierce Biotechnology, Inc. following the recommendations of the supplier. The conjugate was mixed with incomplete Freunds adjuvant and injected subcutaneously into a rabbit, with a booster inoculation after four weeks. A sample of serum was collected after 5 weeks and the remainder was taken after 7 weeks. IgG was precipitated from rabbit anti-SsrA antiserum by mixing with an equal volume of saturated ammonium sulfate on ice for 5 min. The precipitated protein was collected by centrifugation and dissolved by addition of 1.4 ml phosphate buffered saline (PBS). The precipitation was repeated another two times with 45% saturated ammonium sulfate by the addition of saturated ammonium

sulfate and water. The final precipitate (~20 mg protein) was dissolved in PBS containing 10% (v/v) glycerol in a volume of 1 ml, frozen on dry ice, and stored at -80 °C.

Affinity purification of anti-SsrA antibody. λ CI-SsrA (7) was covalently linked to AminoLink Plus resin (Pierce) by mixing 1.1 mg of λ CI-SsrA with 1 ml resin. After the coupling, blocking, and washing steps, the λ CI-SsrA-resin was suspended in 5 ml of PBS containing 0.05% (w/v) sodium azide and stored at 4 °C. For antibody purification, the λ CI-SsrA-resin in a small column was equilibrated with PBS at room temperature. A fraction of purified IgG (1 ml, 20 mg/ml) was loaded onto the column, the column ends were capped, and the mixture was left at room temperature for 1 hour. The resin was then washed with 12 ml of PBS, the bound anti-SsrA IgG was eluted with 0.1 M glycine/HCl, pH 2.5, and the solution was neutralized by addition of Tris base. Purified antibody was stored at –80 °C after addition of glycerol to 10% (v/v).

To map the epitope recognized by the antibody we used a nitrocellulose membrane containing a peptide array in which each position of the 11 amino acid SsrA peptide was changed to each of the twenty amino acids (Jerini Peptide Technologies, Berlin, Germany). The blot was incubated with the affinity-purified antibody, washed, and bound antibody was detected by immunochemical detection as described above. The results confirmed that the antibody specifically recognized the SsrA peptide (Fig. S1). Changes to residues 4-7 of SsrA (DENY) resulted in severely impaired binding of the antibody, while positions 8-10 allowed only conservative substitutions. Because of the C-terminal mode of attachment of the peptide, we could not evaluate the requirement for the terminal alanine residues by this method. Subsequent analysis of mutant SsrA-tagged proteins indicates that the penultimate alanine is also required for recognition (data not shown).

Pulse-chase and immunoprecipitation of GDH-SsrA. L-[^{35}S]-Methionine was from Perkin Elmer (1175 Ci/mmol, 11.0 mCi/ml). Pansorbin cells were from Calbiochem. On the day of use, Pansorbin cells were washed three times with 1X-TBS-T, suspended in their original volume using 1X-TBS-T, and kept on ice.

Cells were grown on M63 minimal media containing glycerol (0.2% (w/v)), thiamine (1 μg/ml), and ampicillin (50 μg/ml) and maintained at 37 °C with shaking at 300 rpm throughout the procedure. When the culture reached OD_{600} ~ 0.2, 0.2% (w/v) arabinose was added and growth was continued for one hour. A small volume of the culture was transferred to a sterile, pre-warmed tube and pulsed for one minute with L-[^{35}S]-methionine at a final concentration of 55 μCi/ml, followed by the addition of unlabeled L-methionine to a final concentration of 1 mM. Samples of 1 ml were retrieved at regular intervals, and the protein was collected by TCA precipitation.

The washed and dried TCA precipitates were dissolved in 40 μl buffer (2% (w/v) SDS, 15% (v/v) glycerol, 50 mM Tris-Cl, pH 7.5, and 2 mM EDTA), boiled 5 minutes, and centrifuged for 5 minutes at maximum speed in a bench top microcentrifuge to remove any debris. Supernatants were diluted with 1 ml of TBS-T. To pre-adsorb contaminating material, Pansorbin (0.1 ml) was then added, and the mixture was incubated at room temperature with rotary mixing for 1 hour. The Pansorbin cells were removed by centrifugation for 5 minutes, and the clarified supernatants were transferred to new tubes. Anti-SsrA serum (5 μl) was added, and the solution was incubated at room temperature for one hour. Pansorbin (0.1 ml) was added and the mixture was incubated at room temperature for one hour with rotary mixing. The Pansorbin was collected by centrifugation for 5 minutes, and the supernatants were discarded. The pellets were washed three times with 1 ml of TBS-T with care taken to remove every trace of

supernatant after the last wash. SDS-PAGE sample buffer (60 μl) was added and the pellets were left at room temperature overnight, after which the samples were vortexed vigorously, boiled 5 minutes, centrifuged briefly to collect condensate, vortexed again, and finally centrifuged for 5 minutes. A set volume (10 μl) of each supernatant was loaded onto a 10% (w/v) acrylamide gel and subjected to SDS-PAGE. After electrophoresis, proteins were fixed in the gel for one hour with 10% acetic acid/20% methanol, equilibrated into mounting solution (25%(v/v) nethanol/2.5% (v/v) glycerol for one hour, sandwiched between two sheets of cellophane, and air-dried. Radioactive bands were visualized by placing the air-dried gels against a phosphor capture screen for 2 days and activating with a Storm Imager (Molecular Dynamics).

Part 2. Supplementary Data

Quantitation of total SsrA tagged protein levels. The linear response for detection of ssrA tagged proteins occurs over a narrow range of protein loading per lane. We chose two strains in which SsrA-tagged proteins accumulate to assess our dynamic range and to estimate the relative amount of endogenous SsrA-tagged proteins in wild type and mutant cells. Deletion of *clpX* alone leads to the single largest increase in accumulated SsrA-tagged proteins relative to wild type (10-20-fold increase) and underscores the primary role ClpX has in degradation of SsrA-tagged proteins. The *clpX* mutant gives rise to a modest steady-state level of SsrA-tagged proteins, because degradation by other proteases, though slower than the ClpXP-mediated degradation, eliminates 30-70% of the accumulated proteins. Maximum stabilization in our experiments occurred in the triple mutant, *clpP clpX lon*. As seen in Fig. S2, this strain accumulates about three times the level of SsrA-tagged proteins as the *clpX* strain, as can be ascertained by noting that 2, 4, and 6 μg protein from the *clpX clpP lon* extract gives the same density as 6, 12, and 18 μg protein from the *clpX* extracts. These fold differences in accumulated SsrA-tagged proteins are very close to that predicted based on degradation rates, which are 84% for the *clpX clpP lon* mutant and 30% for the *clpX* mutant.

Accumulation and degradation of SsrA-tagged proteins during stationary phase. SsrA-tagged proteins could not be detected in cells with ClpXP. In order to determine the conditions giving maximum accumulation of SsrA-tagged proteins we monitored their levels in cells from early in growth until several hours after the end of exponential growth. SsrA-tagged proteins reached a maximum in both *clpX* and *clpX clpA* cells just at the transition to stationary phase (~1.5 h after mid-exponential phase) (Fig. S5, A and B). The levels in *clpX clpA* cells (Fig. S5, B) were about 3-times those in the *clpX* mutant (Fig. S5, A). SsrA-tagged protein levels

decreased slowly for the first two hours in both strains. The decrease most likely reflects degradation of tagged proteins, which outpaces the reduced rate of new protein synthesis in stationary phase. The apparent degradation rates were 0.023 min^{-1} in the *clpX* mutant and 0.01 min^{-1} in the double mutant. The actual rate could be a bit higher, because incorporation of new SsrA tags was very slow but not completely blocked during the first few hours of stationary phase. These rates are very similar to the rate of decay after chloramphenicol addition (see main text, Fig. 9). The ClpA-dependent rate thus appears to be similar to that observed in exponential phase cells, and we can conclude that degradation independent of ClpX does not show a sharp increase at this time. At later times, the rates appear to decrease even more (compare the 255 and 315 min times in panels A and B of Fig. S5).

To monitor the efficiency of ClpXP in removing SsrA-tagged proteins and preventing their accumulation, we took samples from cells with multiple mutations at the start of stationary phase and again after one hour and measured the amounts of SsrA-tagged proteins. The cells were grown to the end of exponential phase (A_{600} 1.5 - 2.0) and samples were removed. The culture was continued for 1 h and new samples were taken. Aliquots of constant culture volume were loaded in adjacent lanes of the gel (Fig. S5), which will show if any loss of SsrA-tagged proteins from the cells occurred. SsrA-tagged proteins were visible only in strains lacking ClpX (lanes 1-4) or ClpP (lanes 7-8). Importantly, no tagged proteins were visible in wild type cells (data not shown) or in a *clpA lon* mutant (*lane 6*). Thus, ClpXP activity alone is sufficient to keep steady state pools of SsrA-tagged proteins at very low levels after cell growth has slowed. Introducing the *clpA* (Fig. S5, *lane 3*) or *lon* (*lanes 2 and 8*) or both (*lane 4*) mutations into the *clpX* strain resulted in higher levels of SsrA-tagged proteins, indicating that ClpAP and Lon can degrade these proteins if they are present at high concentrations in the cell, although such levels

are achieved only in the absence of ClpXP. The highest levels occurred in cells with combined deficiencies in ClpX, ClpA, and Lon ((Fig. S5, *lane 4*) or when both ClpP and Lon were missing (*lane 8*). Interestingly, the levels were higher in the *clpX clpA* mutant than in the *clpP* mutant (Fig. S5, *lane 3 vs lane 7*), consistent with direct measurements showing that degradation is faster in the *clpP* mutant compared to the *clpX clpA* mutant (Table 2). This difference implies that ClpX or ClpA can affect degradation independently of ClpP. In each case only a small decrease in SsrA-tagged proteins was observed after one hour. These data confirm that in the absence of ClpXP degradation occurs at a slow rate during stationary phase. SsrA-tagged proteins were nearly absent from *clpP lon* mutant cells after overnight culture (Fig. 8, *lane 4*), again pointing to a slow degradation pathway in stationary phase cells. The increase in levels of tagged proteins in cells lacking ClpA was greater than expected from the measured rate of ClpA-dependent degradation. We do not have an explanation for this effect, which suggests that ClpA might affect more than just the degradation of these proteins, possibly suppressing their synthesis. We are investigating the role of ClpA in more detail.

Effects of SspB on degradation of SsrA-tagged proteins in stationary phase cells. The previous experiment showed that no SsrA-tagged proteins accumulate in stationary phase cells when ClpXP is present. To determine if ClpXP alone is sufficient to degrade all the SsrA-tagged proteins in stationary phase cells, as we observed in exponential phase cells, we measured tagged proteins in *clpX* cells that lacked *sspB* and both *clpA and sspB* about 2 h after the end of exponential growth by which time the accumulated SsrA-tagged proteins had declined by >75% in *clpX* mutant cells. Fig. S6 shows the results with stationary phase cell extracts along with exponential phase cell extracts for comparison. No SsrA-tagged proteins were detected in wild type, *clpA*, or *sspB* strains during exponential or stationary phase (Fig. S6, lanes 1, 2, 9, and 10),

and no SsrA-tagged proteins accumulated in cells with mutations in both *clpA* and *sspB* at either time Fig. S6, lanes 5, 6, 13, and 14). Thus, ClpA is not responsible for the elimination of SsrA-tagged proteins in stationary phase. Moreover, even without SspB as an adaptor, ClpXP can degrade SsrA-tagged proteins down to very low levels in both growth phases. ClpA degrades SsrA-tagged proteins that accumulate in the absence of ClpX as indicated by increased accumulation when *clpA* is also mutated (Fig. S6, lanes 4 vs 3 and 12 vs 11). There was 2-fold reduced accumulation in stationary phase when SspB was absent, suggesting that SspB might interfere with degradation of the tagged proteins by ClpAP. The effect was seen also in the *clpX clpA* mutant, indicating that the SspB influences degradation by Lon as well as by ClpA, or that SspB affects accumulation of SsrA-tagged proteins by other mechanisms besides blocking the access of other proteases to the tagged proteins. In a separate experiment we measured the degradation rates of SsrA-tagged proteins in *clpX* and *clpX sspB* mutants and found that the rate in the presence of SspB was >70% that seen in its absence (see main text).

Over expression of tmRNA does not allow detection of SsrA-tagged proteins in cells with ClpXP. To determine if increasing the amount of tmRNA in cells would allow detection of SsrA-tagged proteins in the presence of ClpXP, we introduced a plasmid expressing tmRNA encoding a wild-type SsrA tag. The plasmid complemented the chromosomal *ssrA* mutation by restoring SsrA-tagged proteins in a *clpP* mutant background (Fig. S7, *lanes 4 and 8*). However, expression of tmRNA from the plasmid did not produce levels of SsrA-tagged proteins that could be detected in cells containing ClpXP (Fig. S7, *lanes 1, 3, 5, and 7*). Also, excess tmRNA did not increase the amounts of SsrA-tagged proteins seen in *clpP* mutant cells compared to cells with a single endogenous copy of tmRNA (data not shown) and did not prevent the decrease in SsrA-

tagged proteins that occurs in stationary phase (Fig. S7, *lanes 5-8*). These results indicate that SsrA tagging is not limited by tmRNA.

Supplementary figures.

Figure S1. Affinity purified anti-SsrA recognizes the sequence DENY in the SsrA-peptide. A membrane-bound peptide array containing single amino acid variants of the SsrA peptide (Jerini Peptide Technologies, Berlin, Germany) was probed with affinity purified anti-SsrA antibody followed by probing with horseradish peroxidase labeled second antibody and chemiluminescence detection. The columns are labeled with the one-letter amino acid code to indicate the amino acid substituted for the wild type residue at each position. The rows are ordered top to bottom according to the SsrA sequence, and the label adjacent to each row indicates the wild type SsrA residue that was mutated in that row. The peptides in each row are identical in all but a single position, which was filled with a different amino acid in each of the 20 spots. A wild-type peptide is found on the columns in which the inserted amino acid residue matches the residue being replaced in that row.

Figure S2. Detection limits of the affinity-purified antibody. Wild type, *clpX*, *clpP clpX lon*, and *ssrA* cells were grown to mid-exponential phase. Total cellular protein was collected by TCA precipitation and prepared for SDS-PAGE. The first two lanes contain 20 and 100 μg of total cellular protein from the wild type strain (MG1655). Each subsequent lane contains a serial dilution of 20 μg total cellular protein made from a mixture of extracts from the strain of interest and the *ssrA* mutant (ML30020). The number above the lane is the micrograms of protein from the strain of interest, the remainder being made up with total cellular protein from the *ssrA* mutant. For example, in the lane labeled 18, there is 18 μg of total protein from the strain of interest and 2 μg of protein from the *ssrA* mutant. Top panel, *clpX* (ML30035); bottom panel, *clpP clpX lon* (ML30014).

Figure S3. Endogenous SsrA-tagged proteins decreased when new protein synthesis was blocked by addition of spectinomycin. This figure contains two western blots from two representative experiments used to determine rate of degradation. Overnight cultures of ML30035 and YN-594 were diluted 1000-fold into fresh, warmed media and grown to mid-exponential phase, at which time the cultures were split in half. One half received spectinomycin (+ *spectinomycin*) diluted to a final concentration of 0.2 mg/ml from a freshly prepared stock solution and the other half was allowed to continue growing without treatment (- *spectinomycin*). Samples from each culture were withdrawn immediately prior to addition of spectinomycin (-), at the time of addition (*t=0*), and at regular time intervals thereafter. Total protein from each sample was precipitated with cold TCA, and samples were prepared for SDS-PAGE and Western blotting as described above. Protein from constant culture volumes were loaded in the lanes; lane 1 has ~10 μg total protein in both gels. The strains were: top panel, *clpX* (ML30035); bottom panel, *clpP* YN-594.

Figure S4. Progress curves for degradation of SsrA-tagged proteins. The decline in SsrA-tagged proteins following blockage of protein synthesis was used as a measure of protein degradation. Briefly, strains were grown to mid-exponential phase, 200 μg/ml spectinomycin or 100 μg/ml chloramphenicol was added, and samples collected at intervals. Protein was collected by TCA precipitation, separated by SDS-PAGE, and proteins were detected by chemiluminescence after Western blotting with anti-SsrA anti-body. The density in each lane was measured, and the normalized values from several different experiments were plotted versus time. The data were fit to a first-order exponential decay to determine the degradation rate constants shown in Table 1.

Figure S5. Accumulation of SsrA-tagged proteins during stationary phase occurs only when ClpX is absent. A and B, ML30035 (Δ*clpX*) (**A**) and ML30060 (Δ*clpA* Δ*clpX*) (**B**), were inoculated into LB and grown at 37 °C. The times and cell densities when samples were removed are shown above each lane. Gel lanes were loaded with equal amounts of protein (~10 μg) and SsrA-tagged proteins were detected by Western blotting. **C**, Cells were grown in LB at 37 °C, and samples were removed when the culture reached an A_{600} of 2.0; another set of samples was removed 60 min later to determine the extent to which the pools of SsrA-tagged proteins declined in 1 hour. Total protein was isolated by TCA precipitation, and gel loading was arranged so that each lane received proteins from a constant volume of culture. SsrA-tagged proteins were detected by Western blotting with anti-SsrA antibody. Adjacent gels lanes show samples from the same culture taken at 0 min (A_{600} ~2.0) and at 60 min (A_{600} >3.0). The strains used were: lane 1, *clpX* (ML30035); lane 2, *clpX lon* (ML30064); lane 3, *clpX clpA* (ML30060); lane 4, *clpX clpA lon* (ML30067; lane 5, *clpX clpA lon ssrA* (ML30213); lane 6, *clpA lon*, (ML30065); lane 7, *clpP* (YN594); lanes 8, *clpP lon* (ML30010); lane 9, *clpP ssrA* (SG30088).

Figure S6. SspB has minor effects on degradation of SsrA-tagged proteins in exponential or stationary phase. Accumulation of SsrA-tagged proteins was measured in mid-exponential phase (**A**) and 3 h later when cells were in stationary phase (A_{600} ~ 3.5) (**B**). All lanes were loaded with ~10 μg of total cell protein. The data in panel A is identical to the data shown in the main text (Fig. 4A, right panel) and is included here for ease of comparison with the stationary phase data. The genotypes are listed above the lanes. The strains used were: lanes 1 and 9, wild type (MG1655); lanes 2 and 10, *clpA* (SK1004); lanes 3 and 11, *clpX* (ML30035); lanes 4 and 12, *clpA clpX* (ML30060); lanes 5 and 13, *sspB* (ML30018); lanes 6 and 14, *clpA*

sspB (ML30055); lanes 7 and 15 *clpX sspB* (ML30054); and lanes 8 and 16. *clpA clpX sspB* (ML30061).

Figure S7. Endogenous tmRNA is not limiting for synthesis of SsrA-tagged proteins. Four strains, wild type MG1655, YN594 (*clpP*), ML30020 (*ssrA*), and SG30088 (*clpP ssrA*) were transformed with a plasmid expressing tmRNA encoding wild type SsrA tag. Cells were grown at 37 °C in LB and samples were removed in mid-exponential phase (*four left lanes*) and after four hours in stationary phase (*four right lanes*). Lanes are labeled with the relevant genotype of the host and contained ~10 μg of total cell protein collected by TCA precipitation. SsrA-tagged proteins were detected by Western blotting.

Table S1. Strains used in this study.

Strain	Genotype	Comments, reference, or source
MG1655	wild type	
C600	F$^-$ *thr-1 leuB6 thi-1* l*acY1 supE44 rfbD1 fhuA2 mcrA1*	(8)
NM200	DJ480 mini-λ-Cm^R	S. Gottesman
SG22602	MC4100 i^{21} *dsrB-lac argE::Tn10*	S. Gottesman
SG22183	MC4100 *ssrA::cat*	S. Gottesman
ML13	NM200 Δ(*clpX lon*)*::kan*	this paper
ML14	NM200 Δ(*clpP-clpX*)*::kan*	his paper
ML19	NM200 *glnD::kan*	this paper
ML29	C600 *argE::Tn10*	C600 + P1(SG22602)
ML33	NM200 *clpX::kan*	this paper
ML34	NM200 Δ(*clpP-clpX-lon*)*::kan*	this paper
ML35	NM200 *lon::kan*	this paper
ML36	NM200 *sspB::kan*	this paper
YN594	MG1655 *clpP::cat*	S. Gottesman
CAG39499	MG1655 Δ*lac(mlu) sfhC21 zad-220::Tn10*	C. Herman
CAG39502	MG1655 Δ*lac(mlu) sfhC21zad-220::Tn10* Δ*ftsH3::kan*	C. Herman
SG30069	MG1655 *clpA::kan*	S. Gottesman
SG30070	MG1655 *clpP::cat clpX::kan*	S. Gottesman
SG30071	MG1655 *clpP::cat clpA::kan*	S. Gottesman
SG30073	MG1655 *clpP::cat clpA::kan clpX::kan*	S. Gottesman
SG30088	MG1655 *clpP::cat ssrA::cat*	S. Gottesman
SK1002	MG1655 Δ*clpS*	S. K. Singh, this lab
SK1004	MG1655 Δ*clpA*	S. K. Singh, this lab
SK1020	MG1655 Δ*clpS clpX::kan*	S. K. Singh, this lab
ML30008	MG1655 *lon::tet*	S. K. Singh, this lab

ML30009	MG1655 *clpQ::cat*	S. K. Singh, this lab
ML30010	MG1655 *clpP::cat* Δ*lon*	S. K. Singh, this lab
ML30013	MG1655 *clpX::kan*	MG1655 + P1(ML33)
ML30014	MG1655 Δ(*clpP-clpX-lon*)*::kan*	MG1655 + P1(ML34)
ML30015	MG1655 *lon::kan*	MG1655 + P1(ML35)
ML30018	MG1655 *sspB::kan*	MG1655 + P1(ML36)
ML30020	MG1655 *ssrA::cat*	SG22183 + P1(ML20012)
ML30022	MG1655 Δ*clpA clpX::kan*	SK1004 + P1(ML30013)
ML30035	MG1655 Δ*clpX*	ML30013 transformed with pCP20
ML30037	MG1655 Δ*clpX ssrA::cat*	ML30035 + P1(ML30020)
ML30038	MG1655 Δ*sspB*	ML30018 transformed with pCP20
ML30039	MG1655 Δ*clpA clpX::kan ssrA::cat*	ML30022 + P1(ML30020)
ML30054	MG1655 Δ*clpX sspB::kan*	ML30035 + P1(ML30018)
ML30055	MG1655 Δ*clpA sspB::kan*	SK1004 + P1(ML30018)
ML30056	MG1655 Δ*clpA* Δ(*clpP-clpX*)*::kan*	SK1004 + P1(ML14)
ML30060	MG1655 Δ*clpA* Δ*clpX*	ML30022 transformed with pCP20
ML30061	MG1655 Δ*clpA* Δ*clpX sspB::kan*	ML30060 + P1(ML30018)
ML30062	MG1655 Δ(*clpP-clpX*)*::kan*	MG1655 + P1(ML14)
ML30064	MG1655 Δ(*clpX-lon*)*::kan*	MG1655 + P1(ML13)
ML30065	MG1655 Δ*clpA lon::kan*	SK1004 + P1(ML30015)
ML30067	MG1655 Δ*clpA* Δ(*clpX-lon*)*::kan*	SK1004 + P1(ML30064)
ML30068	MG1655 *glnD::kan*	ML30000 + P1(ML19)
ML30076	MG1655 *clpQ::cat argE::Tn10*	ML30009 + P1(ML29)
ML30078	MG1655 *clpP::cat glnD::kan*	ML30005 +P1(ML30068)
ML30094	MG1655 *clpP::cat sfhC21*	ML30078 + P1(CAG39499)
ML30102	MG1655 *clpP::cat sfhC21* Δ*ftsH3::kan*	ML30094 +P1(CAG39502)
ML30110	MG1655 *clpP::cat clpQ::cat argE::Tn10*	YN-594 + P1(ML30076)
ML30122	MG1655 *clpP::cat sspB::kan*	ML30018 + P1(YN-594)

ML30165	MG1655 Δ*clpS* (Δ*clpX-lon*)*::kan*	SK1002 + P1(ML30064)
ML30213	MG1655 Δ*clpA* Δ(*clpX-lon*)*::kan ssrA::cat*	ML30067 + P1(ML30020)
ML30218	MG1655 Δ(*clpP-clpX-lon*)*::kan ssrA::cat*	ML30014 + P1(ML30020)
ML30220	MG1655 *clpA::kan* Δ*lon clpP::cat*	ML30010 + P1(ML30004)
ML30256	MG1655 Δ*clpA lon::tet*	SK1004 + P1(ML30008)
ML30260	MG1655 Δ*sspB* Δ(*clpX-lon*)*::kan*	ML30038 + P1(ML30064)
ML30264	MG1655 Δ*sspB lon::tet*	ML30038 + P1(ML30008)
ML30268	MG1655 Δ*sspB lon::tet clpA::kan*	ML30264 + P1(SG30069)
ML30316	MG1655 Δ(*clpP-clpX*)*::kan* Δ*sspB*	ML30038 + P1(ML30062)

Table S2. Primers used in construction of mutant strains and plasmid clones. Deletion mutations were constructed by amplification of a kanamycin cassette flanked by recognition sites for the FLP recombinase (FRT sites) (4) using primers with overhanging sequences homologous to regions flanking the open reading frames of the genes of interest. The newly created strains, the relevant genotypes, and the primers used for amplification of the kanamycin cassette are listed.

Construct	Primer names	Primer sequences
ML33 (NM200*clpX::kan*)	ΔclpX-forward	5'-GTGTGCGGCACAAAGAACAAAGAAGAGGTTTTGACCCATGGTGTAGGCTGGAGCTGCTTC-3'
	ΔclpX-reverse	5'-TTTTTGGTTAACTAATTGTATGGGAATGGTTAATTATTATTCCGGGGATCCGTCGACC-3'
ML35 (NM200 *lon::kan*)	Δlon-forward	5'-ATTACCTGGCGGAAATTAAACTAAGAGAGAGCTCTATGGTGTAGGCTGGAGCTGCTTC-3'
	Δlon-reverse	5'-GCCCTGTTTTTATTAGTGCATTTTGCGCGAGGTCACTATTATTCCGGGGATCCGTCGACC-3'
ML34 (NM200 Δ(*clpPclpXlon*)*::kan*	ΔPXlon-forward	5'-TACAGCAGGTTTTTTCAATTTTATCCAGGAGACGGAAATGGTGTAGGCTGGAGCTGCTTC-3'
	Δlon-reverse	5'-GCCCTGTTTTTATTAGTGCATTTTGCGCGAGGTCACTATTATTCCGGGGATCCGTCGACC-3'
ML36 (NM200 *sspB::kan*)	delta-sspB-F	5'-AGAACGTGAAATGCGTCTGGGCCGGAGTTAATCTGTGTGTAGGCTGGAGC-3'
	delta-sspB-R	5'-AAAAAGACAAAACAGGCCGCCTGGGCCTGTTTTGTAATTCCGGGGATCCGTCGACC-3'

ML19 (NM200 *glnD::kan*)	KO-glnD-forward KO-glnD-reverse	5'-TTAATGCGATAATTTAATCTTATGGGTGGCGCACAG TGTAGGCTGGAGCTGCTTC-3' 5'-CTGCATTGTTAAACTCTTTTCATATCAGTAAACACA ATTCCGGGGATCCGTCGACC-3'
ML13 (NM200 Δ(*clpXlon*)*::kan*)	ΔclpX-forward Δlon-reverse	5'-GTGTGCGGCACAAAGAACAAAGAAGAGGTTTTGAC CCATGGTGTAGGCTGGAGCTGCTTC-3' 5'-GCCCTGTTTTTATTAGTGCATTTTGCGCGAGGTCACT ATTATTCCGGGGATCCGTCGACC-3'
pBAD33-GDH	gdhA-F2 gdhA-R2	5'-AGCTAGC**AAGGAG**TTAACATATGGATCAGACATAT TCTCTGGAGTCATTCC-3' 5'-GCTGCTAAGC**TTA**AATCACACCCTGCGCCAGCA-3'
pBAD33-SspB	sspB-Nde-f sspB-HindIII-r	5'-GCATGCATCAT**ATG**GATTTGTCACAGCTAACACCA-3' 5'-CTAGAAGCTT**TTA**CTTCACAACGCGTAATGCCGG-3'

Supplementary references

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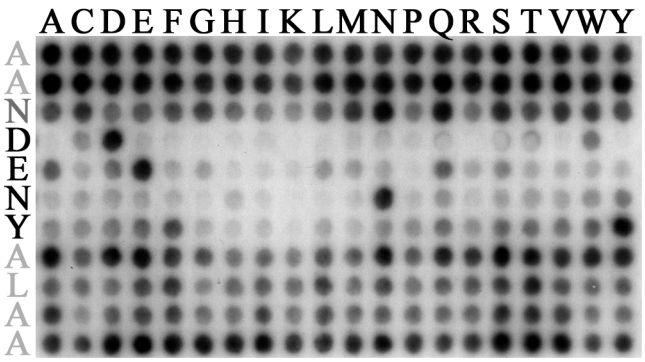

Fig. S1

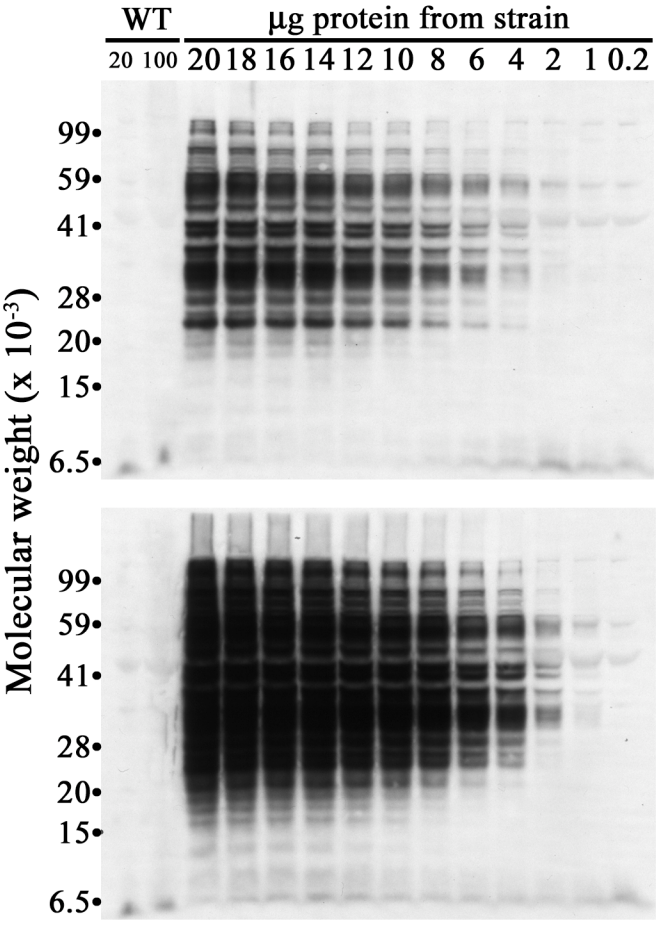
WT
µg protein from strain
20 100 20 18 16 14 12 10 8 6 4 2 1 0.2
Molecular weight (x 10-3)
99
59
41
28
20
15
6.5
99
59
41
28
20
15
6.5

Fig. S2

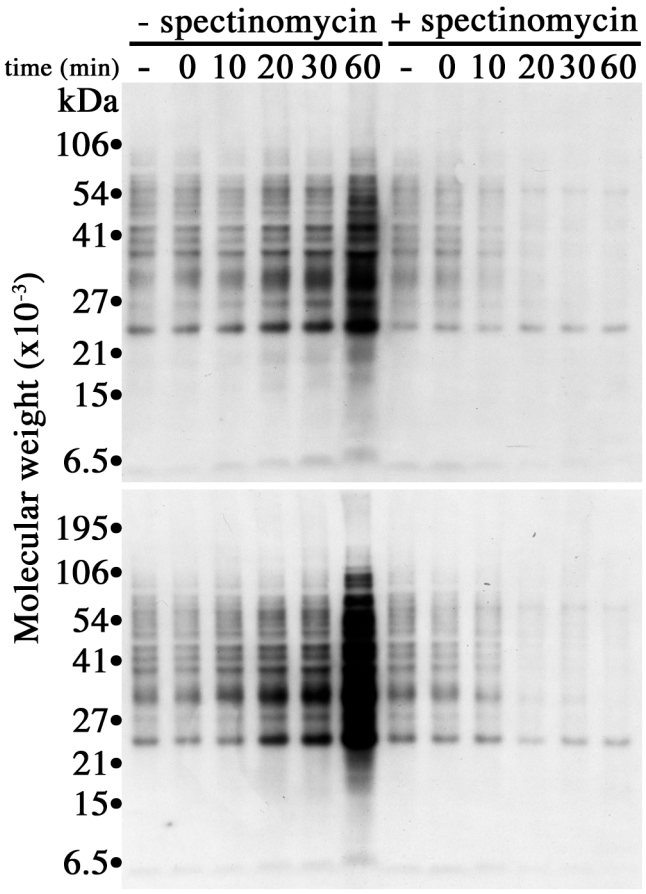
- spectinomycin
+ spectinomycin
time (min)
- 0 10 20 30 60 - 0 10 20 30 60
kDa
106
54
41
27
21
15
6.5
195
106
54
41
27
21
15
6.5
Molecular weight (x10-3)

Fig. S3

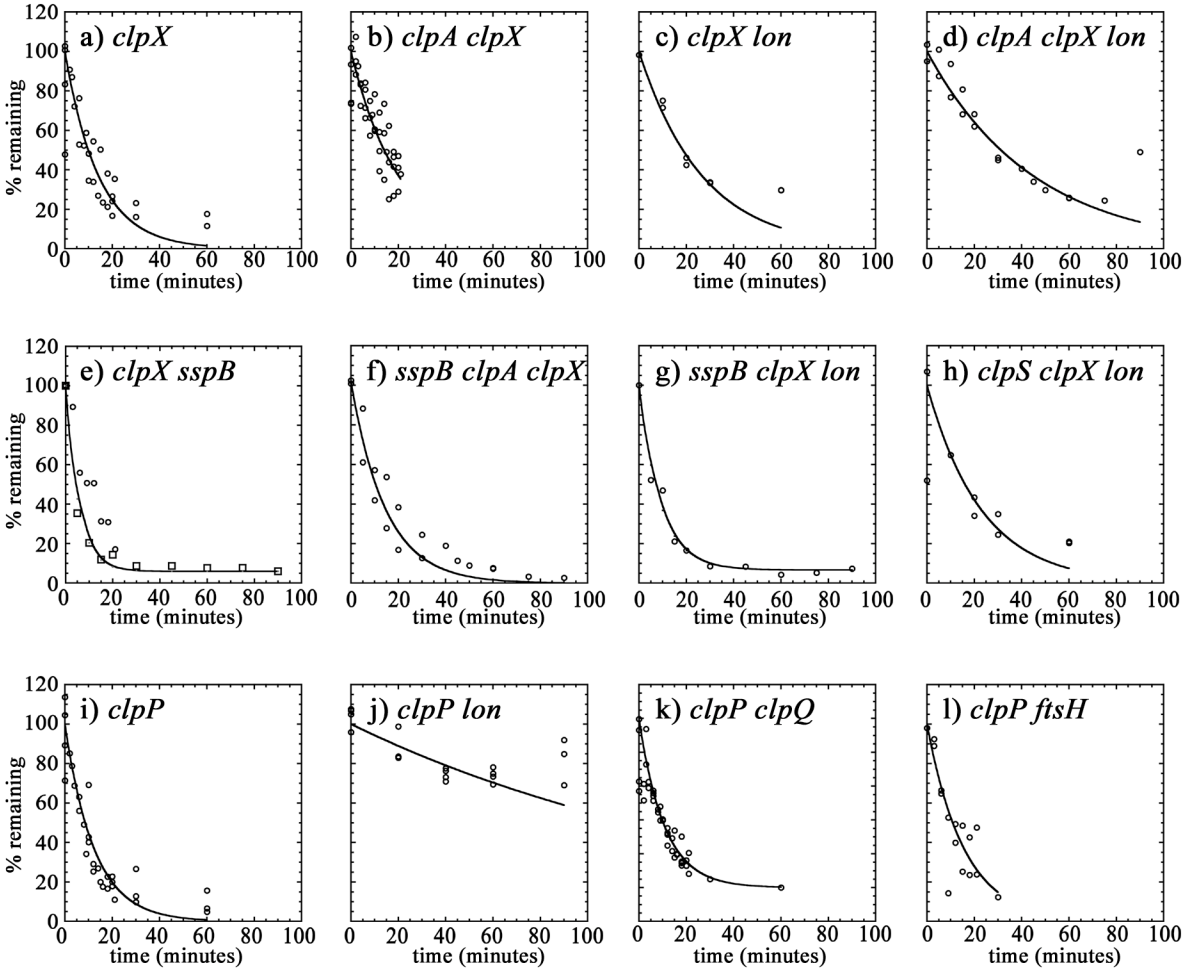
a) clpX
b) clpA clpX
c) clpX lon
d) clpA clpX lon
e) clpX sspB
f) sspB clpA clpX
g) sspB clpX lon
h) clpS clpX lon
i) clpP
j) clpP lon
k) clpP clpQ
l) clpP ftsH
% remaining
time (minutes)

Fig. S4

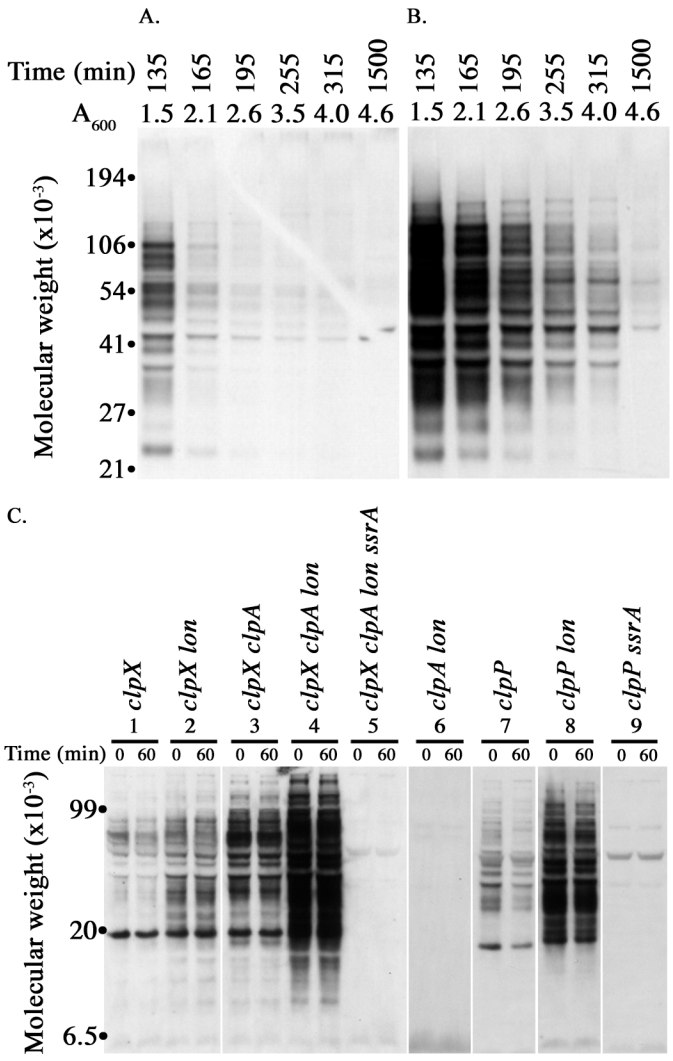
A.
B.
Time (min)
135 165 195 255 315 1500
A600
1.5 2.1 2.6 3.5 4.0 4.6
Molecular weight (x10-3)
194
106
54
41
27
21
C.
clpX
clpX lon
clpX clpA
clpX clpA lon
clpX clpA lon ssrA
clpA lon
clpP
clpP lon
clpP ssrA
1 2 3 4 5 6 7 8 9
Time (min)
0 60
Molecular weight (x10-3)
99
20
6.5

Fig. S5

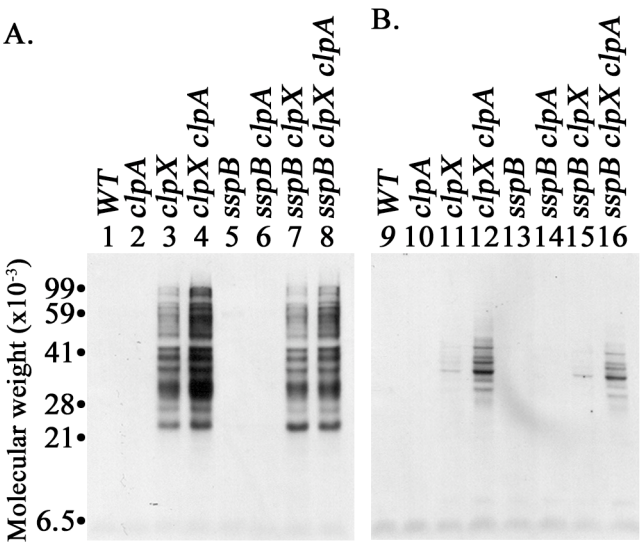
A.
B.
WT clpA clpX clpX clpA sspB sspB clpA sspB clpX sspB clpX clpA
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
Molecular weight (x10-3)
99
59
41
28
21
6.5

Fig. S6

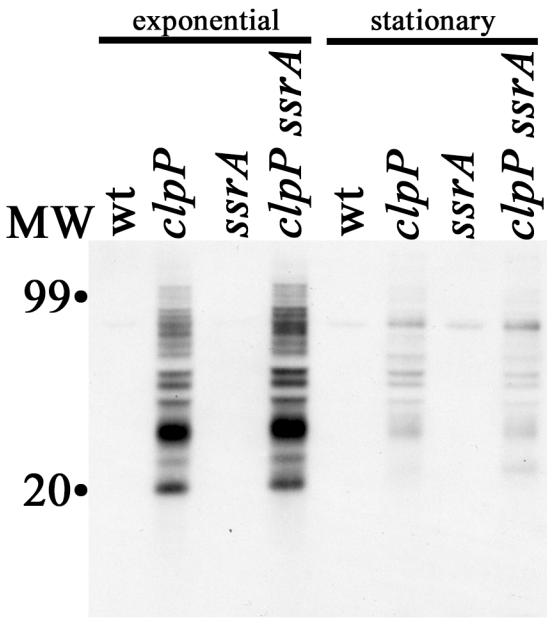
exponential
stationary
MW
wt
clpP
ssrA
clpP ssrA
wt
clpP
ssrA
clpP ssrA
99•
20•

Fig. S7