Plasticity in AAA+ proteases reveals ATP-dependent substrate specificity principles

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Graphical Abstract

In a wildtype cell, AAA+ proteases Lon and ClpXP promote normal growth by degrading distinct substrates. ClpX*P can compensate for the absence of the Lon protease by tuning ClpXP substrate specificity to better degrade Lon-privileged substrates (such as DnaA, SciP, and misfolded proteins) but this comes at the cost of native ClpXP substrates (such as ssrA-tagged proteins and CtrA). We propose that ClpX alternates between a closed and open conformation and promoting one state over the other leads to alterations in substrate specificity. In the presence of ClpX* or in ATP-limited conditions, the open state is favored, allowing capture and recognition of substrates such as casein. The balance shifts to the closed state under high ATP conditions, allowing degradation of substrates such as GFP-ssrA, which preferentially bind the closed state.

Summary

In bacteria, AAA+ proteases such as Lon and ClpXP specifically degrade substrates to promote growth and stress responses. Here, we find that an ATP-binding mutant of ClpX suppresses physiological defects of a Lon-deficient strain by shifting ClpXP protease specificity toward normally Lon-restricted substrates and away from normal ClpXP targets. Reconstitution with purified proteins assigns these effects to changes in direct recognition and processing of substrates. We show that wildtype ClpXP specificity can be similarly altered when ATP hydrolysis is reduced, which unexpectedly accelerates degradation of some substrates. This activation corresponds with changes in ClpX conformation, leading to a model where ClpX cycles between ‘capture’ and ‘processive’ states depending on ATP loading. Limiting ATP binding alters dynamics between states affording better recognition of unorthodox substrates, but worse degradation of proteins specifically bound by the processive state. Thus, AAA+ protease specificities can be directly tuned by differences in ATP hydrolysis rates.

Highlights

- A mutation in the Walker B region of ClpX induces recognition of new substrates.
- Proteases are optimized for specific functions but barrier to recognize new substrates is easily overcome.
- Expanding substrate recognition by a protease comes at the cost of reducing native substrate degradation.
- Decreasing ATP enhances ClpXP mediated degradation of certain classes of substrates.
- ClpX adopts distinct conformational states to favor better recognition of some substrates over others.

Introduction

Energy-dependent protein degradation regulates normal growth and stress responses in all cells. In bacteria, regulated proteolysis is carried out by several energy-dependent AAA+ (ATPases associated with cellular activities) proteases which include Lon, ClpXP,

ClpAP, HslUV, FtsH (Gur et al., 2011; Mahmoud and Chien, 2018). These proteases share a similar general architecture containing an unfoldase domain and a non-specific peptidase (Sauer and Baker, 2011). The two domains can be encoded on a single polypeptide, such as in the case of Lon and FtsH, or they can be encoded by separate proteins, an ATPase, such as ClpX, and a peptidase, such as ClpP (Baker and Sauer, 2012; Langklotz et al., 2012; Lee and Suzuki, 2008). Using the power of ATP hydrolysis, the unfoldase recognizes, unfolds, and translocates substrates into the sequestered peptidase chamber to be degraded. Because they are critical for maintaining proteostasis, loss of these proteases results in defects in growth, cell-cycle progression, stress responses, and pathogenesis (Breidenstein et al., 2012; Howard-Flanders et al., 1964; Jenal and Fuchs, 1998; Rogers et al., 2016).

Despite the similarities in both architecture and mechanism, these proteases generally have distinct niches of substrate preference. For example, in *Caulobacter crescentus*, Lon is the principal protease for degradation of the replication initiator DnaA (Jonas et al., 2013), the methyltransferase CcrM (Wright et al., 1996), and the transcriptional regulator SciP (Gora et al., 2013). By contrast, the ClpXP protease degrades cell-cycle factors like CtrA and TacA during cell-cycle progression through an adaptor hierarchy (Joshi et al., 2015). Lon is also responsible for misfolded protein degradation (Goldberg, 1972; Gur and Sauer, 2008; Jonas et al., 2013). However, while a handful of target substrates for these AAA+ have been identified, it remains unclear how proteases are able to discriminate among protein targets and recognize distinct substrates for irreversible degradation.

In this work, we find that a variant of ClpX (*clpX**) can compensate for the absence of the Lon protease. Expression of *clpX** suppresses defects in motility, growth, filamentation, and sensitivity to stress normally seen in a Δlon strain. In addition to this phenotypic rescue, degradation of normal Lon substrates is restored *in vivo* and *in vitro* by ClpX*P. This increased ability to degrade noncanonical substrates comes at the cost of reduced degradation of ClpXP-specific substrates, resulting in fitness defects in otherwise wildtype strains expressing the *clpX** allele. Further mechanistic

characterization shows that ClpX* has reduced catalytic efficiency for ATP hydrolysis, suggesting a connection between ATP utilization and substrate specificity. Consistent with this, limiting ATP for wildtype ClpXP shifts substrate preferences to that of ClpX*P. Limited proteolysis shows that the ClpX unfoldase adopts distinct conformations under these ATP limiting or saturating conditions. Taken together, our work demonstrates that ATP-dependent dynamics between conformational states are important for substrate recognition by AAA+ unfoldases.

Results

A suppressor screen identifies a *clpX* mutant which rescues Δlon

We reasoned that a suppressor screen would allow us to identify novel Lon-related interactions and used a transposon library to identify mutations that restore motility to a Δlon strain. We isolated a high motility mutant with a transposon insertion in CCNA_00264; however transduction experiments revealed that this transposon alone was not able to rescue motility (Figure S1A). Sequencing the genome of the suppressor strain showed a point mutation in *clpX*, which resulted in a single amino acid change from glycine 178 to alanine (*clpX**). This glycine is highly conserved among ClpX homologs and immediately adjacent to the Walker B motif (Figure S1C). By generating the *clpX** allele in a Δlon background, we found that *clpX** on its own partially restores motility (Figure 1A).

We were intrigued that a single mutation in *clpX* could restore motility to cells lacking Lon and chose to further characterize this strain. Cells lacking Lon show growth defects that exhibit as an extended lag phase and reduced cell mass accumulation in stationary phase (Figure 1A). The *clpX** allele rescues the mass accumulation defect, but not the extended lag (Figure 1A). Δlon strains also have significant morphological deficiencies including elongated cell length and longer stalks, external polar organelles characteristic of *Caulobacter*. We found that the *clpX** mutant restored the cell length of Δlon strains to near wildtype levels (Figure 1B, 1C). However, stalks remained elongated in the Δlon *clpX** strain (Figure 1B, 1C).

We next wondered if, in addition to suppression of Δlon morphological and growth defects, sensitivity to certain stressors would be suppressed by this $clpX^*$ allele. One of the originally described phenotypes for *E. coli lon* mutants is DNA damage sensitivity (Mizusawa and Gottesman, 1983; Witkin, 1946) and *Caulobacter* Δlon strains are also highly sensitive to various DNA damaging agents (Zeinert et al., 2018), including mitomycin C (Figure 1C). We found that $\Delta lon clpX^*$ was 100-fold more resistant to MMC than Δlon alone (Figure 1C).

As mentioned previously, Lon is the major protease responsible for the degradation of the replication initiator DnaA and Δlon strains accumulate excess chromosome content (Jonas et al., 2013; Wright et al., 1996). Like the morphological abnormalities, overreplication is suppressed in the $\Delta lon clpX^*$ strain which shows similar chromosome content as wildtype cells (Figure 1D, S1D). To determine if this rescue extended to a systems-level correction, we compared RNA-seq profiles of wildtype, Δlon and $\Delta lon clpX^*$ strains. As expected, many genes (435) are differentially expressed upon loss of Lon (Figure 1E), while the $\Delta lon clpX^*$ strain shows fewer differences (119) with only 85 genes overlapping between these sets. Our interpretation is that expression of $clpX^*$ in a Δlon background largely shifts the phenotypes and transcriptional landscape to more wildtype profiles.

Figure 1

Figure 1: *clpX mutant suppresses *Δlon* phenotypes**

A. Growth curves of *wild type* (*wt*), *Δlon*, and *Δlon clpX** cells grown in PYE. Biological triplicate experiments are shown. Error bars represent 95% confidence interval. Inset shows motility assays as measured by growth in 0.3% PYE agar.

B. Representative phase-contrast microscopy images of *wt*, *Δlon*, and *Δlon clpX** cells grown in PYE during exponential phase. Quantification of cell length and stalk length for three biological replicates of *n*=100 cells.

C. Serial dilution assays comparing colony formation of strains in PYE and PYE supplemented with mitomycin C. Spots are plated 10-fold dilutions of exponentially growing cells from left to right.

D. Flow cytometry profiles showing chromosome content of indicated strains after three-hour treatment with rifampicin. Cells were stained with SYTOX Green to measure DNA content. The fluorescent intensities corresponding to one chromosome (1) and two chromosomes (2) are indicated. Experiment was performed two times. Representative data from one of the biological replicates is shown here.

E. Venn diagram summarizing the number of differentially expressed genes with an FDR cutoff < 0.01 from RNA seq performed with stationary phase cells. Venn diagram created by BioVenn (Hulsen et al., 2008).

See also Figure S1.

ClpX* restores degradation of Lon substrates *in vivo*

Because cells lacking Lon would accumulate normally degraded substrates, we hypothesized that the rescue we observed in the Δlon *clpX** strain was likely due to lower levels of Lon substrates, such as DnaA, CcrM, and SciP (Figure 2A) (Gora et al., 2013; Jonas et al., 2013; Wright et al., 1996). As expected, in unsynchronized cells, DnaA and CcrM levels are higher in the Δlon strain than in wildtype (Figure 2B). Similarly, SciP levels are higher in Δlon swarmer cells in comparison to wildtype (Figure 2D). Interestingly, levels of DnaA and SciP are restored to wildtype levels in the Δlon *clpX** strain; however, CcrM remained at higher levels (Figure 2B). The reduced levels of DnaA likely explain the rescue of replication defects and enrichment analysis of the RNA-seq results shows that the SciP-controlled regulon and cell-cycle gene expression are largely restored in the Δlon *clpX** strain (Figure S2). We reasoned that expression of *clpX** rescues normal growth and critical stress responses in a Δlon strain through restoring wildtype levels of key proteins.

Next, we explored the mechanism by which a mutation in ClpX is able to restore steady state levels of DnaA and SciP. Because DnaA and SciP are controlled by proteolysis, we suspected that protein turnover may be affected. As expected, DnaA is largely stabilized in Δlon strains (Figure 2C; Jonas, et al. 2013); however, we found that DnaA degradation was restored in the Δlon *clpX** strain, with a half-life similar to that of wildtype (Figure 2D). SciP protein dynamics and cell-cycle phase specific levels were also restored in Δlon *clpX** cells (Figure 2D). By contrast, CcrM degradation was still

solely dependent on Lon, even when *clpX** was present, in both unsynchronized cells (Figure 2C) and during cell-cycle progression (Figure S3A).

We considered that changes in protein turnover could be either due to a gain-of-function in the *clpX** mutant or by indirect cellular effects associated with a loss-of-function in *clpX*. To test this, we created a merodiploid Δlon strain expressing a second copy of *clpX* or *clpX** under control of the native promoter along with a normal chromosomal copy of *clpX*. We reasoned that if the effect was direct, then expression of *clpX** would be sufficient to restore DnaA degradation. If the effect was indirect, then DnaA would remain stable. Consistent with a gain-of-function phenotype for the *clpX** allele, we found that DnaA turnover increased upon expression of *clpX** but not wildtype *clpX* (Figure 2E). Similarly, the phenotypic rescue we observed in the Δlon *clpX** strain was also due to a gain-of-function activity as the merodiploid strain expressing *clpX** suppressed Δlon defects, such as growth, motility, and genotoxic stress tolerance (Figure S3B-D). Because ClpXP is a AAA+ protease, we hypothesized that ClpX*P (the protease complex of the variant ClpX* with wildtype ClpP) was now able to recognize and degrade substrates normally degraded by Lon.

Figure 2

Figure 2: *clpX mutant restores levels of Lon substrates through degradation**

A. Lon degrades DnaA, SciP, and CcrM in *C. crescentus* (Wright *et al.*, 1996; Gora *et al.*, 2013; Jonas *et al.*, 2013).

B. Western blot showing DnaA and CcrM levels in wt, Δlon , and $\Delta lon clpX^*$ cells.

Lysates from an equal number of exponential phase cells were probed with anti-DnaA or anti-CcrM antibody. ClpP was used as a loading controls. A representative image and quantifications of triplicate experiments are shown.

C. Antibiotic shutoff assays to monitor DnaA and CcrM stabilities in wt, Δlon , and $\Delta lon clpX^*$ cells.

Chloramphenicol was added to stop synthesis and lysates from samples at the indicated time points were used for western blot analysis. Quantifications of triplicate experiments with substrate shown relative to ClpP control levels are shown to the right. Error bars represent SD.

D. Western blot showing SciP levels in synchronized populations of wt, Δlon , and $\Delta lon clpX^*$ cells. Swarmer cells were isolated using a density gradient and an equal number of cells were released into fresh PYE medium. Samples were withdrawn at the indicated time points and probed with anti-SciP.

E. DnaA stability is measured in Δlon strains expressing an extra copy of wildtype *clpX* or *clpX**. Experiment was performed four times. Representative data from one of the biological replicates is shown here. Quantifications of experiments shown to the right. Error bars represent SD.

See also Figure S2 and S3.

ClpX*P directly degrades Lon substrates *in vitro*

As a direct test of our hypothesis, we purified the ClpX* variant and reconstituted degradation *in vitro*. Upon initial characterization of purified ClpX*, we found that it formed an active ATPase, but hydrolyzed ATP three times faster than wildtype (Figure S4A). Consistent with the fact that Lon is the principal protease for DnaA degradation (Jonas et al., 2013; Liu et al., 2016), we found that wildtype ClpXP poorly degraded DnaA *in vitro* (Figure 3A). However, purified ClpX*P could degrade DnaA four-fold faster than ClpXP (Figure 3A). Similarly, SciP was degraded three-fold faster by ClpX*P in comparison to ClpXP (Figure 3A). Finally, as predicted from our *in vivo* results, CcrM was not degraded by either ClpX*P or ClpXP (Figure 3A, Figure S4B), but was well degraded by Lon (Figure S4B). Thus, ClpX* appears to have an expanded substrate profile which includes some Lon substrates, but others, such as CcrM, remain exclusive to Lon.

Lon recognizes exposed hydrophobic amino acids (Gur and Sauer, 2008) and is best known as a quality control protease that eliminates mis/unfolded proteins (Goff et al., 1984). Given its altered specificity, we considered if ClpX* could now recognize these substrates. Casein is commonly used as an *in vitro* model substrate for mis/unfolded proteins. Lon robustly degrades fluorescently labeled casein, whereas wildtype ClpXP poorly degrades this substrate (Figure 3B). Intriguingly, ClpX*P degrades FITC-casein more than twice as fast as ClpXP (Figure 3B, Figure S4C). Similar to casein, ClpX*P degrades another unfolded Lon substrate, a chemically denatured domain of titin tagged with the hydrophobic sequence β 20, better than wildtype ClpXP (Figure S4D).

Because cells lacking Lon are sensitive to proteotoxic stresses that create misfolded proteins and because ClpX*P is better at degrading mis/unfolded substrates than wildtype ClpXP, we wondered if the Δlon clpX* strain would be more resistant to stressors that induce cellular protein misfolding. Indeed, the presence of the clpX* allele protected Δlon cells from hypersensitivity to the proteotoxic amino acid L-canavanine

(Figure 3B). We conclude that ClpX^{*} has an altered substrate preference, that includes DnaA, SciP, and unfolded proteins, whose degradation are normally governed by the Lon protease.

Figure 3

Figure 3: ClpX^{*}P degrades some Lon substrates faster than ClpXP in vitro.

A. *In vitro* degradation of DnaA, SciP, or CcrM. Assays were performed with 0.1 μM ClpX₆ or ClpX₆^{*}, and 0.2 μM ClpP₁₄. Substrate concentrations were 1 μM DnaA, 5 μM SciP, 0.5 μM CcrM. Quantification of triplicate experiments shown. Error bars represent SD.

B. *In vitro* fluorescence degradation assay of FITC-casein in the presence of ClpXP or ClpX^{*}P or Lon. Degradation assays were performed with 10 μg/mL FITC-Casein, 0.1 μM ClpX₆ or ClpX₆^{*} and 0.2 μM ClpP or 0.1 μM Lon₆. Serial dilution assays comparing colony formation of strains in PYE and PYE supplemented with L-canavanine. Spots are plated 10-fold dilutions of exponentially growing cells from left to right.

See also Figure S4.

ClpX^{*}P is deficient in degradation of native ClpXP substrates

Mutations in ClpX that improve recognition of some substrates can have negative consequences on others (Farrell et al., 2007). GFP-ssrA is a well-characterized ClpXP substrate that is directly bound by the pore loops of ClpX (Fei et al., 2020; Gottesman et al., 1998). We purified GFP fused to the *Caulobacter* ssrA tag (Chien, et al. 2007), determined degradation rates by ClpX^{*}P and ClpXP, and fit these rates to the Michaelis-Menten equation. We found that ClpX^{*}P degrades GFP-ssrA more poorly

than wildtype ClpXP, principally lowering the turnover rate (Figure 4A, Figure S5A), suggesting that either ClpX* fails to bind the ssrA tag as readily or is unable to translocate the reporter protein as well as ClpX. This effect was not due to the GFP as an ssrA-tagged unfolded titin domain was also degraded more slowly by ClpX*P (Figure S5B). Given that the β 20-tagged version of this same construct was degraded more rapidly, we conclude that the altered specificity of ClpX* must rely on the recognition tag identity, rather than on changes in substrate translocation

CtrA is a master regulator in *Caulobacter* that inhibits replication initiation and regulates the transcription of many cell-cycle genes (Laub et al., 2002; Wortinger et al., 2000). *In vivo*, CtrA is degraded during the G1-S transition by ClpXP through the use of an adaptor hierarchy (Domian et al., 1999; Jenal and Fuchs, 1998; Joshi et al., 2015; Quon et al., 1996); however, *in vitro* CtrA can also be directly recognized by ClpXP as its C-terminus resembles the ssrA tag (Chien et al., 2007). Consistent with a reduced ability to recognize ClpXP substrates, ClpX*P degrades isolated CtrA more poorly than wildtype (Figure 4B) and also shows reduced degradation of a CtrA-derived GFP reporter (Smith et al., 2014) in the presence of the full adaptor hierarchy (Figure S5C). We conclude that ClpX*P overall has a diminished ability to recognize and degrade native substrates normally degraded by ClpXP.

Deficiencies of ClpX* revealed when Lon is present

The prior *in vitro* data suggests that rather than just expanding specificity, this mutation has allowed ClpX*P to degrade Lon-dependent substrates better but at the cost of degrading ClpXP-dependent ones. If this is true, while *clpX** is beneficial in a Δlon background, we reasoned that it may be detrimental to *lon*⁺ cells.

To test this hypothesis, we generated a strain with *clpX** as its sole copy. We first examined ssrA tag turnover in this strain by using an ssrA derivative that relies on the SspB adaptor for degradation (eGFP-ssrA(DAS)) (Chowdhury et al., 2010) so that degradation would be sufficiently slowed to be visualized by western blotting. Consistent with our *in vitro* experiments, we observed stabilization and dramatically

higher levels of eGFP-ssrA (DAS) in the *clpX** strain in comparison to wildtype (Figure 4C). We observed reduced CtrA degradation in the *clpX** strain, consistent with our *in vitro* observations (Figure 4C). Finally, DnaA degradation is accelerated in the *clpX** strains compared to wildtype – consistent with our findings that both Lon and ClpX⁺P are able to degrade DnaA (Figures 4C).

We assayed the overall physiological consequence of ClpX⁺ using competitive fitness in mixed cultures. We grew cultures of wildtype *clpX* or the *clpX** strain together with wildtype strains constitutively expressing the Venus fluorescent protein (Persat et al., 2014). We found that while nonfluorescent wildtype cells could slightly outcompete the constitutive Venus expressing cells, the *clpX** strain showed a substantial competitive disadvantage (Figure 4D). We also tested for stress tolerances and found that *clpX** strains fail to survive genotoxic stress as robustly as wildtype (Figure 4E). Our conclusion is that reduced fitness of the wildtype *clpX** strain is likely due to reduced degradation of normal ClpXP substrates (such as CtrA) and prolific degradation of normal Lon substrates (such as DnaA) that together reduce overall fitness. Collectively, this supports our understanding that AAA⁺ proteases have been optimized for specific priorities in a cell – expansion of their substrate profile, as seen with *clpX**, can compensate for loss of other proteases, but at the cost of their ability to recognize their normal substrates.

Figure 4

Figure 4: ClpX* mutant is deficient in degradation of native ClpXP substrates

A. Michaelis-Menten plot showing the rate of degradation as a function of GFP-ssrA concentration by ClpXP and ClpX*P. Inset displays kinetic parameters. Assays were performed with 0.1 μ M ClpX or ClpX* hexamer, 0.2 μ M ClpP, ATP regeneration system, and the indicated concentrations of GFP-ssrA. Data was fitted to Michaelis-Menten equation. Triplicate experiments are shown.

B. *In vitro* degradation assay of His-CtrA in the presence of ClpXP or ClpX*P. Degradation assays were performed with 3 μ M His-CtrA, 0.1 μ M ClpX or ClpX* hexamer, 0.2 μ M ClpP, and ATP regeneration system. Quantification of duplicate experiments is shown below. Error bars represent SD.

C. *In vivo* degradation assay showing eGFP-ssrA (DAS), DnaA, and CtrA stability in *wt* and *clpX** cells. The plasmid encoded M2FLAG-eGFP-ssrA (DAS) construct was induced with the addition of 0.2% xylose. Chloramphenicol was used to inhibit protein synthesis. Samples were withdrawn at the indicated time points and quenched in SDS lysis buffer. Lysate from an equal number of cells was used for western blot analysis and probed with anti-M2, or anti-CtrA, and anti-DnaA antibody. Quantification of triplicate experiments is shown below. Error bars represent SD.

D. Competition assay with wildtype cells harboring *xylX::Plac-venus* (constitutive venus expression) and nonfluorescent *wt* or *clpX** strains. exponential phase cells were mixed 1:1, diluted, and allowed to outgrow for 12 doublings. . Quantification of triplicate experiments is shown below. Error bars represent SD.

E. Serial dilution assays comparing colony formation of *wt* and *clpX** cells in PYE and PYE supplemented with MMC. Spots are plated 10-fold dilutions of exponentially growing cells from left to right.

See also Figure S5.

Limiting ATP alters wildtype ClpXP substrate specificity

To understand how this mutation in ClpX leads to enhanced degradation of certain substrates, we further characterized the ATPase activity of ClpX*. While ClpX* hydrolyzes ATP three-fold faster than wildtype ClpX at saturating ATP concentrations (Figure S4A), Michaelis-Menten experiments showed that the K_M increases by 5-6 fold and the catalytic efficiency for ATP hydrolysis decreases by approximately 2 fold, suggesting that ClpX* has a different relationship with ATP compared to wildtype ClpX (Figure 5A). As seen previously, degradation of GFP-ssrA was precipitously lost below a minimum threshold of ATP (Martin et al., 2008), with the ClpX*P threshold being higher than that of wildtype, consistent with the higher K_M for ATP described above (Figure S6A). For ClpX*P, casein degradation reduced monotonically as ATP concentration was lowered (Figure 5B). In striking contrast, we found that for wildtype ClpXP, casein degradation was increased at intermediate nucleotide concentrations compared to saturating ATP (Figure 5B). Kinetic analysis shows that at these lower ATP concentrations ClpXP degrades casein with a lower K_M and a similar V_{max} compared to saturating ATP conditions (Figure 5C). Since $K_M = K_D + k_{cat}/k_{on}$, the lower

K_M rate suggests that, at a minimum, casein must bind better to ClpX at these concentrations.

These results suggest that ClpX specificity depends on ATP concentration in an unexpected fashion, with lower levels of ATP facilitating ClpX recognition of some substrates. We reasoned that this could explain the altered specificity of the ClpX* mutant, as its increased K_M for ATP may allow ClpX* to resemble this low ATP state even under saturating ATP conditions (Figure 5A). Remarkably, this enhanced degradation at low ATP extended to other ClpX* substrates, such as DnaA and SciP (Figure 5D and S6B). We conclude that ClpX preference depends on ATP state, with its specificity expanded when not fully loaded with ATP.

This *in vitro* data suggests that ClpXP may play a role in better degrading some substrates such as DnaA *in vivo* under ATP limiting conditions. To test this, we monitored degradation of DnaA during starvation using a $\Delta lon \Delta clpA$ strain which should eliminate any non-ClpX dependent degradation (Liu et al., 2016). Glucose starvation has been shown to accelerate DnaA degradation (Gorbatyuk and Marczyński, 2005) which we found true for wildtype cells as well (Figure 5E). Importantly, starving $\Delta lon \Delta clpA$ also markedly increased DnaA degradation (Figure 5E) concurrent with a reduction in ATP levels (Figure S6C).

We considered conformational changes in ClpX that might explain the altered specificity at low ATP. Using limited proteolysis to probe conformational differences we found that ClpX at low ATP was more protease accessible than at high ATP (Figure 5F). Consistent with the shared altered specificity, protease accessibility of ClpX* at high ATP was more similar to wildtype ClpX at low ATP than high (Figure 5F). This suggests that a more open structure of ClpX is present at low ATP and with ClpX* that allows it to recognize some substrates more readily. We hypothesize that this low ATP-induced conformational difference may reflect the observed changes in AAA+ structures seen in recent cryo-EM studies and discuss their ramifications below.

Figure 5

Figure 5: Limiting ATP alters wildtype ClpXP substrate specificity

A. Michaelis-Menten plot showing the rate of ClpX/ClpX* catalyzed ATP hydrolysis as a function of ATP concentration. Inset displays kinetic parameters. Assays were

performed with 0.1 μM ClpX or ClpX* hexamer, in the presence and absence of 0.2 μM ClpP. Data was fitted to Michaelis-Menten equation. Quantification of duplicate experiments.

B. FITC-Casein degradation by ClpXP or ClpX*P as a function of ATP concentration. Assays were performed with 0.1 μM ClpX or ClpX* hexamer, 0.2 μM ClpP, ATP regeneration system, and 50 $\mu\text{g/mL}$ FITC-Casein. Quantification of duplicate experiments.

C. Michaelis-Menten plot showing the rate of degradation as a function of FITC-Casein concentration by ClpXP and ClpX*P under low and saturating ATP conditions. Inset displays kinetic parameters. Assays were performed with 0.1 μM ClpX or ClpX* hexamer, 0.2 μM ClpP, ATP regeneration system. Data was fitted to Michaelis-Menten equation. Quantification of triplicate experiments

D. *In vitro* degradation of DnaA by ClpXP under low and saturating ATP conditions. Assays were performed with 0.1 μM ClpX₆ or ClpX₆*, and 0.2 μM ClpP₁₄ and 0.5 μM DnaA. Quantification of triplicate experiments.

E. Antibiotic shutoff assays to monitor DnaA under ATP limiting conditions in *wt* and $\Delta lon \Delta clpA$ strains. Chloramphenicol was added to stop synthesis and lysates from samples at the indicated time points were used for western blot analysis. Quantifications of triplicate experiments shown to the right. Error bars represent SD.

F. Limited proteolysis assay to probe conformational states. ClpX was incubated with Chymotrypsin in Hepes buffer (20 mM Hepes pH 7.5, 100 mM KCl, 10 mM MgCl₂, 10% glycerol) at 12 μM ATP and 4 mM ATP in the presence of ClpP and ATP regeneration system at 25 °C for the indicated time points. ClpX* was incubated with Chymotrypsin in Hepes buffer (20 mM Hepes pH 7.5, 100 mM KCl, 10 mM MgCl₂, 10% glycerol) at 4 mM ATP in the presence of ClpP and ATP regeneration system at 25 °C for the indicated time points. ClpX was detected using anti-ClpX antibodies.

See also Figure S6.

Discussion

Because proteolysis is irreversible, degradation of substrates must be carefully monitored to avoid toxic consequences. In bacteria, different energy dependent proteases have distinct substrate preferences that allows them to collectively regulate proteome dynamics. However, how these energy-dependent proteases choose their target substrates from a pool of cellular proteins remains poorly understood. Certain determinants that dictate specificity include degrons, or degradation tags, such as the *ssrA* tag which marks proteins for degradation by ClpXP or ClpAP (Keiler, 2015; Keiler et al., 1996). Specificity can be augmented through adaptor proteins that help deliver substrates to proteases. For example, degradation of CtrA in *Caulobacter* is restricted to the ClpXP protease via a highly regulated series of adaptors (Joshi et al., 2015).

In this work, we identified a mutant allele of *clpX* (*clpX*^{*}) that compensates for the loss of Lon through expanding ClpXP substrate specificity. The variant ClpX^{*}P protease complements the growth, motility, replication status, and morphology defects of a Δlon strain (Figure 1A-1C) by restoring levels and degradation of the Lon substrates DnaA and SciP (Figure 2), which we confirm with biochemical experiments (Figure 3). Interestingly, ClpX^{*}P has an improved ability to degrade unfolded protein substrates, a feature that likely explains the increased proteotoxic tolerance of Δlon *clpX*^{*} strains (Figure 3). Finally, this *clpX* variant is deficient in proteolysis of normal ClpXP substrates, such as CtrA and *ssrA*-tagged proteins, suggesting a shift in substrate preference rather than just an expansion (Figure 4). Mechanistic enzymology revealed that ClpX^{*} requires ~5-fold more ATP for saturation (Figure 5A), leading us to explore the role of ATP levels in controlling specificity. Surprisingly, our *in vitro* studies found that wildtype ClpXP is better at degrading unfolded proteins, DnaA, and SciP in ATP limiting conditions, but worse at degrading *ssrA*-tagged proteins, similar to that seen with ClpX^{*}P in saturating ATP (Figure 5B, 5D, S6B). We recapitulate this effect *in vivo*, showing that in starvation conditions that deplete ATP, DnaA degradation by wildtype ClpXP is accelerated (Figure 5E). Our interpretation is that the ClpX oligomer adopts distinct conformations in the ATP-saturated and ATP-limited conditions, as suggested by limited proteolysis (Figure 5F).

How does altered ATP loading alter substrate recognition? Our working model is that ClpX samples a 'open' state and a 'closed' state (Figure 6). Substrates can be captured during the open-closed transition (as we propose for casein) or directly by the closed state (as proposed for the *ssrA* tag and ClpX (Hersch *et al.*, 2005; Fei *et al.*, 2020). Once a closed state engages a substrate, the unfoldase can processively translocate the substrate to ClpP in the case of ClpXP (Figure 6). Recent cryo-EM structures reveal shared features of substrate-bound AAA⁺ complexes, namely a shallow right-handed ring with pore loops gripping the substrate and at least 4 ATP-bound protomers (Fei *et al.*, 2020; Gates *et al.*, 2017; Lopez *et al.*, 2020; Ripstein *et al.*, 2020; Shin *et al.*, 2020). Consistent with our sampling model, substrate-free forms of these machines exhibit

more open, often left-handed, spiral structures (Shin et al., 2020; Yokom et al., 2016). Interestingly, the original crystallization of ClpX shows a left-handed open spiral crystal packing (Kim and Kim, 2003), similar to these substrate-free cryo-EM structures described for other AAA+ machines. We propose that mutations or limiting ATP can shift the open/closed balance to the more open state, which allows ClpX to better capture certain substrates, such as unfolded proteins, which may not have as strict sequence requirements for recognition. However, degrons such as the ssrA tag which bind selectively to the closed conformation (Fei et al., 2020; Hersch et al., 2005) would be poorly recognized in these conditions.

Prior work has shown that limiting nucleotide leads to loss of substrate degradation by ClpXP (Martin et al., 2008), as would make sense for an ATP-fueled unfoldase. However, we note that these studies exclusively used ssrA-tagged substrates, which requires an ATP-bound (and presumably closed) form of ClpX for recognition. Thus, the equivalent effects we are seeing here for casein, DnaA, and SciP would not have been previously observed and also highlights the importance of using a range of substrates in understanding activity. The open-capture / closed-processive model described here also has precedence in the conformational states and activity of Hsp104 (Gates et al., 2017; Lee et al., 2019; Ye et al., 2020; Yokom et al., 2016), where doping in nonhydrolyzable ATP analogs results in enhanced unfolding activity (Doyle et al., 2007). In our current work, we have directly shown how different states of ClpX, favored by either limiting ATP or mutation, can result in meaningful biological consequences, for example by compensating for the loss of the Lon protease. Overall, we favor the interpretation that exclusive substrate profiles for each AAA+ system are not hard-wired but can be altered not only by mutations but also through modulation of ATP hydrolysis, demonstrating a plasticity in these machines that yield flexibility in maintaining cellular proteostasis.

Figure 6

Figure 6: Model of ATP-dependent changes in ClpXP substrate specificity
ClpX exists in an equilibrium between an open and closed conformation. Under high ATP conditions, the closed conformation is favored, allowing ClpX to recognize and processively degrade certain substrates, such as GFP-ssrA, which makes sequence-specific contacts with pore loops of ClpX in a specific conformation (shown in yellow). GFP-ssrA can then be unfolded and translocated to ClpP for irreversible degradation. Under low ATP conditions or in the presence of ClpX*, the balance shifts towards the open state, allowing ClpX to capture non-traditional classes of substrates, such as casein. ClpX can then switch to the closed state allowing processive degradation, unfolding, and translocation of captured substrate.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in supplementary file 2.

Caulobacter crescentus strains were grown in PYE medium (2g/L peptone, 1g/L yeast extract, 1 mM MgSO₄, and 0.5 mM CaCl₂) at 30°C. To generate strains, antibiotics were added to plates at the following concentrations: kanamycin (25 µg/ml), spectinomycin (100 µg/ml), and oxytetracycline (2 µg/ml). After initial selection steps for strain construction (excluding plasmids), antibiotics were excluded for all assays. For *C.*

crescentus motility assays, PYE with 0.3% agar was used and a single colony was stabbed into the agar using a sterile tip and left to incubate at 30°C for 2 to 3 days.

Escherichia coli strains were grown in LB (10g/L NaCl, 10g/L tryptone, 5g/L yeast extract) and supplemented with antibiotics at the following concentrations: ampicillin (100 ug/ml), oxytetracycline (15 µg/ml), spectinomycin (50 µg/ml), kanamycin (50 µg/ml), gentamycin (20 µg/ml). For solid medium, 1.5% agar was used regardless of bacterial species. For all strains, optical density was measured at 600 nm.

For induction purposes, the 477 plasmids were induced with xylose (0.2%) and repressed with glucose (0.2%). For protein expression, 0.4 M IPTG was used for induction.

Cloning and strain constructions

All *Caulobacter* strains were derived from CPC176, an isolate of NA1000. To generate the Δlon *clpX*^{*} strain, a two-step recombination protocol with a sucrose counter selection was utilized (Skerker et al., 2005). The vector pNPTS138 was digested with HindIII and EcoRI. A PCR product of the *clpX*^{*} sequence was amplified from the original motility suppressor and Gibson assembly was then used to generate pNPTS138_*clpX*^{*}. Following transformation with pNPTS138_*clpX*^{*} into NA1000 or Δlon , primary selection was on PYE supplemented with kanamycin. Primary colonies were grown overnight without selection and overnight cultures were played on PYE agar supplemented with 3% w/v sucrose. To validate allelic swap, strains were tested for sensitivity to kanamycin. Δlon *clpX*^{*} was also screened by motility and sequencing of the *clpX* locus validated candidate clones.

ClpX or *clpX*^{*} merodiploid strains were generated by Gibson assembly of PCR product and double digested plasmids of pMCS-2. The plasmids were then electroporated into Δlon and selected onto kanamycin plates. Strains were validated by anti-ClpX westerns.

eGFP-ssrA(DAS) induction strains were generated by electroporating eGFP-ssrA(DAS) 477 plasmid into wildtype and wildtype *clpX** and selecting on spectinomycin. Strains were validated by anti-M2 westerns.

Motility Suppressor screen

Transposon libraries were generated for Δlon . Two-liter PYE cultures were grown to mid exponential phase, pelleted, and washed with 10% glycerol. Competent cells were electroporated with Ez-Tn5 <Kan-2> transposome (Lucigen, Madison, WI). Cells recovered for 90 minutes at 30 °C and then played on PYE plates supplemented with kanamycin. Libraries were grown for 7 days. Colonies were then scraped from the surface, combined, and resuspended to form a homogenous solution of PYE + 20% glycerol.

The Tn library was thawed out and diluted into a flask containing two-liter 0.3% agar. The cell agar mixture was plated and grown at 30 °C for 3 to 5 days. Candidates that appeared motile were validated by innoculating single colonies into motility agar on the same plate as NA1000 as a positive control and Δlon as a negative control and incubating plates for 2-3 days at 30 °C.

Whole genome sequencing

Genomic DNA was extracted using the MasterPure Complete DNA and RNA purification kit (Epicenter Biotechnologies, Madison, WI). A Qubit Fluorometer (ThermoFisher Scientific, Waltham, MA) was utilized to assess DNA concentration. Illumina libraries were generated from the extracted genomic DNA using the NexteraXT (Illumina, San Diego, CA) protocol. Libraries were multiplexed and sequenced at the University of Massachusetts Amherst Genomics Core Facility on the NextSeq 500 (Illumina). Single nucleotide polymorphisms (SNPs) were detected using breseq (Deatherage and Barrick, 2014).

RNA sequencing

RNA was extracted from stationary phase cells. Libraries were generated from the extracted RNA using the NEB Next RNA Library Prep kits (NEB, Ipswich, MA). Libraries were sequenced at the University of Massachusetts Genomics Core Facility on the NextSeq 500 with single end 75 base reads. Reads were mapped to the *Caulobacter* genome using BWA (Li and Durbin, 2009) and sorted with Samtools (Li et al., 2009). To obtain the number of reads per gene, *bedtools map* (Quinlan and Hall, 2010) was used. Rstudio and EdgeR was used to identify differentially expressed genes (R Core Team, 2019; Robinson et al., 2009).

Raw counts were normalized using the counts-per-million (CPM) method, and the *edgeR* package in R was used to perform differential gene expression analysis, KEGG pathway analysis, and FRY (Wu et al., 2010) gene set analysis. We filtered out any gene that had less than 30 normalized counts across 3 or more samples. To test for gene expression differences and identify differentially expressed genes across experimental groups, we used the quasi-likelihood F-test. The over-representation analysis for KEGG pathways done using the *kegga* function. FRY gene set analysis done using the *fry* function. The CcrM, DnaA, and SciP regulons were obtained from (Gonzalez et al., 2014; Hottes et al., 2005; Tan et al., 2010).

Plating viability and drug sensitivity

All *Caulobacter* strains were grown overnight in liquid PYE media. After overnight growth, cells were back diluted to OD₆₀₀ 0.1 and outgrown to mid-exponential phase before being normalized to OD₆₀₀ 0.1 and 10-fold serially diluted on to media. For experiments using mitomycin C (Sigma, St. Louis, MO) and L-canavanine (Sigma), drugs were prepared at a stock concentration of 0.4 µg/ml mitomycin C, and 100 mg/ml L-canavanine and filter sterilized. PYE agar was cooled before the drugs were added and plates were left to air dry prior to serial dilution plating. All plates were incubated at 30 °C for 2-3 days and imaged with a gel doc.

In vivo assays

The stability of proteins *in vivo* was determined by inhibiting protein synthesis upon addition of 30 µg/ml chloramphenicol to cells in exponential phase. At each time point, 1ml of culture was removed and centrifuged at 15,000 rpm for 2 minutes. The supernatant was removed and pellets were flash frozen in liquid nitrogen. Pellets were thawed, resuspended in 2x SDS dye, and normalized to the OD₆₀₀ of the lowest sample. Samples were boiled for 10 minutes and centrifuged for 10 minutes at 15,000 rpm. Extracts were run on 10% Bis-Tris gels for 1 hour at room temperature at 150 V. Gels were then transferred to nitrocellulose membranes for 1 hour at room temperature at 20V. Membranes were blocked with 3% milk in Tris-based saline with 0.05% Tween-20 (TBST) for 1 hour. Membranes were probed with primary antibody in 3% milk in TBST at 4°C overnight with following dilution factors: 1:5000 dilution of DnaA, 1:5000 SciP, 1:5000 CcrM, 1:5000 ClpP, 1:2500 Lon, and 1:2000 M2. Membranes were washed with 1x TBST for 5 minutes three times and then probed with Licor secondary antibody with 1:10,000 dilution in 1x TBST at room temperature for 1 hour. The protein was visualized using Licor Odyssey CLx. Bands were quantified using imageJ and degradation rates were plotted using Prism.

To synchronize *Caulobacter* strains, cells were grown to an OD₆₀₀ of 0.3-0.5 in PYE and swarmer cells were isolated using Percoll density centrifugation. Cells were released into PYE.

Flow cytometry was used to measure DNA content in rifampicin-treated cells as previously described (Chen et al., 2009). Cells were rifampicin treated for three hours prior to fixing in 70% ethanol.

Carbon Starvation

Cells were grown in M2G media (6.1 mM Na₂HPO₄, 3.9 mM KH₂PO₄, 9.3 mM NH₄Cl, 0.5 mM MgSO₄, 0.5 mM CaCl₂, 10 µM FeSO₄ (EDTA chelate), 0.2% glucose). Cells were then washed twice in cold M2G media (+glucose samples) or in M2 media (-glucose samples) and resuspended in pre-warmed M2G or M2 media.

For bulk measurements of intracellular ATP, the Bactiter-Glo microbial cell viability assay kit (Promega) was used. Cells were mixed with an equal volume of BacTiter-Glo reagent in 384-well microtiter plates and incubated for five minutes before luminescence was measured.

Growth competition assay

Overnight cultures of CPC798 were mixed with either *NA1000* or *clpX** at a 1:1 ratio. Mixed strains were diluted into fresh PYE and allowed to outgrow for 12 doublings overnight. The initial populations were verified by phase contrast and fluorescent microscopy. Final ratios were normalized to their starting ratios. Data was plotted using GraphPad Prism.

Microscopy

Phase contrast images of logarithmically growing cells were taken by Zeiss AXIO Scope A1. Cells were mounted on 1% PYE agar pads and imaged using a 100X objective. MicrobeJ (Ducret et al., 2016) for ImageJ (Schneider et al., 2012) was utilized to quantify cell lengths. Stalk lengths were quantified using ImageJ. Prism was utilized for representations of cell and stalk length measurements. Representative images of the same scale were cropped to display morphological defects.

Protein Purification and Modification

Untagged Lon, untagged ClpX and ClpX*, and his-tagged ClpP were purified as previously described (Chien et al., 2007; Goldberg et al., 1994; Gur and Sauer, 2008; Levchenko et al., 2000). Titin-I27-β20 and Titin-I27-ssrA were purified as previously described (Gur and Sauer, 2008; Kenniston et al., 2003) and labeled with Fluorescein-5-maleimide (Thermo Scientific™) under guanidine hydrochloride denaturation. The modified protein was buffer exchanged into H-buffer (20 mM Hepes pH 7.5, 100 mM KCl, 10 mM MgCl₂, 10% glycerol) and stored at 4°C. His₆SciP and his₆CtrA were purified as described in (Gora et al., 2013). DnaA and CcrM were purified as his₆SUMO tagged proteins, followed by tag cleavage as described (Jonas et al., 2013; Liu et al., 2016; Wang et al., 2007). GFP-ssrA was purified as previously described (Yakhnin et

al., 1998). CtrA-RD+15 was purified as described (Smith et al., 2014). Detailed purification protocols are available upon request.

In vitro assays

Degradation assays were performed at 30°C and monitored using SDS-PAGE gels as described previously (Bhat et al., 2013). The final concentrations used can be found in the figure legends. Densitometry was performed with ImageJ and degradation rates were plotted using Prism.

Degradation of FITC-Casein, FT-Titin- β 20, and FT-Titin-ssrA was monitored as an increase in fluorescence over time. GFP-CtrARD+15 degradation was observed as a loss of fluorescence over time as described previously (Smith et al., 2014).

ATP hydrolysis for ClpXP and ClpX*P was measured using a coupled kinase assay as previously described (Burton et al., 2003)

Limited Proteolysis

To perform limited chymotrypsin digestion, 0.004 mg/mL chymotrypsin was added to 0.01 mg/mL ClpX or ClpX* in the presence of ClpP and ATP regeneration system and incubated at 25°C for indicated time points. To quench the reactions, 5X SDS dye supplemented with 5 mM Protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to quench the reactions. The samples were run on a 10% Bis-Tris gel and transferred to nitrocellulose as described above. Membranes were probed with primary antibody in 3% milk in TBST at 4°C overnight with following dilution factors: 1:5000 dilution of ClpX. Membranes were washed with 1x TBST for 5 minutes three times and then probed with Licor secondary antibody with 1:10,000 dilution in 1x TBST at room temperature for 1 hour. The protein was visualized using Licor Odyssey CLx.

Acknowledgements

We thank the Chien, Strieter, Stratton, and Serio lab members for helpful comments and discussion on the manuscript. S.A.M. and B.A. were supported in part through the UMass NIH Chemistry Biology Interface Training Program (NIH [T32GM008515](#)) and S.A.M. in part by the HHMI Gilliam Fellowship program. Work in the Chien lab is funded by the NIH (R35GM130320). We thank the Genomics Resource Laboratory (Institute for Applied Life Sciences; University of Massachusetts Amherst) and the Mass Spectrometry Core Facilities for services and equipment support.

Author Contributions: Conceptualization: S.A.M. and P.C.; Investigation, S.A.M. and B.A.; Writing, S.A.M and P.C.; Funding Acquisition, P.C. Supervision, P.C.

Declaration of Interests: The authors declare no competing interests

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Supplemental Figure Legends

Figure S1: Validation of motility screen suppressor

(A-B) Motility assay on 0.3% PYE agar of indicated strains.
C. Sequence alignment of ClpX homologs. Conserved glycine residue and walker B motif are marked.
D. Quantification of the percent of cells with DNA content of 1N, 2N, and >2N in *wt*, Δlon , and Δlon *clpX** strains.

Figure S2: RNA-seq gene set analysis

(A-B) Volcano plot showing the transcriptional differences of Δlon compared to *wt* (left) and Δlon *clpX** compared to *wt* (right), as measured by RNA-seq. The negative $\log_{10}$ of the p value is plotted against $\log_2$ of the fold change mRNA counts. Genes that are marked in red have an FDR <0.05. Transcripts that are associated with either the SciP regulon (Tan et al., 2010) or cell cycle (KEGG pathways) are highlighted in blue.
C. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and FRY gene set analysis. FRY gene set analysis performed using *fry* function from *edgeR* package in R (Robinson et al., 2009; Wu et al., 2010). The CcrM, DnaA, and SciP regulons were assigned according to (Gonzalez et al., 2014; Hottes et al., 2005; Tan et al., 2010). Over-represented pathways in KEGG pathway database identified using the *kegga* function from *edgeR* package in R.

Figure S3: *clpX** mutant is dominant

A. Western blot showing CcrM levels in synchronized populations of *wt*, Δlon , and Δlon *clpX** cells. Swarmer cells were isolated using a density gradient and an equal number of cells were released into fresh PYE medium. Samples were withdrawn at the indicated time points and probed with anti-CcrM.
B. Growth curves of *wild type* (*wt*), Δlon , and Δlon merodiploid with second copy of *clpX** at native locus. Cells grown in PYE.
C. Spot assays comparing colony formation of strains in PYE and PYE supplemented with mitomycin C.
D. Motility assay on 0.3% PYE agar of indicated strains

Figure S4: *In vitro* characterization of ClpX*

A. ATPase activity of 0.1 μ M ClpX/ClpX* plus 0.2 μ M ClpP is displayed. Triplicate experiments are shown.
B. *In vitro* degradation of CcrM by 0.2 μ M Lon is shown as a control. The bottom gel shows CcrM degradation assay in the presence of ATP, with no regeneration mix.
C. FITC-Casein titration in the presence of either ClpXP or ClpX*P. Data was fit to the Michaelis-Menten equation. ClpX*P degrades FITC-Casein 2-3 fold better than ClpXP. Inset shows residual plot for Michaelis-Menten fit
F. Fluorescein-*Titin*-I27- β 20 titration in the presence of either ClpXP or ClpX*P. Data was fit to the Michaelis-Menten equation. V_{max}/K_M was 30% higher for ClpX*P in comparison to ClpXP. Inset shows residual plot for Michaelis-Menten fit.

Figure S5 ClpX*P is deficient in native substrate degradation

A. *In vitro* degradation of GFP-ssrA.

B. Fluorescein-*Titin*-I27-ssrA titration in the presence of either ClpXP or ClpX*P. Data was fit to the Michaelis-Menten equation. V_{max}/K_M was 30% lower for ClpX*P in comparison to ClpXP. Inset shows residual plot for Michaelis-Menten fit.

C. *In vitro* fluorescence degradation assay of eGFP-CtrA-RD+15 in the presence of adaptors. Degradation assays were performed with 1 μ M eGFP-CtrA-RD+15, 0.1 μ M ClpX or ClpX* hexamer, 0.2 μ M ClpP, 2 μ M CpdR, 1 μ M RcdA, 1 μ M PopA, and 20 μ M cyclic di-GMP, and ATP regeneration system.

Figure S6: Modulating ATP changes substrate preferences

A. GFP-ssrA degradation by ClpXP or ClpX*P as a function of ATP concentration. Assays were performed with 0.1 μ M ClpX or ClpX* hexamer, 0.2 μ M ClpP, ATP regeneration system, and 10 μ M GFP-ssrA.

B. *In vitro* degradation of SciP by ClpXP under low and saturating ATP conditions. Assays were performed with 0.1 μ M ClpX₆ or ClpX₆*, and 0.2 μ M ClpP₁₄ and 5 μ M SciP. Quantification of triplicate experiments shown.

C. Relative intracellular ATP concentrations measured using a luciferase-based assay during the course of antibiotic shutoff assay.

Figure S1 - related to figure 1

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Figure S2- Related to Figure 2

Figure S3- Related to Figure 2

Figure S4- Related to Figure 3

Figure S5- Related to Figure 4

Figure S6- Related to Figure 5