A mutation in the N domain of E. coli Lon stabilizes dodecamers and selectively alters degradation of model substrates

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Running Title: Lon<sup>E240K</sup> dodecamers have selective degradation defects
Abstract

*E. coli* Lon, an ATP-dependent AAA+ protease, recognizes and degrades many different substrates, including the RcsA and SulA regulatory proteins. More than a decade ago, the E240K mutation in the N domain of Lon was shown to prevent degradation of RcsA but not SulA *in vivo*. Here, we characterize the biochemical properties of the E240K mutant *in vitro* and present evidence that the effects of this mutation are complex. For example, Lon<sub>E240K</sub> exists almost exclusively as a dodecamer, whereas wild-type Lon equilibrates between hexamers and dodecamers. Moreover, Lon<sub>E240K</sub> displays degradation defects *in vitro* that do not correlate in any simple fashion with degron identity, substrate stability, or dodecamer formation. The Lon sequence segment near residue 240 is known to undergo nucleotide-dependent conformational changes, and our results suggest that this region may be important for coupling substrate binding with allosteric activation of Lon protease and ATPase activity.
Introduction

AAA+ proteases play important biological roles in all organisms (1). Inhibition of the AAA+ Lon protease reduces or eliminates virulence in several pathogenic bacteria and is toxic to human lymphoma cells, whereas Lon overexpression improves the lifespan of some fungi but kills Escherichia coli (2-7). Like most AAA+ proteases, bacterial Lon recognizes substrates by binding to specific amino-acid sequences called degrons or degradation tags (8-9). In E. coli, Lon degrades native regulatory proteins, including the RcsA transcription factor and the SulA inhibitor of cell division, but also appears to be responsible for degrading the majority of misfolded proteins, including β-galactosidase (10-12). The degron that targets RcsA for Lon degradation is unknown, whereas degrons for SulA (called sul20) and β-galactosidase (called β20) have been indentified (8,13). The binding of different degrons stabilize enzyme conformations with high or low protease activities, and the latter state may allow Lon to function as a chaperone (14).

E. coli Lon subunits assemble into a homohexamer, which appears to be the minimal unit of proteolytic function (15). Each subunit contains an N-terminal domain, a central AAA+ module, and a C-terminal peptidase domain (16-17). The active sites for peptide-bond cleavage are sequestered within a bowl-like chamber formed by the peptidase domains of the hexamer (17-18). The hexameric AAA+ ring of Lon appears to regulate access to this chamber by coupling ATP hydrolysis to conformational changes that unfold and translocate substrates through an axial pore and into the chamber (1). The N domain of Lon is required for stable hexamer formation (19-20), and recent studies suggest it also stabilizes a dodecamer, with hexamers and dodecamers being populated at physiological Lon concentrations (21). The dodecamer degrades certain substrates...
as well as the hexamer but degrades other substrates, including casein, at substantially reduced rates (21). Although crystal structures are known for most individual domains of Lon and a low-resolution EM structure of the dodecamer has been reported (21-24), high resolution structures of full-length hexamers or dodecamers have not been solved and it is not known how the N domain stabilizes hexamers or dodecamers or regulates the activities of the Lon catalytic domains.

The E240K mutation in the N domain was isolated in a genetic screen for E. coli Lon variants that inactivated SulA but not RcsA (25). Cells expressing LonE240K from the chromosome showed normal degradation of SulA but displayed reduced degradation and overexpression of RcsA (25). These results are consistent with a model in which the region around residue 240 functions as an RcsA binding site. However, experiments using hydrogen-deuterium exchange and limited proteolysis show that this region undergoes nucleotide-dependent changes in conformation (26-27), and thus the effects of the E240K mutant could be indirect. Here, we characterize the assembly state and activities of LonE240K. This mutant forms a stable dodecamer, which degrades casein as well as the wild-type Lon hexamer and much faster than the wild-type dodecamer. We find that LonE240K binds a peptide mimic of the sul20 degron as well as wild-type Lon but displays severe defects in degrading certain sul20-tagged substrates and modest defects in degrading other sul20-tagged substrates. Similar variability was observed in LonE240K degradation of β20-tagged substrates. The E240K mutation also affects the ability of substrates to stimulate ATP hydrolysis by Lon. These results suggest that the Lon dodecamer can exist in multiple conformations with distinct functional properties. However, LonE240K suppresses proteotoxic stress in vivo as well as wild-type Lon, demonstrating that a dodecamer can mediate this biological activity. In combination, our results suggest that specific substrate properties in
addition to the identity of the degron play important roles in determining how these proteins interact with Lon and allosterically control ATP hydrolysis and proteolysis.

Materials and Methods

Variants of *E. coli* Lon were cloned into pBAD33. For proteotoxic-stress assays (28), the chloramphenicol resistance marker of pBAD33 was replaced with an ampicillin resistance marker from pSH21. Degron-tagged variants of the human titin\(^{I27}\) domain (8,14,29,30) were cloned into a pSH21 vector with an N-terminal His\(_6\) tag. \(\beta20\)-cp6-\(^{SF}\)GFP and cp6-\(^{SF}\)GFP-sul20 (31) were cloned into a pCOLADuet1 vector with an N-terminal His\(_6\) tag followed by a PreScission protease site. Mutations were generated by QuickChange PCR (Stratagene) or standard PCR techniques. Lon variants, cp6-GFP-sul20, \(\beta20\)-cp6-GFP, and titin\(^{I27}\) variants were expressed, purified, and carboxymethylated (if applicable) as described (14,29,31). Following purification, protein preparations had less than 5% contaminants based on Coomassie Blue staining after SDS-PAGE. Assays for degradation and ATP hydrolysis were performed as described (8,14,30,31). Binding of a fluorescently labeled sul20 peptide to proteolytically inactive Lon\(^{S679A}\) was assayed by changes in fluorescence anisotropy. We constructed a His\(_{6}\)-tagged fusion of maltose-binding protein and RcsA (His\(_{6}\)-MBP-RcsA). This fusion protein expressed well and was soluble but did not bind to a Ni\(^{2+}\)-NTA column. A small fraction bound to amylose resin (New England Biolabs), and subsequently chromatographed near the excluded volume of a Superdex S75 gel-filtration column.

Prior to ultracentrifugation, Lon variants were dialyzed overnight against 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 µM EDTA, and 100 µM TCEP. Immediately before loading samples into
dual-sector charcoal-filled epon centerpieces, 1 mM MgCl$_2$ and 100 µM ATPγS were added.

Sedimentation-velocity analysis was performed at 16,000 rpm and 20 °C in a Beckman OptimaXL-1 analytical ultracentrifuge (Biophysical Instrumentation Facility, MIT) using an An60-Ti rotor. SEDFIT (32) was used to calculate the continuous distribution of sedimentation coefficients from 0 to 60 $S$ at a resolution of 200 scans per concentration with a confidence level of 0.95. Calculations were performed using a Lon partial specific volume of 0.7431 mL/g, a density of 1.00831 g/mL, and a viscosity of 0.010475 poise (21).

For the mucoidy assay, E. coli W3110 lon::kan$^8$ cells were transformed with pBAD33 vectors expressing Lon variants from an arabinose promoter. Liquid cultures were grown in Luria-Bertani (LB) broth until early-log phase, diluted 1000-fold in LB broth, and then spread onto minimal media plates containing 0.4% glycerol (33), 25 µg/mL kanamycin, and 10 µg/mL chloramphenicol. Cells were grown at 30 °C for 48 h. The proteotoxic-stress assay tests the ability of Lon variants to support 42 °C growth of cells lacking the chromosomal lon, clpX, and clpP genes and expressing low levels of the DnaJ and DnaK chaperones (28). Anti-Lon antibodies were a gift from E. Vieux (MIT).

**Results**

**Lon$^{E240K}$ forms a stable dodecamer.** We expressed and purified Lon$^{E240K}$ and performed sedimentation-velocity ultracentrifugation to characterize its oligomeric state. Strikingly, Lon$^{E240K}$ sedimented almost exclusively at a position expected for a dodecamer at concentrations in hexamer equivalents ranging from 0.5 to 3 µM (Fig. 1A). At the lowest concentration, Lon$^{E240K}$ was greater than 95% dodecameric, allowing calculation of an upper limit of 2.6 nM for
the equilibrium constant for dodecamer-hexamer dissociation. Under the same conditions, wild-type Lon sediments as a mixture of hexamers and dodecamers (21). Thus, the E240K mutation in the N domain of Lon stabilizes dodecamers relative to hexamers.

The wild-type Lon dodecamer degrades FITC-casein very poorly compared to the hexamer, and thus the enzyme-normalized rate of degradation decreases at higher concentrations where the fraction of dodecamer increases (21). By contrast, we found that the normalized rate of degradation of FITC-casein by Lon\textsuperscript{E240K} remained relatively constant over a 20-fold range of enzyme concentration (Fig. 1B). At a concentration of 0.3 µM, where wild-type Lon is less than 30% dodecameric (21), it degraded FITC-casein with a $V_{\text{max}}$ of 4.4 min\textsuperscript{-1} enz\textsuperscript{-1} and a $K_{M}$ of 16 µM (Fig. 1C). At the same concentration, where Lon\textsuperscript{E240K} is more than 90% dodecameric, it degraded FITC-casein with roughly similar values of $V_{\text{max}}$ and $K_{M}$ (Fig. 1C). Thus, with respect to degradation of this substrate, the Lon\textsuperscript{E240K} dodecamer behaves like the wild-type hexamer rather than the wild-type dodecamer. These results suggest that the wild-type dodecamer and the E240K dodecamer adopt different conformations, with the identity of residue 240 being an important factor in dictating which of these conformations are populated. As assayed by changes in fluorescence anisotropy, Lon\textsuperscript{E240K} and wild-type Lon bound to a fluorescent sul20 peptide with similar affinity (Fig. 1D), indicating that the binding site for the sul20 degron is not occluded or altered substantially in the mutant dodecamer. The maximum anisotropy was higher for Lon\textsuperscript{E240K}, consistent with slower tumbling of a stable dodecamer.

Lon\textsuperscript{E240K} has selective defects in degrading model substrates. We attempted to purify His\textsuperscript{6}-MBP-RcsA for biochemical experiments, but it ran in the excluded volume of a gel-filtration
column, suggesting that it forms soluble aggregates, and was not degraded by wild-type Lon. It is possible that aggregation prevents recognition by Lon, or that another cellular protein is required for Lon degradation of RcsA.

To determine if the E240K mutation affected degradation of other model substrates, we initially tested degron-tagged titin\textsuperscript{I27} proteins that had been unfolded by carboxymethylation (CM) of cysteines normally buried in the hydrophobic core (29). \( V_{\text{max}} \) values for Lon\textsuperscript{E240K} degradation of CM-titin\textsuperscript{I27}-sul20 and CM-titin\textsuperscript{I27}-β20 were 45% and 70%, respectively, of the values for wild-type Lon degradation, and the mutant \( K_M \) values were similar to or higher than the wild-type values (Fig. 2A, 2B; Table 1). Thus, the E240K mutation causes modest defects in the degradation of unfolded titin\textsuperscript{I27} substrates with sul20 or β20 degrons.

Next, we tested Lon\textsuperscript{E240K} degradation of two degron-tagged native substrates, cp6-\textsuperscript{SF}GFP-sul20 and β20-cp6-\textsuperscript{SF}GFP (Fig. 2C, 2D). cp6-\textsuperscript{SF}GFP is a circularly permuted variant of superfolder GFP (31). Strikingly, both substrates were degraded very poorly by Lon\textsuperscript{E240K} with \( V_{\text{max}} \) values reduced by 10-fold or more relative to wild-type Lon and with \( K_M \)'s similar to or higher than the wild-type values (Fig. 2C, 2D; Table 1). The severe defect in degradation of cp6-\textsuperscript{SF}GFP-sul20 by the Lon\textsuperscript{E240K} dodecamer is not a property shared by the wild-type dodecamer, as enzyme-normalized rates of cp6-\textsuperscript{SF}GFP-sul20 degradation were relatively constant over concentrations of wild-type Lon from 0.125 to 2 µM (Fig. 2E), where the ratio of wild-type dodecamers to hexamers has been shown to increase substantially (21).
Does the E240K mutation cause a general defect in protein unfolding? To test this possibility, we assayed Lon and Lon\textsuperscript{E240K} degradation of a set of sul20-tagged variants of native titin\textsuperscript{I27} with different thermodynamic and kinetic stabilities (Fig. 3). $V_{\text{max}}$ for Lon\textsuperscript{E240K} degradation of titin\textsuperscript{I27}-sul20, the most stable variant, was ~50% of $V_{\text{max}}$ for wild-type Lon, a value similar to the Lon\textsuperscript{E240K} defect in degradation of unfolded CM-titin\textsuperscript{I27}-sul20. Surprisingly, however, Lon\textsuperscript{E240K} showed larger defects relative to wild-type Lon in degrading the Y9P, V13P, and V15P titin\textsuperscript{I27}-sul20 variants (Fig. 3). Because the native structures of these substrates are less stable than the structure of titin\textsuperscript{I27}-sul20 (28), Lon\textsuperscript{E240K} appears to have greater difficulty in unfolding certain substrates in a way that does not correlate simply with their thermodynamic or kinetic stabilities.

**Substrate-stimulated ATP hydrolysis by Lon\textsuperscript{E240K}**. To address the linkage between substrate degradation and ATP turnover, we measured stimulation of Lon\textsuperscript{E240K} ATP hydrolysis by CM-titin\textsuperscript{I27}-sul20 or CM-titin\textsuperscript{I27}-\beta20 and observed near wild-type levels of ATP hydrolysis at saturating substrate (Figs. 4A, 4B; Table 1). However, saturation required substantially higher concentrations of these substrates in comparison to wild-type Lon, even though a sul20 peptide bound Lon\textsuperscript{E240K} with an affinity similar to wild-type Lon. By contrast, the maximal level of cp6-GFP-sul20 or \beta20-GFP-cp6 stimulation of ATP hydrolysis by Lon\textsuperscript{E240K} was reduced markedly in comparison with wild-type Lon (Figs. 4C, 4D; Table 1). This very low level of ATPase stimulation by native GFP substrate could be the reason for their very slow degradation, or the slow rate of degradation could be responsible for the low level of ATPase stimulation. In combination, these results show that the E240K mutation impairs degradation and substrate stimulation of ATPase activity in a manner that depends on the detailed properties of the substrate.
Activity of Lon$^{E240K}$ in vivo. We cloned Lon, Lon$^{E240K}$, and proteolytically inactive Lon$^{S679A}$ into low-copy plasmids under control of an arabinose-inducible promoter, transformed *E. coli* strains harboring a deletion of the chromosomal *lon* gene, and tested for a phenotype that depends upon RcsA-activated transcription of enzymes that synthesize capsular polysaccharide (34,35). Cells without Lon secrete excess polysaccharide and are mucoid. As expected (12,25), strains expressing wild-type Lon formed non-mucoid colonies, whereas cells expressing Lon$^{E240K}$, or harboring an empty vector were mucoid (Fig. 5A). Cells expressing proteolytically inactive Lon$^{S679A}$ were also mucoid, suggesting that active degradation is needed to maintain low RcsA levels in this assay. In these experiments, intracellular Lon levels were minimized by the absence of arabinose and were only slightly higher than the normal level of chromosomal Lon as judged by Western blots (not shown). We next tested if Lon$^{E240K}$ could support the growth of cells subjected to proteotoxic stress by a lack of the ClpXP protease, low levels of the DnaK and DnaJ chaperones, and growth at 42 °C (28). Importantly, Lon$^{E240K}$ expressed from the low-copy plasmid without arabinose induction supported growth as well as wild-type Lon, whereas cells with the empty vector died (Fig. 5B). These results suggest that the Lon activities required to suppress proteotoxic stress can be performed by a dodecamer.

Discussion

Wild-type Lon is a complex allosteric enzyme that is active both as a hexamer and as a dodecamer (21). However, unlike the Lon hexamer, the dodecamer fails to efficiently degrade the IbpB heat-shock protein and FITC-casein. The dodecamer contains ~45 Å portals that are absent in the hexamer, and it has been proposed that these portals exclude FITC-casein and IbpB,
which behave as large complexes in solution (21). Lon\textsuperscript{E240K} was originally isolated in a genetic
screen as a variant that failed to degrade RcsA but did degrade SulA (25), a phenotype consistent
with a simple RcsA-binding defect. However, we find that Lon\textsuperscript{E240K} assembles as a stable
dodecamer. If RcsA were present as a large complex in the cell, then it might be excluded by the
portals of the stable Lon\textsuperscript{E240K} dodecamer. Interestingly, however, Lon\textsuperscript{E240K} can degrade FITC-

-casein efficiently and thus must differ in some way from the wild-type dodecamer. One

possibility is that Lon\textsuperscript{E240K} adopts an altered dodecamer conformation with portals large enough
to accommodate FITC-casein. Alternatively, FITC-casein might not be excluded by the entry
portals of the wild-type dodecamer but fail to bind because a docking site is occluded in this
structure. By this model, the E240K mutation could unmask a casein-binding site in the
dodecamer. Either of these models requires somewhat different structures for the wild-type and
E240K dodecamers.

We find that Lon\textsuperscript{E240K} degrades many model substrates more slowly than wild-type Lon.

Interestingly, however, the magnitude of these defects is highly substrate dependent. For
example, \( \nu_{\text{max}} \) for Lon\textsuperscript{E240K} was \(~70\%\) of the wild-type value for CM-titin\textsuperscript{I27}-\( \beta_{20} \), \(~60\%\) for
titin\textsuperscript{I27}-SulA, \(~44\%\) for CM-titin\textsuperscript{I27}-Sul20, \(~35\%\) for Y9P-titin\textsuperscript{I27}-Sul20, \(~22\%\) for V13P-titin\textsuperscript{I27}-
Sul20, \(~12\%\) for V15P-titin\textsuperscript{I27}-Sul20, \(~8\%\) for \( \beta_{20}\)-cp6-SF\textsuperscript{GFP}, and \(~5\%\) for cp6-SF\textsuperscript{GFP}-Sul20.

Defects occurred for substrates bearing \( \beta_{20} \) and SulA degrons and for denatured and native
substrates. Moreover, the size of the defect did not correlate with native thermodynamic stability.

Because AAA+ proteases forcibly unfold native substrates, the mechanical stability of the
substrate protein near the point of attachment to the degradation tag is likely to be more
important than thermodynamic stability (36,37).
The ability of substrates to stimulate ATP hydrolysis by Lon\textsuperscript{E240K} also differs from wild-type Lon. For example, Lon\textsuperscript{E240K} has wild-type levels of ATP hydrolysis when stimulated by CM-titin\textsuperscript{127}-sul20 and CM-titin\textsuperscript{127-β20}, but ~10-fold higher substrate concentrations are required for maximal stimulation. By contrast, the maximal levels of cp6-GFP-sul20 or β20-GFP-cp6 stimulation of ATP hydrolysis by Lon\textsuperscript{E240K} are much lower than those for wild-type Lon. These results suggest that the detailed properties of different substrates play important roles in determining how they interact with and control proteolysis by wild-type and mutant Lon enzymes. Such properties could include the geometric relationship between the position of the primary degron and other regions of the substrate that might interact with different Lon sites, including the axial pore.

The E240K mutation falls within a coiled-coil region of the N domain (residues 232-250) that undergoes nucleotide-dependent changes in conformation (25,26). Because the E240K mutation does not affect binding of a peptide mimic of the sul20 degron but does alter degradation of several sul20-tagged substrates, this sequence change may alter allosteric communication between the sul20 binding site and the sites in the AAA+ module of Lon that bind and hydrolyze ATP to power translocation and unfolding. Indeed, we recently characterized a set of mutations (R33A/E34A/K35A) in the N domain of Lon that prevent binding of a sul20 peptide (38). Interestingly, these mutations cause $V_{\text{max}}$ defects in degradation of sul20-tagged substrates very similar to those observed for Lon\textsuperscript{E240K}. For example, both Lon\textsuperscript{E240K} and Lon\textsuperscript{R33A/E34A/K35A} degrade cp6-GFP-sul20 with $V_{\text{max}}$ values 5-10% of wild-type Lon but degrade CM-titin\textsuperscript{127}-sul20 and titin\textsuperscript{127}-sul20 with $V_{\text{max}}$ values 40-60% of wild-type Lon. Similarly, CM-titin\textsuperscript{127}-sul20 stimulates
ATP hydrolysis by Lon$^{E240K}$ and Lon$^{R33A/E34A/K35A}$ to maximal levels similar to wild-type Lon but 10-fold more substrate is needed to reach these levels for both mutants.

Although the phenotype originally described for Lon$^{E240K}$ by Ebel et al. (25) is consistent with a simple change in substrate discrimination, the biochemical properties of the purified enzyme strongly support a model in which the E240K mutation stabilizes Lon dodecamers, reduces the ability of Lon to degrade many model substrates, and alters allosteric communication between substrate binding and regulation of ATP hydrolysis and proteolysis. Nevertheless, Lon$^{E240K}$ suppresses proteotoxic stress in vivo as well as wild-type Lon, suggesting that Lon$^{E240K}$ dodecamers can execute this biological role. We anticipate that structural studies of the Lon$^{E240K}$ dodecamer will provide further insight into the relationship between different Lon conformations and machine function.

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References


FIG 1. Biochemical properties of Lon\textsuperscript{E240K}. (A) Sedimentation velocity analytical ultracentrifugation of Lon\textsuperscript{E240K} at different concentrations (listed as hexamer equivalents). Traces were normalized to have a maximum signal equal to 1 and offset on the y-axis for clarity. Experiments were performed at 20°C and 16,000 rpm in 50 mM HEPES (pH 7.5), 150 mM NaCl, 0.01 mM EDTA, 0.1 mM Tris (2-carboxyethyl) phosphine, 1 mM MgCl\textsubscript{2}, and 0.1 mM ATP\textsubscript{γS}. (B) Initial rates of degradation of FITC-casein type II (Sigma, 50 µM) by different concentrations of Lon\textsuperscript{E240K} were determined by changes in fluorescence (excitation 490 nm; emission 525 nm). Values are means ± SEM (n ≥ 3). (C) Substrate dependence of degradation of FITC-casein type II by wild-type Lon or Lon\textsuperscript{E240K} (0.3 µM hexamer equivalents). Values are means ± SEM (n = 3). Solid lines are fits to the Hill form of the Michaelis-Menten equation: rate = \( V_{\text{max}} \left( 1 + \left( \frac{[S]}{K_M} \right)^n \right) \). (D) Binding of fluorescein-labeled sul20 peptide (200 nM) by wild-type Lon or Lon\textsuperscript{E240K} was assayed by changes in fluorescence anisotropy (excitation 494 nm; emission 521 nm). Values are means ± SEM (n ≥ 2) after subtraction of the anisotropy of the free peptide. Solid lines are fits to a hyperbolic binding equation with fitted \( K_D \)'s of 4.7 ± 0.2 µM (wild-type Lon) and 2.4 ± 0.1 µM (Lon\textsuperscript{E240K}).
FIG 2. Lon and Lon<sup>E240K</sup> degradation of degron tagged CM-titin<sup>I27</sup> or cp6-SF-GFP proteins. (A) Degradation of CM-titin<sup>I27</sup>-sul20. (B) Degradation CM-titin<sup>I27</sup>-β20. (C) Degradation of cp6-SF-GFP-sul20. (D) Degradation of β20-cp6-SF-GFP. In panels A-D, values are means ± SEM (n ≥ 2), lines are fits to the Hill form of the Michaelis-Menten equation, and rates were determined by acid-soluble radioactivity (for <sup>35</sup>S-labeled CM-titin<sup>I27</sup> substrates) or changes in fluorescence (for cp6-SF-GFP substrates). In panels A-D, the Lon or Lon<sup>E240K</sup> enzymes were present at concentrations of 0.3 µM (hexamer equivalents). (E) Initial rates of degradation of cp6-SF-GFP-sul20 (20 µM) by different concentrations of wild-type Lon were determined by changes in fluorescence (excitation 467 nm; emission 525 nm). Values are means ± SEM (n=3).
FIG 3. Degradation of stability variants of titin^{127}-sul20 by wild-type Lon and Lon^{E240K} (0.3 µM hexamer equivalents). $V_{\text{max}}$ values were determined by fitting the substrate dependence of initial degradation rates (n=3; determined by acid-soluble radioactivity using $^{35}$S-labeled proteins) to the Hill form of the Michaelis-Menten equation; bars represent the error of non-linear-least-squares fitting.
FIG 4. Substrate stimulation of ATP hydrolysis by Lon or Lon$^{E240K}$ (0.15 µM hexamer equivalents). (A) CM-titin$^{I27}$-sul20 stimulation. (B) CM-titin$^{I27}$-β20 stimulation. (C) cp6-$^{{}^{SF}}$GFP-sul20. (D) β20-cp6-$^{{}^{SF}}$GFP. Values are means ± SEM (n ≥ 3). Lines are fits to the equation rate $= V_{max}$ = basal + amp. 

rate = basal + amp/(1+(K_M/[S])^n); V_{max} = basal + amp.
FIG 5. Assays of activity in vivo. (A) Colony morphology of *E. coli* W3110 *lon::kan* cells transformed with pBAD33 plasmids expressing Lon, Lon\(^{E240K}\), Lon\(^{S679A}\), or the empty parental vector. (B) Expression of Lon or Lon\(^{E240K}\) from pBAD33 plasmids but not the empty vector allowed growth at \(42^\circ\)C of a strain expressing reduced levels of DnaK and DnaJ and lacking the chromosomal genes encoding ClpX, ClpP, and Lon.
Table 1. Steady-state kinetic parameters.

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<th>$K_M$ (µM)</th>
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<td>240 ± 20</td>
<td>16 ± 2</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Y9P-titin-β20</td>
<td>Lon</td>
<td>6.7 ± 0.6</td>
<td>38 ± 6</td>
<td>1.6 ± 0.2</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Y9P-titin-β20</td>
<td>Lon</td>
<td>1.5 ± 0.1</td>
<td>34 ± 4</td>
<td>1.8 ± 0.2</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>V15P-titin</td>
<td>Lon</td>
<td>3.0 ± 0.1</td>
<td>29 ± 2</td>
<td>1.2 ± 0.1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>V15P-titin-β20</td>
<td>Lon</td>
<td>1.0 ± 0.1</td>
<td>53 ± 8</td>
<td>1.3 ± 0.1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>titin-β20</td>
<td>Lon</td>
<td>5.5 ± 0.4</td>
<td>55 ± 8</td>
<td>1.3 ± 0.1</td>
<td>nd</td>
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<tr>
<td>titin-β20</td>
<td>Lon</td>
<td>0.65 ± 0.06</td>
<td>31 ± 5</td>
<td>2.3 ± 0.6</td>
<td>118 ± 5</td>
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<tr>
<td>titin-β20</td>
<td>Lon</td>
<td>2 ± 0.1</td>
<td>29 ± 3</td>
<td>1.5 ± 0.2</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>titin-β20</td>
<td>Lon</td>
<td>1.2 ± 0.1</td>
<td>38 ± 3</td>
<td>1.4 ± 0.1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

Errors are from non-linear-least-squares fitting. * Data taken from reference 31. nd – not determined.