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# Distinct regulatory mechanisms balance DegP proteolysis to maintain cellular fitness during heat stress

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Intracellular proteases combat proteotoxic stress by degrading damaged proteins, but their activity must be carefully controlled to maintain cellular fitness. The activity of *Escherichia coli* DegP, a highly conserved periplasmic protease, is regulated by substrate-dependent allosteric transformations between inactive and active trimer conformations and by the formation of polyhedral cages that confine the active sites within a proteolytic chamber. Here, we investigate how these distinct control mechanisms contribute to bacterial fitness under heat stress. We found that mutations that increase or decrease the equilibrium population of active DegP trimers reduce high-temperature fitness, that a mutation that blocks cage formation causes a mild fitness decrease, and that combining mutations that stabilize active DegP and block cage formation generates a lethal rogue protease. This lethality is suppressed by an extragenic mutation that prevents covalent attachment of an abundant outer-membrane lipoprotein to peptidoglycan and makes this protein an inhibitor of the rogue protease. Lethality is also suppressed by intragenic mutations that stabilize inactive DegP trimers. In combination, our results suggest that allosteric control of active and inactive conformations is the primary mechanism that regulates DegP proteolysis and fitness, with cage formation providing an additional layer of cellular protection against excessive protease activity.

[*Keywords*: protein quality control; proteolysis; cellular fitness; DegP; HtrA; Lpp]

Supplemental material is available for this article.

Protein degradation is an essential cellular function. In the absence of degradation, toxic misfolded proteins or aggregates can accumulate after heat shock or other environmental stresses, resulting in reduced viability or cell death. Protein quality control (PQC) proteases play critical biological roles by degrading diverse misfolded proteins but also require careful regulation because proteolysis of functional proteins can be cytotoxic. Intracellular proteases whose activities are allosterically regulated have evolved to meet these conflicting needs.

DegP is a member of the highly conserved HtrA family of proteases, is overexpressed at high temperatures as the major PQC protease in the periplasmic compartment of *Escherichia coli* and many other Gram-negative bacteria, and is implicated in the virulence of several pathogens (Lipinska et al. 1989; Strauch et al. 1989; Pallen and Wren 1997; Clausen et al. 2011). Each subunit of DegP contains a trypsin-like protease domain and two PDZ domains

(Krojer et al. 2002, 2008b; Kim et al. 2011). The protease domains of three subunits pack together to form a stable trimer, which can switch between a proteolytically active conformation with high substrate affinity and a proteolytically inactive conformation with low substrate affinity (Fig. 1A; Kim et al. 2011). DegP trimers display positively cooperative substrate binding to the active enzyme, a hallmark of Monod-Wyman-Changeux (MWC) allostery, which allows proteolysis to be controlled. Substrate binding also triggers the assembly of DegP trimers and hexamers into large polyhedral cages that restrict the access of native proteins to the active sites within these cages (Fig. 1A; Jiang et al. 2008; Krojer et al. 2008b; Kim et al. 2011).

Distinct modes of substrate binding control DegP activation and assembly. In unfolded or misfolded substrates, two degron sequences in a single polypeptide interact with DegP to promote allosteric activation (Kim et al. 2011). Substrate residues flanking scissile peptide bonds bind to

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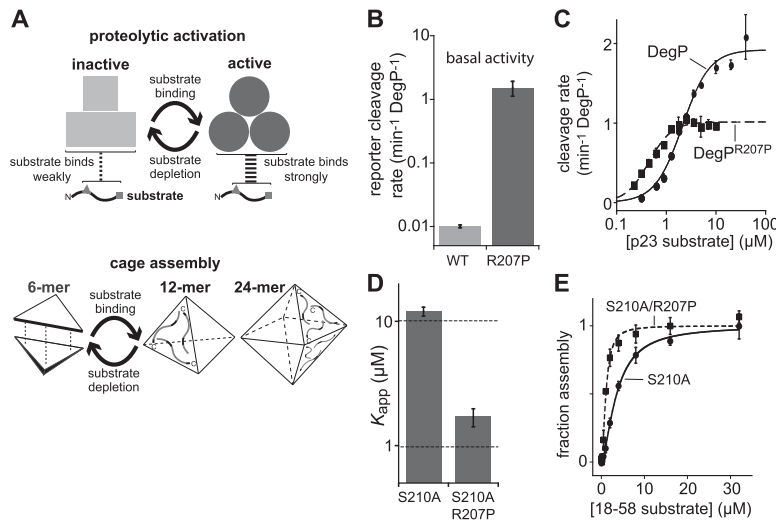[2]Corresponding author
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**Figure 1.** The R207P mutation stabilizes the high-affinity active conformation of DegP. (*A*) Substrate-binding triggers proteolytic activation and cage assembly. The fundamental unit of DegP structure is a trimer, which reversibly transforms between an inactive conformation with low substrate affinity and an active conformation with high substrate affinity. DegP trimers and hexamers also assembled into cages with 12 or 24 subunits. (*B*) Basal cleavage of 100 μM Abz-KASPVSLGY$^{NO2}$D ([Abz] 2-amino-benzoic acid; [Y$^{NO2}$] 3-nitrotyrosine), a poor substrate, by 10 μM DegP or 1 μM DegP$^{R207P}$. Values are averages ± 1 SD ($n = 3$). (*C*) Cleavage rates of the p23 substrate by DegP or DegP$^{R207P}$ were measured at different substrate concentrations at room temperature (22°C). Values are averages ± 1 SD ($n = 3$). Lines are fits to the Hill equation: rate = $V_{M}/\{1 + (K_{M}/[p23])^h\}$. For DegP, $V_{max} = 1.9 \pm 0.1$, $K_{M} = 2.1 \pm 0.2$, and $h = 1.6 \pm 0.2$ (Kim and Sauer 2012). For DegP$^{R207P}$, $V_{max} = 1.0 \pm 0.03$, $K_{M} = 0.45 \pm 0.03$, and $h = 1.9 \pm 0.2$. (*D*) Apparent dissociation constants ($K_{app}$) of a model substrate ($^{flC}$18–58) for proteolytically inactive DegP$^{S210A/R207P}$ (1.7 μM ± 0.3 μM) or DegP$^{S210A}$ (12 μM ± 1 μM) (Kim et al. 2011) were measured by fluorescence anisotropy. Bars represent the nonlinear least squares fitting error. (*E*) Substrate-dependent assembly of DegP$^{S210A}$ or DegP$^{S210A/R207P}$ cages was monitored by Förster resonance energy transfer (FRET) between proteins labeled with acceptor and donor dyes (Kim et al. 2011). Values are averages ± 1 SD ($n = 3$). Lines are fits to a Hill equation. For DegP$^{S210A}$, $K_{app} = 3.6 \pm 0.2$, and $h = 1.6 \pm 0.1$. For DegP$^{S210A/R207P}$, $K_{app} = 1.1 \pm 0.1$, and $h = 1.9 \pm 0.2$.

the active site cleft and oxyanion hole of the active protease domain, whereas a hydrophobic residue at the substrate C terminus binds to a site in the PDZ1 domain. These linked interactions also result in the assembly of trimers into hollow polyhedral cages with 12, 18, or 24 subunits by stabilizing interactions between the PDZ1 domains of one trimer and the PDZ2′ domains of neighboring trimers (Kim and Sauer 2012). Substrate cleavage eventually generates peptide products that bind too weakly to maintain DegP in the active conformation, resulting in cage disassembly and a return to the inactive conformation. Thus, bivalent substrate binding helps ensure that DegP is activated and assembled only in the presence of appropriate substrates.

Several observations demonstrate the coupling of DegP activation and cage assembly. Crystal structures are known for a DegP hexamer in which trimers assume an inactive conformation with a malformed Ser–His–Asp catalytic triad and oxyanion hole and for substrate-bound cages with 12 or 24 subunits in which trimers assume an active conformation (Krojer et al. 2002, 2008b; Kim et al. 2011). In solution, proteolytic activation and cage assembly of DegP also occur synchronously (Krojer et al. 2008b; Kim et al. 2011), which led to the proposal that cage assembly was the molecular switch for activation (Krojer et al. 2008b). We found, however, that cage assembly was not required for either proteolytic activity in vitro or suppression of proteotoxic heat stress in vivo (Kim and Sauer 2012).

At present, it is unclear how allosteric activation and cage assembly contribute to DegP function in vivo and cellular fitness. Here, we show that mutations that increase or decrease the equilibrium population of active DegP trimers result in small decreases in high-temperature

fitness. Combining a mutation that allosterically activates DegP with one that prevents cage formation converts the enzyme into a lethal rogue protease. This lethality appears to be caused by excessive proteolysis of proteins associated with cell envelope maintenance and can be relieved by an extragenic suppressor that alters the cell envelope and directly inhibits the rogue protease or by intragenic mutations that stabilize inactive DegP trimers. We propose that substrate-dependent allosteric control of the conformation and activity of DegP trimers represents the primary form of proteolytic regulation, whereas cage assembly protects cells from promiscuous proteolysis that would otherwise be triggered under heat stress and other conditions that result in protein misfolding and allosteric activation of DegP.

## Results

### A mutation that stabilizes active DegP

To test the consequences of increasing the equilibrium population of functional DegP enzymes, we sought to identify a mutation that preferentially stabilizes the active state. Because DegP mainly adopts an inactive conformation without substrate, mutational stabilization of the active conformation should result in higher basal proteolytic activity and tighter substrate binding (Fig. 1A). In DegS, a trimeric DegP paralog, the H198P mutation results in exactly this phenotype by stabilizing the active conformation of the oxyanion hole (Sohn and Sauer 2009; Sohn et al. 2010). Modeling suggested that the homologous R207P mutation in DegP could also stabilize the active conformation, and thus we constructed and purified DegP$^{R207P}$ for biochemical characterization.

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Several results established that the R207P mutation increased the equilibrium fraction of active enzymes compared with wild-type DegP. First, DegP$^{R207P}$ had substantially higher basal activity in cleaving a poor peptide substrate (Fig. 1B). This reporter peptide binds weakly to active DegP and converts very little wild-type enzyme into the active conformation (Kim et al. 2011). Second, DegP$^{R207P}$ cleaved a good peptide substrate derived from lysozyme (p23) (Kim and Sauer 2012) with a lower $K_M$ (~0.5 μM) than wild-type DegP (~2 μM) (Fig. 1C), as expected if more DegP$^{R207P}$ trimers assume the active high-affinity conformation in the absence of substrate. At saturating concentrations of p23, DegP$^{R207P}$ had lower maximum cleavage activity than wild-type DegP (Fig. 1C), establishing that the higher basal cleavage activity of DegP$^{R207P}$ is not a result of intrinsically increased proteolytic activity. Third, DegP$^{S210A/R207P}$ bound a fluorescent peptide derived from residues 18–58 of lysozyme ($^{flC}$18–58) more tightly (dissociation constant [$K_{app}$] ~1.7 μM) than DegP$^{S210A}$ ($K_{app}$ ~12 μM) (Fig. 1D; Kim et al. 2011). In this assay, the S210A mutation was used to remove the nucleophilic serine from the catalytic triad and prevent degradation. Fourth, the R207P mutation resulted in cage formation at lower concentrations of substrate than required for cage assembly of otherwise identical proteins lacking R207P (Fig. 1E), as assayed in the S210A background by Förster resonance energy transfer (FRET) between trimers labeled with donor dyes and trimers labeled with acceptor dyes (Kim et al. 2011).

## A dominant lethal DegP protease

To test the cellular effects of R207P and DegP mutations that affect cage formation, we constructed E. coli strains with mutant alleles in chromosomal degP and assayed bacterial growth on Luria-Bertani (LB) agar plates at 37°C

or 45°C. The latter temperature induces heat stress and high-level production of DegP. As expected from previous studies (Lipinska et al. 1989; Strauch et al. 1989), the ΔdegP strain grew at 37°C but not at 45°C (Fig. 2A). Both the degP$^+$ strain and the otherwise isogenic degP$^{R207P}$ strain grew reasonably well at both temperatures (Fig. 2A), although the degP$^{R207P}$ strain showed reduced viability at 47°C (Supplemental Fig. S1A). DegP$^{Y444A}$, in which a mutation in the PDZ2 domain prevents cage assembly and makes the enzyme constitutively trimeric, supported growth at 37°C, 45°C, and 47°C (Fig. 2A; Supplemental Fig. S1A; Kim and Sauer 2012). Notably, however, the double-mutant degP$^{R207P/Y444A}$ strain was inviable at 45°C and 47°C but not at 37°C (Fig. 2A; Supplemental Fig. S1A). Purified DegP$^{R207P/Y444A}$ efficiently cleaved the p23 substrate at a rate faster than DegP$^{R207P}$ but slower than wild-type DegP or DegP$^{Y444A}$ at 26°C, 37°C, and 47°C (Fig. 2B), suggesting that the high-temperature inviability of strains expressing DegP$^{R207P/Y444A}$ is not caused by the absence of proteolytic activity. Moreover, wild-type DegP, DegP$^{R207P}$, DegP$^{Y444A}$, and DegP$^{R207P/Y444A}$ were expressed at similar levels at high temperature (Supplemental Fig. S1B), and thus the inability of the double mutant to support growth at 45°C is unlikely to be caused by a reduced concentration of enzyme.

To test for dominance, we cloned genes encoding DegP, DegP$^{R207P}$, DegP$^{Y444A}$, DegP$^{R207P/Y444A}$, and DegP$^{R207P/S210A/Y444A}$ into plasmids under transcriptional control of an arabinose-inducible $P_{BAD}$ promoter and transformed E. coli W3110, a strain that expresses wild-type DegP from the chromosome. Strains were grown overnight at 37°C without arabinose and diluted in medium containing 0.2% arabinose (except for one control culture), and growth at 37°C was monitored by OD$_{600}$ (Fig. 2C). All strains grew at similar rates for the first ~2.5 h, but the cell density of the strain expressing
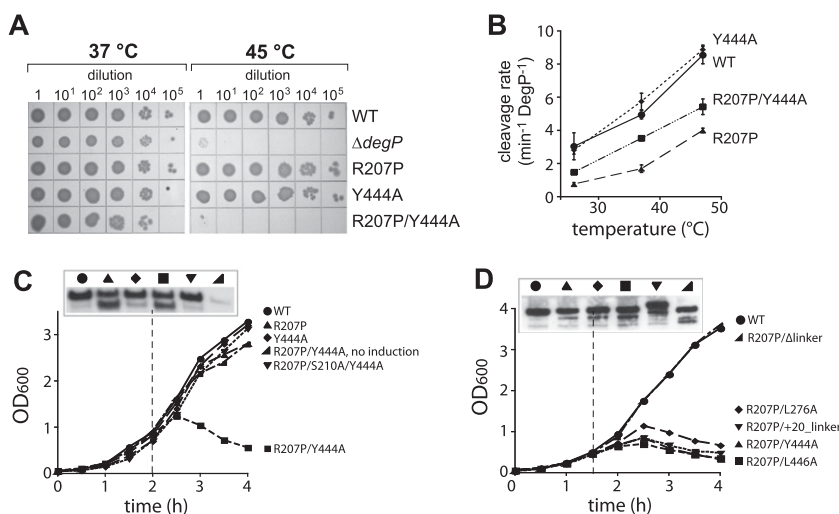


**Figure 2.** DegP$^{R207P/Y444A}$ is a rogue protease that dominantly kills cells. (A) Strains with different chromosomal degP alleles were grown to log phase in LB broth, serially diluted, spotted on LB agar plates, and grown overnight at 37°C or 45°C. (B) Cleavage rates of a saturating concentration of the p23 substrate (40 μM) by different DegP variants (0.5 μM) were determined at 26°C, 37°C, and 47°C. Values are averages ± 1 SD (n = 3). (C,D) A degP$^+$ E. coli strain (W3110) was transformed with plasmids expressing wild-type DegP or variants under arabinose control. Overnight cultures grown in LB broth plus 10 μM/mL chloramphenicol were diluted 100-fold in the same medium plus or minus 0.2% arabinose, and growth at 37°C was monitored by OD$_{600}$. The panels above the growth curves show DegP expression levels after 2 h (C) or 1.5 h (D), as determined by SDS-PAGE of equal quantities of cells and Western blotting using an anti-DegP antibody. The top band is full-length DegP; the bottom band arises from DegP autocleavage that does not affect activity.

DegP$^{R207P/Y444A}$ then decreased, an indication of cell lysis. In contrast, no major growth defects were observed for strains expressing DegP, DegP$^{R207P}$, or DegP$^{Y444A}$ from the plasmid or for a strain in which expression of DegP$^{R207P/Y444A}$ was not induced (Fig. 2C). Expression of proteolytically inactive DegP$^{R207P/S210A/Y444A}$ also did not interfere with growth, indicating that proteolysis is required for the dominant lethality of DegP$^{R207P/Y444A}$. Thus, combining a mutation that stabilizes the active conformation of DegP (R207P) with a mutation that prevents cage assembly (Y444A) results in dominant lethality, which appears to be caused by excessive proteolysis of one or more cellular proteins.

We also tested for toxic effects of the activating R207P mutation in combination with other cage-destabilizing mutations (L276A, L446A, and +20_linker) or a cage-stabilizing mutation (Δlinker) (Kim and Sauer 2012). Plasmid-mediated expression of DegP$^{R207P/L276A}$, DegP$^{R207P/L446A}$, or DegP$^{R207P/+20\_linker}$ in E. coli W3110 resulted in dominant lethality at 37°C, whereas cells expressing DegP$^{R207P/\Delta linker}$ grew normally (Fig. 2D; Supplemental Fig. S2). Thus, all cage-deficient mutants tested resulted in toxicity in the R207P background, supporting a model in which cage assembly prevents excessive proteolysis by activated DegP.

## Excessive proteolysis results in cell envelope defects

Periplasmic proteins are required for essential reactions in cell envelope maintenance and cell division. To test whether DegP$^{R207P/Y444A}$ expression interfered with either process, we examined cells expressing this double mutant for abnormal morphology. After a temperature shift to 45°C, chromosomal expression of DegP$^{R207P/Y444A}$ resulted in dramatically bloated, elongated, and irregular cells (Fig. 3A). This morphology suggests severe defects in cell wall biogenesis and was different from ΔdegP cells
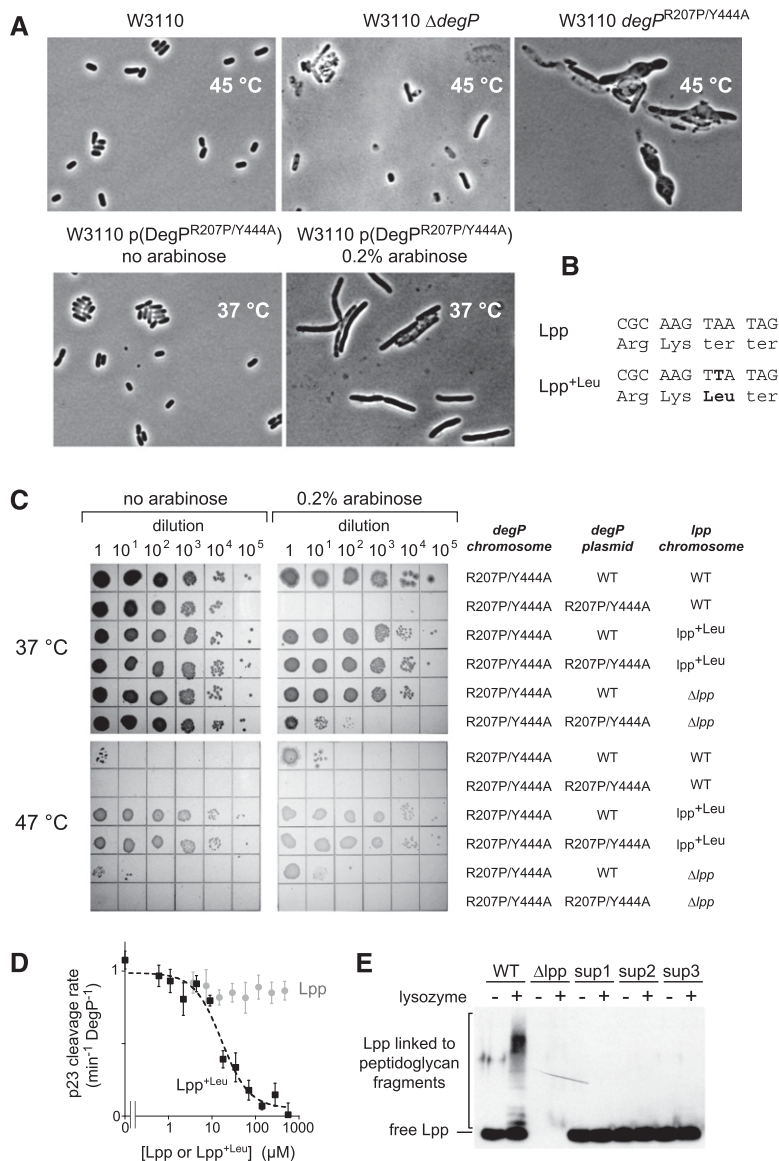


**Figure 3.** Proteolysis by DegP$^{R207P/Y444A}$ damages the cell envelope and is relieved by an lpp mutation. (A) Micrographs of E. coli W3110 strains with different degP alleles after 3–4 h of growth at 45°C or 37°C following dilution from overnight cultures. (B) Gene and C-terminal protein sequences for wild-type Lpp and Lpp$^{+Leu}$ from extragenic suppressor strains. (C) Strains SK339 (degP$^{R207P/Y444A}$/lpp$^{+}$), SK373 (degP$^{R207P/Y444A}$/lpp$^{+Leu}$), and SK367 (degP$^{R207P/Y444A}$/Δlpp) expressing plasmid-borne DegP or DegP$^{R207P/Y444A}$ under arabinose control were serially diluted and grown on LB agar plates at 37°C or 47°C. The lethality of DegP$^{R207P/Y444A}$ expression was largely suppressed in the degP$^{R207P/Y444A}$/lpp$^{+Leu}$ strain at both temperatures and partially suppressed in the degP$^{R207P/Y444A}$/Δlpp strain at 37°C. (D) The rate of cleavage of 2 μM p23 substrate at 37°C by 0.2 μM DegP$^{R207P/Y444A}$ was assayed in the presence of increasing quantities of purified Lpp or Lpp$^{+Leu}$. Values are averages ± 1 SD (n = 3). (E) SDS-PAGE and Western blots using anti-Lpp antibody were used to assay covalent attachment of Lpp to peptidoglycan. Following treatment of cells with lysozyme, a portion of wild-type Lpp electrophoreses as a ladder of higher-molecular-weight species, indicating covalent linkage to peptidoglycan. These larger species were not observed for Lpp$^{+Leu}$ from extragenic suppressor strains.

(Fig. 3A). In contrast, plasmid-mediated overproduction of DegP$^{R207P/Y444A}$ at 37°C resulted in elongated cells, as expected for cell division defects (Fig. 3A). It is possible that additional cell envelope proteins are degraded at the higher temperature or that degradation of one or more proteins is simply more extensive at the higher temperature.

### An extragenic suppressor of toxicity

To obtain additional information concerning the lethality of DegP$^{R207P/Y444A}$, we isolated suppressor mutations that restored growth to cells producing this mutant from the chromosome and from a multicopy plasmid at 47°C (Supplemental Fig. S3A), a temperature even more restrictive than 45°C. After eliminating strains with additional mutations in degP, we obtained three isolates. In each isolate, whole-genome sequencing revealed a common mutation in the stop codon of the lpp gene that results in an Lpp variant with one additional leucine at the C terminus (Lpp$^{+Leu}$) (Fig. 3B). Lpp, a trimeric coiled coil, is one of the most abundant E. coli proteins; a fatty acid attached to the N terminus of Lpp inserts into the outer membrane, whereas the ε-amino group of the C-terminal lysine is covalently attached to cell wall peptidoglycan, linking these structures and helping to maintain cell envelope integrity (Braun and Rehn 1969; Braun 1975; DiRienzo et al. 1978; McLachlan 1978). We reconstructed a strain with chromosomal lpp$^{+Leu}$ and degP$^{R207P/Y444A}$ mutations and transformed it with DegP or DegP$^{R207P/Y444A}$ plasmids. The lpp$^{+Leu}$ strain grew equally well when DegP$^{R207P/Y444A}$ or DegP was overproduced from the plasmid at 37°C and/or from the chromosome and plasmid at 47°C, whereas the lpp$^{WT}$ strain grew poorly under all conditions where DegP$^{R207P/Y444A}$ was overproduced (Fig. 3C). The viability of the reconstructed strain was comparable with the three original suppressor strains (Supplemental Fig. S3B), indicating that the lpp$^{+Leu}$ allele is the major determinant of lethality suppression.

Because the PDZ1 domain of DegP preferentially binds C-terminal hydrophobic residues (Krojer et al. 2008a; Kim et al. 2011), we tested the possibility that the Lpp$^{+Leu}$ protein might be a substrate or inhibitor of DegP$^{R207P/Y444A}$. We detected no cleavage of Lpp$^{+Leu}$ by DegP$^{R207P/Y444A}$ (Supplemental Fig. S3C). Notably, however, purified Lpp$^{+Leu}$ inhibited DegP$^{R207P/Y444A}$ cleavage of the p23 substrate, whereas purified Lpp did not inhibit cleavage (Fig. 3D). Lpp$^{+Leu}$ also inhibited wild-type DegP proteolysis, albeit substantially less efficiently than it inhibited DegP$^{R207P/Y444A}$ (Supplemental Fig. S3D). Thus, Lpp$^{+Leu}$ probably directly reduces the intracellular activity of the DegP$^{R207P/Y444A}$ protease. We also tested whether the C-terminal leucine of Lpp$^{+Leu}$ affected covalent attachment to peptidoglycan. Approximately one-third of Lpp in wild-type cells is linked to peptidoglycan (Inouye et al. 1972) and forms a ladder of higher-molecular-weight bands on SDS-PAGE after lysozyme treatment (Fig. 3E). In contrast, Lpp$^{+Leu}$ from each suppressor strain showed no evidence of peptidoglycan attachment (Fig. 3E), suggesting that lpp$^{+Leu}$ might be a biochemical loss-of-function allele as well as a gain-of-function allele. Indeed,

we found that deletion of lpp partially suppressed DegP$^{R207P/Y444A}$ toxicity at 37°C (Fig. 3C). These results support a model in which Lpp$^{+Leu}$ relieves the toxic effects of DegP$^{R207P/Y444A}$ by direct inhibition of rogue proteolysis and by restructuring the cell envelope (see the Discussion).

### Intragenic suppressors of toxicity

We also selected intragenic suppressor mutations that relieved R207P/Y444A toxicity and allowed growth at 47°C, a temperature at which DegP-mediated proteolysis is required for survival. Seven amino acid substitutions and a duplication of residues 193–195 (ENY) were obtained and subjected to further analysis. The positions of the suppressors, which were scattered throughout the protease domain and PDZ1 domain (Supplemental Fig. S4A), provided little indication of the suppression mechanism. Each suppressor mutation was reconstructed in the R207P/S210A/Y444A background, and the proteins were purified and tested for substrate-dependent cage assembly by gel filtration (Supplemental Fig. S4B). In no case were cages observed, indicating that these mutations do not restore cage assembly. Next, we purified enzymes containing individual suppressor mutations and the R207P/Y444A mutations and assayed basal cleavage of the reporter substrate. Strikingly, all of the enzymes with suppressor mutations displayed substantially lower levels of basal cleavage than the R207P/Y444A mutant (Fig. 4A, black bars). In contrast, when substrate-activated cleavage of a saturating concentration of p23 was assayed, most suppressor mutations supported rates similar to the parental R207P/Y444A mutant, and one mutation increased the rate (Fig. 4A, gray bars). DegP variants containing just the suppressor mutations also had lower levels of basal activity than wild-type DegP but mediated comparable or higher levels of saturated p23 cleavage (Supplemental Fig. S4C). In combination, these results suggest that the suppressor mutations modify DegP activity independently of the R207P and Y444A mutations and relieve R207P/Y444A toxicity by stabilizing the inactive conformation of DegP and reducing the excessive basal activity caused by the R207P mutation.

To test whether DegP enzymes bearing just the suppressor mutations relieve high-temperature stress, we expressed variants harboring the single mutations from plasmids in ΔdegP cells and monitored growth on LB agar plates at 37°C or 45°C. Seven of the eight mutants resulted in reduced cell viability at 45°C (Fig. 4B). Thus, mutational stabilization of the inactive conformation reduces DegP's ability to combat heat stress caused by protein unfolding or misfolding.

### Single R207P or Y444A mutations reduce high-temperature cellular fitness

Although the individual R207P or Y444A mutations did not substantially alter cell viability in plating or growth assays, we suspected that they might reduce high-temperature cellular fitness. To test this idea, we constructed cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) strains producing wild-type DegP, DegP$^{R207P}$, or DegP$^{Y444A}$ from the chromosome and performed growth competition experiments. Cells producing wild-type
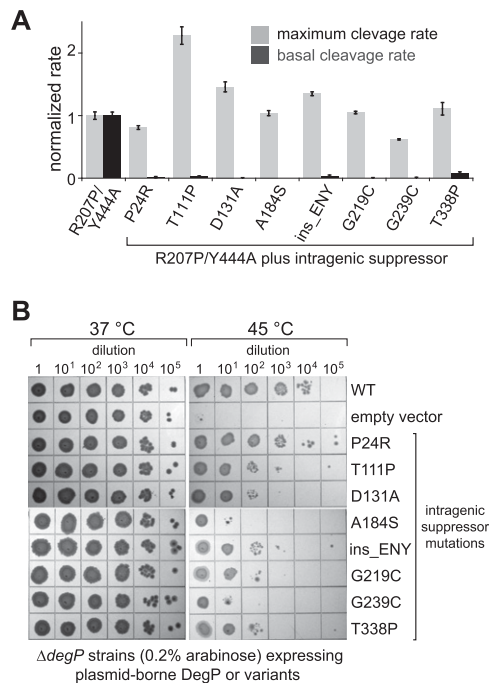
**Figure 4.** Intragenic suppressors of DegP[R207P/Y444A] lethality. (*A*) DegP[R207P/Y444A] variants with or without mutations that suppress lethality were assayed for the maximum rate of cleavage of the p23 substrate by Michaelis-Menten analysis and fitting to the Hill equation (gray bars are the fitted $V_{max}$ normalized to the value for DegP[R207P/Y444A] $\pm$ the error of fitting) and were also assayed for basal cleavage of 100 μM Abz-KASPVSLGY[NO2]D peptide by 10 μM variant (black bars are averages [$n = 3$] normalized to the value for DegP[R207P/Y444A] $\pm$ 1 SD). (*B*) Plasmids expressing DegP or variants containing different intragenic suppressor mutations without the R207P/Y444A mutations were transformed into a Δ*degP* strain, and growth of serial dilutions on LB agar plates was monitored at 37°C or 45°C in the presence of 0.2% arabinose.

DegP and either DegP[R207P] or DegP[Y444A] were initially mixed in a 1:1 ratio, diluted 10⁴-fold in LB broth, and grown at 45°C for ~12 h, and then additional cycles of dilution and growth were carried out. The relative population of each strain at the beginning of the experiment and after each cycle was quantified by CFP and YFP fluorescence. In one set of experiments, the strain expressing wild-type DegP was marked with YFP, and the mutant strain was marked with CFP. In a second set of experiments, the fluorescent markers were reversed to exclude possible effects of the markers on cell fitness. In competitions against DegP[R207P], cells expressing wild-type DegP dominated the population after a single cycle of competition (Fig. 5A, left panels). Cells expressing wild-type DegP also competed effectively against DegP[Y444A] but required five to six cycles to dominate the population (Fig. 5A, right panels). Examination of the morphology of *degP*⁺, *degP*[R207P], and *degP*[Y444A] cells at 45°C revealed that cells expressing DegP[R207P] were highly filamented compared with cells expressing wild-type DegP or DegP[Y444A] (Fig. 5B), suggesting that DegP[R207P] degradation of one or more

cell division factors contributes to the reduced fitness of cells expressing this mutant.

## Discussion

Our results establish that there is a delicate balance between the proteolytic activity of a PQC protease, like DegP, and the fitness of bacterial cells at temperatures that result in protein unfolding or misfolding (Fig. 6). For example, we found that mutations that alter the allosteric equilibrium between the active and inactive conformations of the DegP trimer reduce the fitness of *E. coli* at heat-shock temperatures. Moreover, combining a mutation that allosterically activates DegP with a mutation that prevents formation of DegP cages produces a toxic rogue protease. This toxicity can be relieved by intragenic mutations that restore allosteric balance or by an extragenic mutation that generates an inhibitor and alters the cell envelope. Thus, allosteric activation of DegP appears to be the major mechanism for regulating DegP proteolysis, whereas cage assembly provides a second level
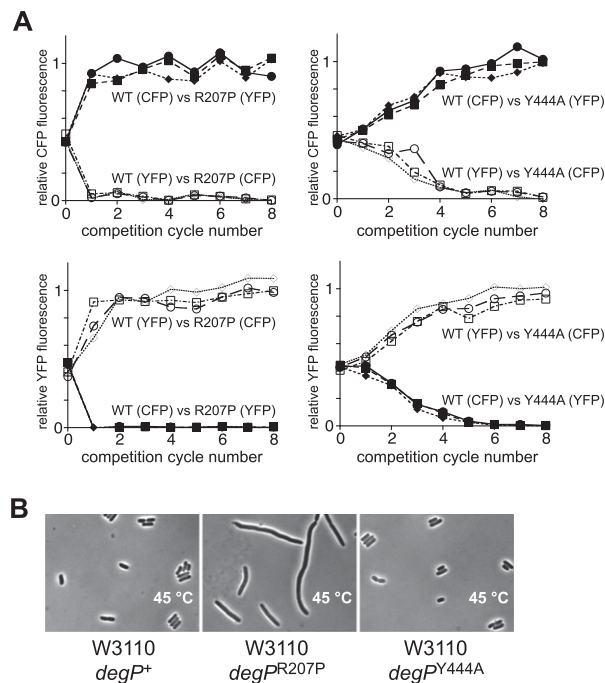


**Figure 5.** Growth competitions between *degP*⁺ cells and *degP*[R207P] or *degP*[Y444A] cells. (*A*) Three independent mixtures with equal initial quantities of a *degP*⁺ strain and a *degP*[R207P] or *degP*[Y444A] strain were subjected to eight cycles of 10⁴-fold dilution in LB broth and subsequent growth for ~12 h at 45°C. At the beginning of the experiment and after each cycle, the fraction of each strain in the population was determined by relative CFP or YFP fluorescence. The *degP*⁺ cells dominated the population after one cycle of competition against *degP*[R207P] cells and after six cycles of competition against *degP*[Y444A] cells when the wild-type strain was marked with CFP and the mutant strain was marked with YFP and vice versa. (*B*) Micrographs of strains expressing chromosomal DegP, DegP[R207P], or DegP[Y444A] at 45°C.
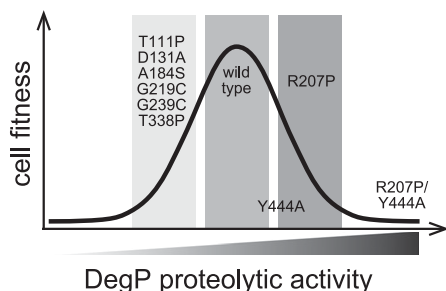
**Figure 6.** Relationship between proteolysis levels and cellular fitness. DegP proteolysis at a high enough level to protect cells from proteotoxic stress is required for fitness, but excessive degradation is harmful.

of protection by limiting the repertoire of substrates degraded by activated DegP.

### A critical allosteric balance

For any allosteric enzyme, the relative populations of inactive and active molecules are determined by a conformational equilibrium constant, the concentrations of substrates or allosteric effectors, and the relative affinities of these molecules for the two enzyme conformations. We found that most mutations that alter the conformational equilibrium constant for DegP reduce cellular fitness. For example, the R207P mutation stabilizes active DegP, increasing basal activity, and cells expressing DegP$^{R207P}$ compete poorly against wild-type cells and show morphologies consistent with defects in cell division. Moreover, in combination with cage-disrupting mutations, R207P results in proteolysis-dependent lethality, which can be suppressed by intragenic mutations that stabilize inactive DegP. In otherwise wild-type DegP, however, these intragenic suppressors reduce basal activity, and most also decrease cellular viability at 45°C. Thus, achieving a proper allosteric balance between active and inactive DegP is critical, as either too little or too much active DegP creates problems for the cell.

Mutations that stabilize inactive DegP are likely to reduce cell viability at 45°C because substrates present at subsaturating concentrations are not degraded sufficiently rapidly. Deleterious proteolysis by DegP$^{R207P}$ could, in principle, arise from a change in substrate specificity, a higher rate of degradation of saturating concentrations of normal substrates, or increased degradation of subsaturating levels of normal substrates. Compared with wild-type DegP, the R207P mutant had ~100-fold higher basal activity but lower maximal activity in degrading model substrates. Moreover, the lethality of the R207P/Y444A variant could be suppressed by many different mutations that stabilize inactive DegP, suggesting that altered substrate specificity is unlikely to lead to degradation of "new" target proteins by DegP$^{R207P}$. Hence, the deleterious effects of this mutant on cell fitness probably arise from increased levels of degradation of low concentrations of normal substrates.

At the position corresponding to residue 207 in *E. coli* DegP, many orthologs have a wild-type proline

(Mohamedmohaideen et al. 2008; Cezairliyan and Sauer 2009). Based on our results, any potential deleterious effects of this "activating" proline are likely to be balanced by one or more "inactivating" residues elsewhere in the structure. Thus, the trimeric architecture of HtrA proteases allows the stabilities of the active and inactive conformations to be tuned to meet cellular requirements.

### Protective effects of cage assembly

Assembly of DegP cages appears to protect cells from excessive or inappropriate proteolysis. For example, cells expressing DegP$^{Y444A}$, which cannot form cages, fared poorly in a competition with cells expressing wild-type DegP at 45°C, indicating that cage assembly provides a fitness advantage at this heat-shock temperature. Moreover, combining the activating R207P mutation with Y444A or other cage-destabilizing mutations resulted in dominant lethality. Why might cage-defective DegP variants mediate inappropriate degradation? Following cage assembly, the proteolytic active sites of DegP are accessible to unfolded substrates that can pass through small openings into the cage lumen, but misfolded substrates bound to chaperones or partially native substrates would be excluded. Once substrates are depleted, cages disassemble, and trimers revert to the inactive conformation. In contrast, the active sites of an activated cage-defective DegP trimer are exposed, and thus any polypeptide sequence with an appropriate P1 residue could be cleaved. Moreover, the binding of one or two good substrates to the active sites and PDZ1 domains of a trimer can trans-activate cleavage of poor substrates by the remaining active sites. Because cage assembly should restrict the normal repertoire of DegP substrates, additional cellular proteins would be expected to be cleaved by cage-defective trimers, especially those that are easily activated, like DegP$^{R207P/Y444A}$. Therefore, sequestering the active sites of DegP within cages allows modulation of proteolysis independently of the allosteric control of active and inactive states (Kim and Sauer 2012).

Based on the finding that outer-membrane proteins (OMPs) copurify with cages of DegP$^{S210A}$, it was proposed that cages of wild-type DegP function as chaperones (Krojer et al. 2008b). However, OMP-associated DegP$^{S210A}$ cages assume a proteolytically active conformation (Krojer et al. 2008b; Kim et al. 2011), and thus any encapsulated partially folded protein in a wild-type DegP cage would have a high probability of being cleaved. Moreover, at 37°C, we found that the dominant lethality of DegP$^{R207P/Y444A}$ overexpression is suppressed by a mutation in the proteolytic catalytic triad, a result inconsistent with loss of chaperone activity being responsible for the deleterious effects of this cage assembly defect.

### Toxicity, suppression of toxicity, and targets of rogue proteolysis

Combining the DegP R207P allosteric activation mutation with the Y444A cage-defective mutation results in rogue proteolysis and cellular toxicity. At heat-shock temperatures, where wild-type DegP is normally expressed

at high levels to combat the stress associated with protein misfolding, high-level expression of DegP[R207P/Y444A] results in cell death and morphologies suggesting damage to proteins involved in cell wall maintenance and/or cell division. High-level expression of DegP[R207P/Y444A] at 37°C, a non-heat-shock temperature, also resulted in cell death.

Intriguingly, the lethality of DegP[R207P/Y444A] is suppressed by adding one additional leucine to the C terminus of Lpp, a lipoprotein that normally cross-links the bacterial outer membrane to the peptidoglycan lattice. Two distinct properties of the Lpp[+Leu] protein appear to mediate suppression of toxicity. First, this protein functions directly as a gain-of-function inhibitor of DegP[R207P/Y444A] proteolysis in vitro (Fig. 3D). Because DegP proteolysis is required for high-temperature survival (Lipinska et al. 1989; Strauch et al. 1989), we suspect that Lpp[+Leu] inhibition in the cell reduces but does not completely eliminate DegP[R207P/Y444A] activity, but the level of such inhibition is unclear. Second, Lpp[+Leu] did not cross-link to peptidoglycan (Fig. 3E), mimicking one cellular effect associated with loss-of-function Lpp mutations. Indeed, deletion of *lpp* partially suppressed toxicity associated with DegP[R207P/Y444A] overexpression at 37°C (Fig. 3C), suggesting that cross-linking of wild-type Lpp to peptidoglycan accounts for some lethal effects of rogue proteolysis. Importantly, previous studies show that depletion or inhibition of *E. coli* proteins needed for lipoprotein biogenesis causes lethality, which can be suppressed by deletion of *lpp* (Zwiebel et al. 1981; Matsuyama et al. 1997; Yakushi et al. 1997; Tajima et al. 1998; Kikuchi et al. 2000; Robichon et al. 2005; Pailler et al. 2012). Deleting *lpp* may suppress lethality by preventing toxic cross-linking of Lpp mislocalized to the inner membrane to peptidoglycan; by reducing the load on lipoprotein translocation machinery, thereby allowing better translocation of other essential lipoproteins; or by outer-membrane leakage that reduces toxic species in the periplasm. The toxicity of DegP[R207P/Y444A] overexpression may arise in part from excessive degradation of one or more proteins involved in lipoprotein biogenesis. Collectively, these results indicate that the toxicity of DegP[R207P/Y444A] is caused by excessive degradation of proteins that function in essential cellular processes in the bacterial periplasm. Thus, regulating wild-type DegP proteolysis by allosteric activation and cage formation limits damage to critical periplasmic proteins while allowing efficient degradation of proteotoxic proteins.

## Protein unfolding and periplasmic degradation

The peptide bonds cleaved by proteases are inaccessible in native proteins, and unfolding or prevention of folding are therefore key steps in degradation. In the cytoplasm, AAA[+] proteases execute most protein degradation (Sauer and Baker 2011). These enzymes use hexameric AAA[+] rings to bind target proteins, unfold them, and translocate the polypeptide into a barrel-like proteolytic chamber in reactions fueled by ATP hydrolysis. Interestingly, the proteolytic barrel of the AAA[+] ClpXP protease becomes a rogue enzyme when ADEP antibiotics bind and alter its

conformation, allowing unfolded substrates to enter the chamber in an unregulated fashion (Brötz-Oesterhelt et al. 2005). Uncontrolled proteolysis by the ADEP•ClpP complex has recently been shown to kill persistent forms of pathogenic bacteria, which are extremely hard to eradicate with conventional antibiotics (Conlon et al. 2013). The periplasmic compartment of bacteria is devoid of ATP, and thus protein degradation largely depends on spontaneous unfolding or misfolding, which is enhanced at elevated temperatures. In *E. coli*, heat shock induces high-level expression of DegP, whose proteolytic activity is essential for cell survival and is regulated by allosteric activation and cage formation to avoid damaging important cellular processes in the periplasm. Thus, small molecule dysregulation of DegP proteolysis may also be a promising route to new antibiotics in the fight against pathogenic bacteria.

## Materials and methods

### Bacterial strains

Supplemental Table S1 lists strains, plasmids, and oligonucleotide primers used in this study. All plasmid constructions and cellular mutations introduced by site-directed mutagenesis were confirmed by DNA sequencing (Genewiz). Two-step recombineering (Davis et al. 2011) was used to replace the wild-type *degP* gene with mutant alleles as described (Kim and Sauer 2012). Briefly, in one recombination step, the *degP* gene in *E. coli* W3110 was replaced by an *mPheS-kan[R]* allele, which was subsequently replaced with a mutant *degP* gene in a second step. DNA fragments for the second recombination step were amplified with two primers, insP_ss_for1 and insP_rev2, from pSK617 (for SK341, which expresses DegP[R207P]) or insP_ss_for1 and insP_rev3 from pSK576 (for SK339, which expresses DegP[R207P/Y444A]).

The *lpp* deletion strain (JW1667-5 from the Keio collection; Coli Genetic Stock Center [CGSC] no. 9417) was obtained from the CGSC (Yale University, CT). The *Δlpp-752::kan* allele in this strain was transferred to SK339 by P1 transduction (Thomason et al. 2007) to make strain SK367. Recombineering was also used to introduce *lpp[+Leu]* into W3110 to make strain SK371; the DNA fragment for the second recombination was amplified from genomic DNA of the suppressor strain with two primers, 5-lpp_up67 and 3-lpp_down75. Strain SK373, harboring both *lpp[+Leu]* and *degP[R207P/Y444A]*, was constructed by transduction of the *mPheS-kan[R]* allele into the *degP* locus and subsequent recombination with the *degP[R207P/Y444A]* allele.

IPTG-inducible genes encoding CFP (*galK::cfp-amp[R]*) and YFP (*galK::yfp-amp[R]*) were amplified with primers 5-galK_80up and 3-galK_80down from parental strains (Hegreness et al. 2006) and recombined into W3110 to generate SK352 and SK353, respectively. The CFP and YFP genes were then transferred to SK326 (*degP[R207P]*) and SK341 (*degP[Y444A]*) by P1 transduction.

### Plasmids

pBAD vectors were used for arabinose-induced expression of proteins (Guzman et al. 1995). Initially, the *degP* gene was amplified with primers 5-degP-NcoI and 3-degP-SalI and inserted between the NcoI and SalI sites of pBAD24, which contains a Shine-Dalgarno (SD) sequence upstream of the NcoI site. The *SD_degP* locus was amplified with primers 5-SacI_SD_degP and 3-SphI_degP from pBAD24_degP and inserted between the SacI and SphI sites in pBAD33 to generate pSK633 (pBAD33_SD_degP).

Plasmids harboring *degP* mutations were constructed by mutagenesis of pBAD24_degP and subsequent transfer of alleles to pBAD33 or by direct mutagenesis of pSK633. Plasmids that overexpress DegP variants were constructed as reported (Kim et al. 2011).

To construct plasmids expressing $His_6$-Lpp or $His_6$-Lpp$^{+Leu}$, a portion of the *lpp* gene missing the signal sequence and $Cys^{21}$ was initially amplified from *E. coli* genomic DNA with primers 5-pET28_lpp-2 and 3-pET28_lpp (for pSK709) or 3-pET28_lpp_stop79L (for pSK710) and inserted between the NdeI and NotI sites of pET28b.

### Proteins, peptides, and biochemical assays

DegP variants, 18–58 peptide, p23 peptide, reporter peptide, and $^{flC}$18–58 (fluorescently labeled 18–58) were prepared as described (Kim et al. 2011; Kim and Sauer 2012). Activity assays, fluorescence anisotropy, FRET, and gel filtration chromatography were performed as described (Kim et al. 2011; Kim and Sauer 2012).

### Bacterial growth and viability assays

To test strain viability, overnight cultures were diluted 1:100 in LB broth and grown to $OD_{600}$ ~0.2 at 37°C, and 5-μL aliquots from a set of 10-fold serial dilutions were spotted for growth on LB agar plates at 37°C. To test growth in liquid culture, overnight cultures were grown in LB broth with 10 μM/mL chloramphenicol at 37°C and diluted 100-fold in fresh medium with or without 0.2% arabinose, and continued growth at 37°C was monitored by $OD_{600}$. To monitor the expression of DegP variants, cells were taken 1.5 h or 2 h after dilution, lysed by boiling in SDS loading buffer, and subjected to SDS-PAGE, and DegP was detected by Western blotting with anti-DegP antibody (a gift from T. Silhavy, Princeton University).

### Microscopy

Cells harboring different chromosomal or plasmid *degP* alleles were grown in LB broth, diluted to $OD_{600}$ 0.05–0.2 with fresh LB, and placed on an LB 1.5% agarose pad. Phase microscopy images were taken using a Zeiss Axiovert 200 microscope with a 100×/1.45 oil immersion objective.

### Isolation of suppressor mutations

SK339 cells encoding chromosomal $degP^{R207P/Y444A}$ were spread on LB agar plates and incubated overnight at 47°C, and colonies that grew were purified by restreaking and growth at 47°C. The *degP* genes of nine candidates contained mutations in addition to R207P/Y444A. Consistent with the absence of extragenic suppressor mutations in these strains, plasmid-mediated overexpression of DegP$^{R207P/Y444A}$ from pSK636 reduced viability at 37°C. Additional intragenic suppressor mutations were identified after selections at 47°C starting with strains SK345(Δ*degP*)/pSK636 or SK339/pSK636 grown on LB agar plates containing 10 μg/mL chloramphenicol and 0.2% arabinose. Three survivors from the two SK339/pSK636 selection experiments contained no additional mutations in either the chromosomal or plasmid *degP* genes, and these strains overexpressed DegP$^{R207P/Y444A}$ at the expected high levels after 47°C heat shock or induction with arabinose at 37°C. To identify extragenic mutations, we isolated genomic DNA from the parental strain and the three suppressor strains and subjected them to Illumina whole-genome sequencing (BioMicro Center, Massachusetts Institute of Technology), which revealed one common suppressor mutation located in the stop codon of *lpp*.

### Peptidoglycan attachment of Lpp

To test the attachment of Lpp or Lpp$^{+Leu}$ to peptidoglycan, overnight cultures were diluted 100-fold in LB broth and grown to log phase at 37°C, and the cells in 1 mL of culture were harvested by centrifugation and resuspended in 200 μL of 10 mM Tris (pH 7.9) and 5 mM EDTA. This material was divided into two 100-μL solutions, and 0.5 mg/mL lysozyme (final) was added to one solution. After 10 min at room temperature, 100 μL of SDS-PAGE sample buffer was added to both solutions, which were heated for 10 min at 100°C and centrifuged at 20,000*g* for 10 min. Aliquots of the supernatants were subjected to SDS-PAGE and Western blot analysis with anti-Lpp antibody (provided by H. Tokuda). The free form of Lpp electrophoreses at a position of the expected molecular weight, whereas peptidoglycan-bound Lpp electrophoreses as a ladder-like pattern of higher-molecular-weight fragments after lysozyme treatment.

### Growth competition

Strains harboring chromosomal *degP*, $degP^{R207P}$, or $degP^{Y444A}$ and IPTG-inducible CFP or YFP were grown overnight at 37°C in LB broth. For competition experiments, equal amounts of a *degP* strain marked with one color and a $degP^{R207P}$ or $degP^{Y444A}$ strain marked with the other color were mixed (cycle #0), diluted $10^4$-fold in LB broth, and grown for ~12 h at 45°C (cycle #1). Seven addition cycles of $10^4$-fold dilution and growth were performed (cycles #2–#8). Cell mixtures were taken after each cycle, mixed with glycerol (15% final concentration), and stored at −80°C for further analysis. Three independent competition experiments were performed with each combination of strains. After all competition cycles were complete, frozen cell mixtures were thawed, diluted 100-fold in M9 minimal medium (1× M9 salts, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.4% glucose) supplemented with 0.1% casamino acid and 1 mM IPTG, and grown for ~3 h at 37°C. M9 supplemented medium was used to reduce background fluorescence, and ~3 h of growth in this medium did not significantly change the initial cell ratios (Fig. 5A shows that the CFP/YFP cell ratios at cycle #0 were consistently 0.4–0.5). One-hundred microliters of cells was transferred to clear 96-well plates (Greiner, catalog no. 655096) to measure $OD_{600}$, CFP fluorescence (excitation at 430 nm; emission at 480 nm; cutoff filter at 455 nm), and YFP fluorescence (excitation at 490 nm; emission at 530 nm; cutoff filter at 515 nm) in a SpectraMax M5 microplate reader (Molecular Devices). Blank signals from medium alone were subtracted from CFP and YFP fluorescence, which were normalized by $OD_{600}$. Data were normalized by the average high fluorescence signals during the final cycles to obtain relative CFP/YFP fluorescence.

### Acknowledgments

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