The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system

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Interruption of translation in Escherichia coli can lead to the addition of an 11-residue carboxy-terminal peptide tail to the nascent chain. This modification is mediated by SsrA RNA (also called 10Sa RNA and tmRNA) and marks the tagged polypeptide for proteolysis. Degradation in vivo of λ repressor amino-terminal domain variants bearing this carboxy-terminal SsrA peptide tag is shown here to depend on the cytoplasmic proteases ClpXP and ClpAP. Degradation in vitro of SsrA-tagged substrates was reproduced with purified components and required a substrate with a wild-type SsrA tail, the presence of both ClpP and either ClpA or ClpX, and ATP. Clp-dependent proteolysis accounts for most degradation of SsrA-tagged amino-domain substrates at 32°C, but additional proteases contribute to the degradation of some of these SsrA-tagged substrates at 39°C. The existence of multiple cytoplasmic proteases that function in SsrA quality-control surveillance suggests that the SsrA tag is designed to serve as a relatively promiscuous signal for proteolysis. Having diverse degradation systems able to recognize this tag may increase degradation capacity, permit degradation of a wide variety of different tagged proteins, or allow SsrA-tagged proteins to be degraded under different growth conditions.

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Certain proteins and protein fragments in Escherichia coli are modified by carboxy-terminal addition of an 11-residue peptide tag (Tu et al. 1995). This tagging process requires functional SsrA RNA (10Sa RNA), which encodes the last 10 residues of the peptide (Tu et al. 1995) and results in rapid degradation of the tagged protein by carboxy-terminal-specific proteases (Keiler et al. 1996). SsrA-mediated tagging of proteins translated from defective messenger RNAs lacking termination codons has been demonstrated, and a model in which SsrA functions both as a tRNA and an mRNA has been proposed (Keiler et al. 1996). The 363-nucleotide SsrA RNA has sequences that form a tRNA-like structure and has been shown to be chargeable with alanine (Komine et al. 1994; Williams and Bartel 1996; Felden et al. 1997). In the model proposed by Keiler et al., when ribosomes stall at the 3' end of the damaged message, SsrA charged with alanine binds to the ribosome like a tRNA and contributes the alanine to the idle nascent chain. Translation then switches from the mRNA to a small open reading frame (ORF) in SsrA that encodes the carboxy-terminal degradation peptide. This system provides both a method to avoid the accumulation of ribosomes stalled at the end of defective messages and a general quality-control mechanism that allows the cell to rid itself of incomplete protein fragments that might have inappropriate cellular activities. Cells devoid of SsrA RNA grow more slowly and show a certain degree of temperature sensitivity (Oh and Apirion 1991; Komine et al. 1994; Trempy et al. 1994). The involvement of carboxy-terminal amino-acid sequences in targeting proteins for rapid degradation was recognized before the discovery of the SsrA-tagging system (Bowie and Sauer 1989; Parsell et al. 1990), and a periplasmic protease (Tsp or Prc) that degrades protein substrates in a carboxy-terminal-specific manner was purified and characterized (Silber et al. 1992). The carboxy-terminal substrate sequences recognized by Tsp are similar to those of the SsrA tag (Keiler et al. 1995; Tu et al. 1995), and Tsp is responsible for degradation of SsrA-tagged proteins that are exported to the periplasm (Keiler et al. 1996). Cytoplasmic proteases with carboxy-terminal degradation sequences, however, are still proteolysed rapidly in cells lacking Tsp (Silber and Sauer 1994; Keiler et al. 1996), indicating that other proteases must be re-
Clp degradation of SsrA-tagged substrates

Essentially all cytoplasmic degradation in prokaryotes, archaea, and eukaryotes is energy-dependent. E. coli, for example, has at least five ATP-dependent proteases [Lon (La); HflB (FtsH); ClpAP; ClpXP; and ClpYQ (HslUV)] (for review, see Gottesman 1996). These enzymes appear to have distinct substrate preferences, as a mutation in a single protease gene is often sufficient to stabilize a specific unstable protein. For example, mutations in Lon lead to stabilization of the N protein of bacteriophage λ, the SulA and RcsA proteins of E. coli, and the CcdA protein of the episomal F factor. HflB appears to be responsible for degradation of the cII protein of λ and the heat-shock σ factor RpoH (Herman et al. 1993, 1995). The principal substrates for ClpYQ degradation have not yet been identified, although this two-component protease has been implicated in degradation of both Lon substrates and HflB substrate in vivo (Missiakas et al. 1996; Kanemori et al. 1997; Khattar 1997; W.-F. Wu and S. Gottesman, unpubl.). ClpAP and ClpXP are two-component proteases that share a common proteolytic subunit, ClpP, but have different ATPase regulatory subunits, ClpA or ClpX. Proteins stabilized by mutations in clpX but not in clpA include λ O, phage Mu repressor variants, and the stationary-phase σ factor, RpoS; clpA but not clpX mutants stabilize certain LacZ fusion substrates, ClpA or ClpX. Proteins stabilized by mutations in clpX but not in clpA include λ O, phage Mu repressor variants, and the stationary-phase σ factor, RpoS; clpA but not clpX mutants stabilize certain LacZ fusion proteins and the MazE protein. ClpB, an ATPase with extensive sequence similarity to ClpA, has not thus far been demonstrated to have a direct role in proteolysis but may act as a chaperone (Squires and Squires 1992).

In the studies presented here, we show that intracellular degradation of variants of the amino-terminal domain of λ repressor containing the SsrA peptide tag is dramatically reduced in cells lacking ClpP or lacking both ClpX and ClpA, and is somewhat reduced in cells lacking ClpX or ClpA only. Purified ClpXP and purified ClpAP degrade SsrA-tagged protein substrates in vitro, suggesting that these ATP-dependent enzymes are directly responsible for degradation of SsrA-tagged proteins in the bacterial cytoplasm.

Results

At sufficiently high concentrations, a protein containing the 93 amino-acid amino-terminal domain of λ cl repressor binds to the λ operators and prevents lytic growth of superinfecting phage (Sauer et al. 1979; Jordan and Pabo 1988). When degradation signals such as the SsrA tag, however, are attached to the carboxyl terminus of this fragment, proteolysis reduces the steady-state quantities of this domain to levels insufficient for function (Parsell et al. 1990; Keller et al. 1996). Therefore, phage immunity provides an assay for the intracellular degradation of tagged amino-terminal domain derivatives.

For the work reported here, we have used three pairs of amino-domain variants (see Fig. 1). In each pair, one variant contains the protease-sensitive SsrA tag (AANDENYALDD) and the other bears a protease-resistant control tag (AANDENYALAA); the pairs differ in the linker regions that connect these carboxyl-terminal tags to the amino-terminal domain. We have also used two phages, λcI−, which produces no functional λ repressor, and the weaker λc17 (see Materials and Methods), which is capable of producing some λ repressor. Wild-type strains expressing the three SsrA-tagged proteins (λN-AA, λN-L1-AA, and λN-L2-AA) were found, as expected, to be sensitive to superinfection by λc1− or λc17, whereas cells expressing the three control-tagged proteins (λN-DD, λN-L1-DD, and λN-L2-DD) were immune.

Immunity in protease-deficient mutants

To test for cytoplasmic proteases required for degradation of SsrA-tagged proteins, we assayed for increased λ immunity in known or suspected protease-defective

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**Figure 1.** Proteins used for degradation studies of SsrA-tagged proteins. Cartoon representation of the structures of hybrid proteins. The names of these proteins and of the plasmids that encode them are indicated to the left. [(M2 Flag) The epitope sequence DYKDDDDK. (6His) The sequence HHHHHHHH. (SsrA tag AA) The sequence AANDENYALAA; (SsrA tag DD) The sequence AANDENYALDD. (trpAt) The AARLMSG sequence encoded by the stem–loop region of the trpAt transcriptional terminator. Note that because of heterogeneity in the position of SsrA tagging (Keller et al. 1996), the λN–trpAt protein may consist of several species, some of which differ by 1 or 2 amino acids from the otherwise identical sequence of λN-L2-AA.

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**Table 1.** Proteins used for degradation studies of SsrA-tagged proteins.

| Plasmid | Tag Type | Description |
|---------|----------|-------------|
| pN-GPT | λN-AA | λ(1-93) ssrA tag RR |
| pN-CPT | λN-DD | λ(1-93) ssrA tag DD |
| pER130 | λN-L1-AA | λ(1-93) M2 Flag 6 His srrA tag RR |
| pER135 | λN-L1-DD | λ(1-93) M2 Flag 6 His srrA tag DD |
| pER161 | λN-L2-AA | λ(1-93) M2 Flag 6 His trpAt srrA tag RR |
| pER163 | λN-L2-DD | λ(1-93) M2 Flag 6 His trpAt srrA tag DD |
| pPW500 | λN-trpAt | λ(1-93) M2 Flag 6 His trpAt srrA tag RR |
strains expressing the λN- AA or λN-L-1-AA proteins. No changes in immunity were observed in strains with an insertion mutation in clpB, an insertion mutation in hflA, a deletion of lon, mutations in lon and clpQ, or a temperature-sensitive mutation in hflB (data not shown). A very different result was observed in cells with defects in the ClpXP or ClpAP proteases. In strains containing insertions in the protease subunit (clpP) or regulatory subunits (clpA or clpX), expression of λN- AA or λN-L-1-AA resulted in immunity to superinfection by λc17 (Fig. 2), suggesting that these proteases are involved in degradation of SsrA-tagged proteins.

Among the different clp mutants, no differences in λc17 immunity were observed, but clear differences in immunity to λc17 were evident (Fig. 2). The patterns of λc17 immunity suggest that the highest steady-state levels of SsrA-tagged amino-domain proteins are achieved when ClpP is defective or when both ClpX and ClpA are inactivated. In contrast, inactivation of ClpX alone in a strain expressing λN- AA resulted in very modest immunity to λc17, and inactivation of ClpA alone gave partial immunity (λN-L-1-AA) or no immunity (λN- AA) to λc17 (see Fig. 2 for quantitation of immunity). In control experiments, we found that expression of the control-tagged λN-DD or λN-L-1-DD proteins provided full immunity [efficiencies of plating (eop) 10^{-4} or lower] to λc17 in both wild-type and clp mutants. Therefore, the Clp-mediated increases in phage immunity are dependent on the presence of λ amino-terminal domain proteins bearing a wild-type SsrA degradation tag at their carboxyl terminus. We interpret these data as suggesting that either ClpXP or ClpAP can degrade and thereby reduce the intracellular levels of the λN- AA and λN-L-1-AA proteins.

Protein turnover in vivo

Degradation of λN- AA or λN-L-1-AA in wild-type and protease-deficient strains was assayed directly by blocking protein synthesis and measuring the disappearance of the protein by Western blotting. Transformed cells expressing either λN- AA or λN-L-1-AA were grown, induced by the addition of IPTG for 30 min, and treated with spectinomycin to block further protein synthesis. Samples were removed during the subsequent incubation and examined by Western blotting for remaining levels of λN- AA or λN-L-1-AA. Figure 3A shows a Western blot of λN- AA turnover in wild-type cells, in which the half-life is <0.1 min, and in clpP mutant cells, in which the half-life is longer than 1 hr. The initial level of λN- AA also was higher in the clpP mutant host, a result consistent with the immunity experiments. In parallel experiments, the λN-DD protein showed no detectable turnover (half life >1 hr) in either the wild-type or clpP strain (data not shown). The rapid turnover of λN- AA and slow turnover of λN-DD had been demonstrated previously in a different wild-type strain background (Kepler et al. 1996).

Figure 3. Clp-dependent turnover in vivo of λN- AA and λN-L-1-AA. (A) Western blot analysis of λN- AA in wild-type (SG22163) or clpP mutant (SG22175) cells after spectinomycin blocking of protein synthesis. Cells were grown to mid-log phase at 32°C and induced with IPTG for 30 min before addition of spectinomycin. Samples were removed at the times shown into cold TCA, precipitated, and resuspended for electrophoresis in 15% SDS–polyacrylamide gels. Gels were blotted and probed with an anti-λ-repressor antibody. (B) Turnover of λN-L-1-AA in wild-type and clp mutants. Experimental conditions were the same as in A except 10%–20% tricine SDS–polyacrylamide gels were used, and Western blots were probed with the M2 anti-FLAG antibody and quantified by the Eagle Eye II gel imaging system. Curves and strains are marked as for Fig. 2.
Figure 3B shows time courses for λN-L1-AA degradation in a set of isogenic clp mutants. As expected from the immunity results, degradation of λN-L1-AA is fastest in the wild-type strain that contains functional ClpAP and ClpXP. A small increase in half life was observed in the ClpA-defective strain, and relatively long half lives were observed in strains lacking ClpP, ClpA and ClpX, or ClpX. We note that in different experiments, the results with clpX mutant hosts were somewhat variable, often showing less stabilization than observed in Figure 3B; we currently have no explanation for this variability. Mutations in lon, clpB, hflA, or fhfB had no detectable effect on λN-L1-AA turnover under these conditions of these experiments (data not shown). Therefore, the high turnover rates of the λN-AA and λN-L1-AA proteins are entirely consistent with the immunity tests. More importantly, the turnover experiments demonstrate that the increased phage immunity and increased steady-state levels of λN-AA and λN-L1-AA in ClpAP-defective or ClpXP-defective strains result mainly from decreased rates of degradation.

Protein degradation in vitro

To test directly for degradation of SsrA-tagged proteins by Clp proteases, 35S-labeled λN-L1-AA and λN-L1-DD were purified and incubated with purified ClpAP or ClpXP. As shown in Figure 4A, both Clp proteases degraded the SsrA-tagged λN-L1-AA protein efficiently but degraded the control-tagged λN-L1-DD protein at rates only 5% to 10% of that seen with λN-L1-AA (Fig. 4A). Degradation required both ClpP and either ClpX or ClpA and, in every case, required ATP (data not shown). These results demonstrate that both ClpXP and ClpAP can degrade proteins with the carboxy-terminal SsrA degradation peptide.

As shown in Figure 4B, λN-L1-AA and λN-L1-DD were equally sensitive to degradation by chymotrypsin, a relatively nonspecific protease (Benyon and Bond 1989), indicating that the SsrA tag does not increase the general protease sensitivity of proteins to which it is attached. Purified λN-L1-AA and λN-L1-DD also had superimposable CD spectra (data not shown) and identical melting profiles in thermal denaturation experiments monitored by changes in circular dichroism (Fig. 4C). These data show that the susceptibility of λN-L1-AA to degradation by ClpXP and ClpAP does not result from major changes in the structure or stability of this tagged protein. Together, these results support a model in which the SsrA tag is recognized directly by ClpXP or ClpAP and argue against models in which the tag destabilizes the substrate, allowing proteases to recognize amino-acid sequences exposed in the denatured state.

Degradation after SsrA tagging in vivo

The experiments described above use amino-terminal domain variants in which the SsrA peptide tag is encoded by the genes for the λN-AA or λN-L1-AA proteins. Using the λN-trpAt system of Keiler et al. (1996), we also examined turnover in vivo of proteins in which the carboxy-terminal degradation peptide is added cotranslationally by the SsrA-mediated tagging system. As diagrammed in Figure 1, the gene for λN-trpAt encodes the amino-terminal domain and amino acids from the trpAt transcription terminator. There is no translation termination codon within this sequence. Therefore, when this message is read, translation ceases without termination and release of the growing polypeptide. Keiler et al. (1996) demonstrated that, under these circumstances, SsrA has an essential role in tagging and allows release of the stalled polypeptide. The λN-L2-AA and λN-L2-DD proteins contain the same amino acids from the trpAt
Turnover experiments for \(\lambda N\)-trpAt and \(\lambda N\)-L2-AA are shown in Figure 5. \(\lambda N\)-trpAt protein, expressed in an ssrA mutant, migrated faster in the gel (as expected for untagged protein) and was proteolytically stable (Fig. 5A; see also Keiler et al. 1996). In the ssrA\(^+\) host, only about half of the \(\lambda N\)-trpAt protein appears to be tagged, but this species was degraded rapidly in the clpP\(^+\) strain and had a longer half life in the clpP\(^-\) mutant (Fig. 5A). The shorter \(\lambda N\)-trpAt protein in the ssrA\(^+\) host appears to be untagged, based on its electrophoretic mobility, and was longer lived than the tagged protein. \(\lambda N\)-L2-AA, which has essentially the same sequence as tagged \(\lambda N\)-trpAt, was turned over rapidly in clpP\(^+\) cells and is significantly stabilized in the clpP\(^-\) mutant (Fig. 5B). These results show that SsrA-tagged \(\lambda N\)-trpAt and \(\lambda N\)-L2-AA are degraded in a clpP-dependent fashion. We note, however, that SsrA-tagged \(\lambda N\)-trpAt was degraded faster than \(\lambda N\)-L2-AA in both clpP\(^+\) and clpP\(^-\) strains (Fig. 5C), indicating that the cotranslational, SsrA-dependent tagging of \(\lambda N\)-trpAt may result in the tagged protein becoming exposed to or more sensitive to other proteases in addition to being substrates for ClpAP and ClpXP.

High-temperature effects on Clp-dependent turnover

The immunity and turnover experiments described above were performed at 32\(^\circ\)C. We observed, however, that cells expressing \(\lambda N\)-AA did not acquire immunity at 39\(^\circ\)C in clpP or clpA clpX mutant strains (Fig. 6). Preliminary turnover experiments indicated that, as above, this lack of immunity reflected rapid degradation of the \(\lambda N\)-AA protein at elevated temperatures, suggesting that proteases other than ClpAP or ClpXP may participate in degradation of this SsrA-tailed protein at 39\(^\circ\)C. C. Herman and R. D’Ari (pers. comm.) have made similar observations and identified HflB as a protease contributing to \(\lambda N\)-AA degradation under these conditions. Unlike \(\lambda N\)-AA, however, we found that \(\lambda N\)-L1-AA was still immune in clpP or clpA clpX mutant strains at high temperature (Fig. 6), suggesting that \(\lambda N\)-L1-AA is not sensitive to HflB or proteases other than ClpXP at 39\(^\circ\)C. Because both of these proteins have the same SsrA tag but only \(\lambda N\)-AA appears to be degraded in a ClpP-independent fashion at 39\(^\circ\)C, factors in addition to the SsrA tag must influence degradation by proteases such as HflB.

Discussion

SsrA-mediated addition of a peptide signal to the carboxyl terminus of incompletely translated protein fragments is a newly discovered way for bacterial cells to mark abnormal proteins for destruction (Keiler et al. 1996). Cytoplasmic proteases that recognize and degrade SsrA-tagged proteins are critical components of this quality-control system and have now been identified.
Our studies show that ClpXP and ClpAP, two related protease complexes, degrade SsrA-tagged proteins in the bacterial cytoplasm. This ATP-dependent degradation of SsrA-tagged substrates can be reproduced with purified components in vitro, and requires a substrate with a wild-type SsrA tail, the presence of both ClpP and either ClpX or ClpA. Keiler et al. (1996) showed previously that periplasmic proteins containing the SsrA-tag are degraded by Tsp (Prc), a protease that does not require or use ATP. There are, therefore, at least three proteases in E. coli, which function in the SsrA quality-control surveillance system. In addition, as discussed below, there are hints that one or more additional cytoplasmic proteases may also degrade SsrA-tagged substrates. This indicates that both carboxy-terminal-specific substrate recognition is relatively common among bacterial proteases and that the SsrA quality-control system has evolved to use a substantial number of different proteolytic systems.

ClpAP and ClpXP have mini-proteasome structures composed of four stacked rings (Kessel et al. 1995; Grimaud et al. 1998), in which ClpP, the proteolytic component, forms the central portion. The crystal structure of ClpP shows a double-ring assembly of heptamers in which the active sites are located in a central proteolytic chamber with small axial entrance pores (Wang et al. 1997). It has been proposed that Clpx or ClpA, which themselves form the outer rings of the proteolytic complex, act in a chaperone-like fashion to help denature substrates and feed them into the active-site chamber of ClpP (Gottesman et al. 1997; Wang et al. 1997). This model is appealing as both ClpX and ClpA, in the absence of ClpP, have been shown to have ATP-dependent chaperone activities that catalyze the disassembly of protein oligomers (Wickner et al. 1994; Levchenko et al. 1995). The ability of ClpA and ClpX to use the energy of ATP hydrolysis to aid in protein degradation may be important to allow SsrA-tagged substrates to be degraded even when these proteins are stably folded, as is the case, for example, for the SsrA-tagged amino-terminal domain variants of λ repressor studied here.

Recognition of protein substrates by ClpXP and ClpAP is mediated by the ClpX and ClpA subunits (Gottesman et al. 1993; Wojtkowiak et al. 1993), and recent protein-dissection studies have shown that ClpX contains modular domains that bind to the carboxy-terminal peptides of several target proteins, including SsrA-tagged Arc repressor (Levchenko et al. 1997). Because both ClpA and Tsp also contain domains homologous to the substrate-binding domains of ClpX (Levchenko et al. 1997), it seems very likely that ClpXP, ClpAP, and Tsp will all recognize SsrA-tagged substrates by a common mechanism that employs independent peptide-binding domains to recognize the carboxy-terminal SsrA tag.

ClpAP degrades certain β-galactosidase fusion proteins in vivo and degrades model peptides and casein in vitro (Gottesman et al. 1990; Tobias et al. 1991). Remarkably, the ClpAP-dependent degradation of certain of these β-galactosidase fusions is controlled by the identity of their amino-terminal amino acids (Tobias et al. 1991). Therefore, there is a somewhat startling difference between our results, which indicate carboxy-terminal recognition by the ClpAP enzyme, and those of Tobias et al. (1991), which suggest amino-terminal recognition. These apparently conflicting results might be reconciled if ClpAP contained a substrate-binding domain that recognized amino-terminal determinants in addition to the peptide-binding domain that apparently recognizes carboxy-terminal determinants.

ClpX recognizes the carboxy-terminal regions of several specific substrates including MuA transposase, Mu repressor, and certain virulent derivatives of Mu repressor (Levchenko et al. 1995; Laachouch et al. 1996; Vogel et al. 1996). Hence, the carboxy-terminal specificity shown here for ClpXP degradation of SsrA-tagged substrates is consonant with prior results. It is important to note, however, that the common specificity that we observe for ClpAP and ClpXP degradation of SsrA-tagged proteins contrasts with previous results, in which little or no cross-reactivity was observed for specific substrates (for review, see Gottesman 1996). It remains to be determined how the ClpA and ClpX enzymes recognize common SsrA-tagged substrates but have distinct specificities in other cases.

ClpXP and ClpAP must be responsible for most of the intracellular degradation of the λN-αα and λN-L1-β-αα proteins in E. coli growing at 32°C, as little residual degradation of either SsrA-tailed protein is observed in cells mutant for clpP or mutant for both clpA and clpX. Several observations, however, suggest that other cytoplasmic proteases are also capable of degrading SsrA-tagged proteins under appropriate conditions. For example, degradation of the λN-β-αα protein at 39°C is not fully eliminated by a clpP mutation. C. Hermann and R. D’Ari (pers. comm.) have found that HflB, a membrane-bound protease of the AAA family (Tomoyasu et al. 1995), has an important role in the high-temperature degradation of λN-β-αα. We note, however, that degradation of λN-L1-β-αα, which has the same SerA tag as λN-αα, remains dependent on ClpP at both high and low temperatures. HflB must not, for some reason associated with the

![Figure 6](image-url)
linker region of $\lambda N$–L1-AA, be capable of degrading this protein efficiently. These differences might arise from linker-mediated alterations in the accessibility of the SsrA tail or possibly from binding interactions of the linker regions that sequester the associated protein from membrane-bound HflB.

The existence of multiple proteases that can function in SsrA quality-control surveillance may be biologically important in providing increased degradation capacity, in allowing degradation of virtually any incomplete translation product that is marked for degradation by this system, in allowing degradation in different cellular compartments, or in ensuring that SsrA-tagged proteins can be degraded under a wide variety of growth conditions. Functional redundancy or overlap of this sort makes biological sense for a general quality-control system but also requires that the SsrA degradation tag be recognized as a ubiquitous signal by proteases that normally have discrete substrate preferences. How this occurs is an intriguing problem in molecular recognition.

There are also clues that SsrA RNA may influence proteolysis of tagged proteins by mechanisms in addition to directing carboxy-terminal addition of the SsrA tag to incomplete proteins. For example, the shorter, presumably untagged species of the $\lambda N$–trpAt protein is degraded faster in ssrA + strains than in ssrA − strains. Moreover, the SsrA-tagged species of $\lambda N$–trpAt in which the tag is added cotranslationally is degraded faster than $\lambda N$–L2-AA, a protein with essentially the same sequence in which the SsrA tail is DNA encoded (Fig. 5). There are a number of ways to rationalize these results. SsrA recognition of stalled ribosomes might recruit proteases or degradation chaperones; the proteolytic susceptibility of proteins synthesized from mRNAs without stop codons might be influenced by changes in the kinetics of translation or protein folding; and/or additional proteolytic activities may be induced in ssrA + strains.

Some of the phenotypes of ssrA mutants suggest effects beyond those caused by Clp-dependent stabilization of protein fragments. ssrA mutants grow slowly under a number of conditions (Oh and Apirion 1991; Komine et al. 1994; Trempy et al. 1994), whereas clpP mutants do not suffer the same growth problems under these conditions (S. Gottesman, unpubl.). Although this may simply indicate that degradation of some SsrA-tagged proteins requires proteases such as Tsp or HflB, it is also possible that the relief of ribosome stalling by SsrA is as important as the subsequent degradation of proteins or that tagging of particular proteins as part of their normal synthesis is important for cell growth. It is worth pointing out that ssrA mutants have been found to have a variety of as yet unexplained phenotypes including induction of a protease activity with Lon-like specificity (Kirby et al. 1994), defects in phage P22 growth (Retallack et al. 1994), and enhancement of the activities of a variety of phage and bacterial repressors (Retallack and Friedman 1995). The extent to which these phenotypes might be independent of the degradation of SsrA-tagged proteins is an important question that remains to be answered.

### Materials and methods

#### Strains and plasmids

Hosts for experiments in vivo were a set of isogenic strains, each containing lacO1 inserted into the malA gene. The parental strain, SG21163, is derived from SG20250 (Gottesman et al. 1985); a malB-lacO1 derivative was constructed as described previously (Jubete et al. 1996). From SG21163, the various protease mutations were introduced by PI transduction and either selection for the inserted antibiotic resistance (SG21164, clpB::kan; SG21165, clpP::cat $\Delta \lambda$on; SG21268, hflA::kan; SG22174, clpP::cat; SG22175, clpP::kan (polon on clpP); SG22176, clpA::kan; and SG22177, clpP::kan), linkage of lon to proc (SG22186, $\Delta$lon rcsA51::kan), or linkage to a marker for HflBts (SG22166, hflBts linked to tet, transduced from SL93, a gift from C. Herman and R. D’Ari (Herman et al. 1993)). To construct the clpX clpA double mutant, SG22178, we first linked zba-1091::tet [from SG1091; (Mauriz et al. 1985)] to clpX::kan and then transduced from this host to the clpA::kan strain, selecting for tetracycline resistance. Transductants were screened initially for increased immunity in the presence of the pAN–SPT plasmid and subsequently checked by back transduction of the tet resistance to a kanamycin sensitive strain and demonstration of ability to transfer the linked clpX::kan marker. Strain GL005 is lon− clpQ− rcsA− (G. Leffers, unpubl.).

The isogenic protease-deficient strains were transformed with the plasmids shown in Figure 1. Plasmids pAN–SPT and pAN–CPT encode residues 1–93 of $\lambda$ repressor (the amino-terminal domain) with the carboxy-terminal extensions AAN DENYAL and AAN DENYALDD, respectively (Keiler et al. 1996). The proteins encoded by these plasmids are referred to as AN–AA and AN–DD, respectively. Plasmids pER130 and pER132 encode similar constructs but have a linker with an M2 FLAG epitope and 6 His sequence inserted residues 1–93 and the carboxy-terminal extensions (Fig. 1); the resulting proteins are referred to as AN–L1-AA and AN–L1-DD. To construct pER130, PCR was used to amplify the carboxy-terminal portion of $\lambda$ repressor amino-terminal domain–M2 FLAG–6 His gene in plasmid pAD103 (A. Davidson, unpubl.) using primers ER27 (5′-TATACCGGGCATTGCCATCAGAAA-3′) and ER30 (5′-CCGATCCCTAATACGCTATAGATATGTTTTGGCAGCGCATTGCGATGATGATGATGCTTGTCA-3′). The resulting product was cut with Nhel and BamHl and ligated to the pAD103 Nhel–BglII backbone fragment. Plasmid pER132 was constructed in the same fashion using primers ER27 and ER32 (5′-CCGATCCCTAATACGCTATAGATATGTTTTGGCAGCGCATTGCGATGATGATGATGCTTGTCA-3′). The expected structure of each construct was verified by restriction mapping and DNA sequencing.

The backbones for pPW500, pER161, and pER163 (Fig. 1) are from pAD100, a pBR322-based plasmid carrying a Ppro promoter (trp/lacUV5 hybrid promoter), lacO1 and an F+ phage origin (Davidson and Sauer 1994). Cloning into pAD100 resulted in a change in the amino-terminal amino acid of $\lambda$ repressor from Ser to Gly. This sequence change, however, does not affect repression activity (Clarke et al. 1991). Construction of pPW500, which contains a trpA terminator following the amino-terminal domain and directs transcription of an mRNA with no in-frame stop codons, has been described previously (see footnote 14 in Keller et al. 1996). Addition of the AAN DENYALDD carboxy-terminal sequence to the AN–trpA protein is dependent on SsrA-mediated addition of the tag sequence in vivo, and therefore there can be untagged or SsrA-tagged versions of AN–trpA. Plasmids pER161 and pER163 encode proteins (AN–L1-AA and AN–L1-DD, respectively) with linkers containing the same
amino acids as those encoded by the trpA terminator sequence. As a result, the SsrA-tagged λN-trpA protein and the λN-L2-AA protein should have the same or very similar amino-acid sequences (see Fig. 1 and legend). Plasmid pER161 was constructed by amplifying plasmid pAD103 with primers ER60 (5'-GGCCATGGGCACAAAAAAGAAACCATTA-3') and ER61 (5'-GGCCATGGGACCTAAAGCGTGATGGTGATGATGGG-3'), cloning the PCR products into plasmid pT7Blue-T (Novagen), and expressing the resulting product in E. coli. Cells containing plasmids encoding λN-L2-DD, λN-L2-AA, λN-trpA were grown without IPTG induction in tryptone broth with vitamin B1, maltose, 0.01 M MgSO4, and ampicillin (100 µg/ml) at 32°C to late log phase. Lawns of cells were plated in top agar on LB or LB-Amp plates, spotted with dilutions of λc12, λc17, λvir, and λimm21cl phases, and incubated at 32°C until indicated. Efficiencies of plating were reported relative to growth on cells with vector alone or with no plasmid; both control strains were cotransformed. Efficiencies of plating are reported relative to growth on cells with vector alone or with no plasmid; both control strains were cotransformed. Efficiencies of plating are reported relative to growth on cells with vector alone or with no plasmid; both control strains were cotransformed.

Steady-state expression and degradation in vivo

Intracellular levels of different amino-terminal domain variants, which reflect a balance between the rates of synthesis and degradation of these proteins, were assayed by measuring immuno-

ity to superinfecting λ phage. Cells containing high levels of the amino-terminal domain of λ repressor are immune, whereas those containing low levels are sensitive to phage in-

fection. Cells containing plasmids encoding λN-AA, λN-DD, λN-L1-AA, λN-L1-DD, λN-L2-AA, λN-L2-DD, and λN-trpA were grown without IPTG induction in tryptone broth with vitamin B1, maltose, 0.01 M MgSO4, and ampicillin (100 µg/ml) at 32°C to late log phase. Lawns of cells were plated in top agar on LB or LB-Amp plates, spotted with dilutions of λc12, λc17, λvir, and λimm21cl phases, and incubated at 32°C until indicated. Efficiencies of plating were reported relative to growth on cells with vector alone or with no plasmid; both control strains were cotransformed. Efficiencies of plating are reported relative to growth on cells with vector alone or with no plasmid; both control strains were cotransformed. Efficiencies of plating are reported relative to growth on cells with vector alone or with no plasmid; both control strains were cotransformed.

Determination of Clp degradation of SsrA-tagged substrates

The λN-L1-AA and λN-L1-DD proteins were expressed in E. coli strain X90 transformed with pER130 or pER132. Cells were grown to mid-log phase at 37°C in M9 minimal medium lacking cysteine and methionine, and protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside to 200 µg/ml. Twenty minutes after induction, [35S]methionine was added (40 µCi/ml culture). After 1 hr at 37°C, the cells were harvested by centrifugation and frozen at −80°C. Frozen cells were thawed, resuspended in Buffer A (6 המ guanidine hydrochloride, 0.1 M NaH2PO4, 0.01 M Tris (pH 8.0)) containing 10 mM imidazole (40 µl/ml culture) and allowed to lyse while rocking at 4°C for 1 hr.

Preparation of 35S-labeled substrate proteins

The λN-L1-AA and λN-L1-DD proteins were expressed in E. coli strain X90 transformed with pER130 or pER132. Cells were grown to mid-log phase at 37°C in M9 minimal medium lacking cysteine and methionine, and protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside to 200 µg/ml. Twenty minutes after induction, [35S]methionine was added (40 µCi/ml culture). After 1 hr at 37°C, the cells were harvested by centrifugation and frozen at −80°C. Frozen cells were thawed, resuspended in Buffer A (6 M м guanidine hydrochloride, 0.1 M NaH2PO4, 0.01 M Tris (pH 8.0)) containing 10 mM imidazole (40 µl/ml culture) and allowed to lyse while rocking at 4°C for 1 hr.

Proteolytic assays in vitro

Proteolytic digestions were performed in a total volume of 20 µl in buffer containing 25 mM HEpes/NaOH (pH 7.5), 25 mM potassium acetate, 5 mM MgC12, 10% glycerol, and 0.02% NP-40. Complete ClpP and ClpXP digestions were performed in 200 µg/ml, 0.1 mg/ml creatine kinase, 10 mM creatine phosphate, 10 pmoles of 35S-labeled protein substrate (λN-L1-AA or λN-L1-DD), 3.5 pmoles of ClpP subunits, and 3 pmoles of ClpXP subunits. Complete chymotrypsin digestions contained 10 pmoles protein substrate and 200 ng of α-chymotrypsin. Digestion solutions were incubated at 30°C for different times, the reactions stopped by the addition of TCA to 10%, and the tubes left on ice. After a minimum of 30 min, the samples were centrifuged for 10 min at 20,000 g and 4°C, and TCA soluble counts were determined by scintillation counting in Ready Safe liquid scintillation cocktail (Beckman). Control 2 hr digestions lacking either ClpX or ClpP, ClpP, or ATP showed an average of 3%–4% of the total 35S counts in the supernatant after TCA precipitation. These average values were subtracted as background from the complete digestion samples containing ClpP/ATP or ClpXP/ATP.

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Circular-dichroism spectra and thermal melts

The circular-dichroism (CD) spectra of the λ-L1-1AA and λN-1L1-DD proteins were recorded at a concentration of 3.8 μM in 25 mM HEPES/NaOH (pH 7.5), 50 mM NaCl at 25°C using an AVIV model 60DS spectropolarimeter. Thermal melts, performed using the same buffer and protein concentrations, were monitored by changes in CD ellipticity at 230 nm. Melts were performed in 1°C increments with a 42-sec equilibration time and a 30-sec averaging time at each temperature.

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The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system

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