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Co-Evolution of Multipartite Interactions Between an Extended tmRNA Tag and a Robust Lon Protease in *Mycoplasma*

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Abstract

Messenger RNAs that lack in-frame stop codons promote ribosome stalling and accumulation of aberrant and potentially harmful polypeptides. The SmpB-tmRNA quality control system has evolved to solve problems associated with nonstop mRNAs, by rescuing stalled ribosomes and directing the addition of a peptide tag to the C-termini of the associated proteins, marking them for proteolysis. In *E. coli*, the ClpXP system is the major contributor to disposal of tmRNA tagged proteins. We have shown that the AAA+ Lon protease can also degrade tmRNA tagged proteins, but with much lower efficiency. Here, we present a unique case of enhanced recognition and degradation of an extended *Mycoplasma pneumoniae* (MP) tmRNA tag by the MP-Lon protease. We demonstrate that MP-Lon can efficiently and selectively degrade MP-tmRNA tagged proteins. Most significantly, our studies reveal that the larger (27 amino acid long) MP-tmRNA tag contains multiple discrete signaling motifs for efficient recognition and rapid degradation by Lon. We propose that higher affinity multipartite interactions between MP-Lon and the extended MP-tmRNA tag have co-evolved from pre-existing weaker interactions, as exhibited by Lon in *E. coli*, to better fulfill the function of MP-Lon as the sole soluble cytoplasmic protease responsible for the degradation of tmRNA tagged proteins.

Keywords

Protein degradation; AAA+ proteases; Lon protease; SmpB; tmRNA; *trans*-translation

INTRODUCTION

Protein degradation has emerged as a key cellular mechanism for regulation of a diverse array of physiological processes. In prokaryotes, energy-dependent proteases play a major role in re-sculpting the bacterial proteome, in maintaining ideal concentrations of critical regulatory proteins, and in the disposal of unwanted, damaged or misfolded proteins. Bacterial energy-dependent proteases are grouped into four families, named after their representative members: ClpXP/ClpAP, HslUV (ClpYQ), FtsH (HflB), and Lon (Gottesman, 1996). The Clp and HslUV proteases are two-component proteases consisting of a chaperone subunit, ClpA, ClpX, or HslU, and a peptidase subunit, ClpP or HslV. The

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ClpA, ClpX, and HslU chaperones are ATPases that are critical for substrate recognition, unfolding and translocation into the ClpP or HslV peptidase. The activities of the ATP-driven chaperone components help determine the substrate range and specificity for ClpAP, ClpXP, and HslUV proteases. Unlike the two-component proteases, the Lon protease forms a hexameric ring, derived from a single polypeptide that carries both the ATP-dependent chaperone and peptidase functions. Each Lon monomer has three distinct domains: the amino-terminal domain that is implicated in substrate binding, the ATPase domain that contains the Walker A and B motifs for ATP binding and hydrolysis, and the peptidase domain located at the carboxyl terminus of the protein.

For the vast majority of proteins, the sequence determinants recognized by energy dependent proteases appear to be present in the primary sequence of each individual substrate, which are either constitutively accessible for protease recognition or become available under specific cellular transitions or environmental conditions (Gottesman, 1996, Gottesman, 2003, Baker & Sauer, 2006). For one group of substrates, the tmRNA tagged proteins, a defined protease recognition module is added to their C-termini. The tmRNA-mediated tagging and ribosome rescue system is the only known biological process that co-translationally appends a degradation module to the C-termini of proteins to target them for directed proteolysis (Dulebohn *et al.*, 2007, Karzai & Sauer, 2001, Keiler, 2008, Keiler *et al.*, 1996, Withey & Friedman, 2003). The idea of a degradative role for tmRNA function originated from the realization that the C-terminal residues of the peptide sequence encoded by the mRNA-like domain of tmRNA are similar to recognition determinants of intracellular proteases (Keiler *et al.*, 1995, Parsell *et al.*, 1990, Silber *et al.*, 1992, Silber & Sauer, 1994). Subsequent studies showed that energy-dependent proteases are important contributors to this process (Gottesman *et al.*, 1998, Herman *et al.*, 1998). Early work on the proteolytic function of the tmRNA-mediated *trans*-translation process had shown that ClpXP, ClpAP, and FtsH were involved in disposal of tmRNA tagged proteins in a tag-specific manner (Gottesman *et al.*, 1998, Herman *et al.*, 1998). In *E. coli*, ClpXP has been established as the principal protease responsible for degradation of tmRNA tagged proteins *in vivo* (Lies & Maurizi, 2008). The inner membrane-bound protease FtsH is thought to have narrower specificity against tmRNA tagged proteins than ClpXP and ClpAP, and is active mainly on unstable (Herman *et al.*, 2003) and locally available substrates (Kihara *et al.*, 1995, Kihara *et al.*, 1999). Recent work from our lab demonstrated a role for the Lon ATP-dependent protease in degradation of tmRNA tagged proteins (Choy *et al.*, 2007). Although Lon contributes more to the *in vivo* degradation of tmRNA tagged proteins than ClpAP or FtsH, its contributions are still far less than those made by the ClpXP system (Choy *et al.*, 2007, Lies & Maurizi, 2008). These findings suggest that *E. coli* ClpXP, in coordination with its SspB cofactor, has much higher affinity for tmRNA tagged proteins than the *E. coli* Lon protease. However, this selective delivery and high affinity for targeted degradation of tmRNA tagged proteins need not be true in all bacterial species. Indeed, recent surveys of protease homologs and orthologs in eubacteria have revealed that the ATP-dependent Lon protease is more strongly conserved than other bacterial energy-dependent proteases, including the Clp family proteases (Tripathi & Sowdhamini, 2008). For instance, the small genome bacterial species *Mycoplasma* lack both the ClpAP and ClpXP protease systems, yet they possess a Lon protease ortholog. In contrast to the variable conservation of bacterial energy-dependent proteases, the SmpB-tmRNA system is strictly conserved and is presumably universally utilized in eubacteria to co-translationally tag proteins for directed proteolysis. This presents an interesting quandary. If the *E. coli* tmRNA tag recognition model for ClpXP and Lon proteases is universally true, then how are *Mycoplasma* tmRNA tagged proteins targeted for efficient degradation?

Despite the significance of Lon in re-sculpting the bacterial proteome, there is a paucity of information about how Lon is targeted to specific substrates, how these substrates are

recognized and degraded, or what sequence or structural features are important for recognition by Lon. We have been interested in understanding how Lon protease recognizes and degrades specific substrates. In particular, we have been interested in the potential link between the Lon protease and the *trans*-translation system of *Mycoplasma* species (*Mycoplasma pneumoniae* and *Mycoplasma genitalium*). We have been especially intrigued by the expanded nature and sequence composition of the *Mycoplasma* tmRNA tag, as it is considerably longer (27 amino acid residues, compared to the 11 amino acid residue *E. coli* tmRNA tag) and contains an unusual preponderance of charged residues (Fig. 1A). Thus, we thought it conceivable that the *Mycoplasma* tmRNA tag and the *Mycoplasma* Lon protease have co-evolved to facilitate rapid and efficient recognition and degradation of tmRNA tagged proteins. In this report, we present evidence to demonstrate that the *Mycoplasma* Lon protease is a highly robust protease that has co-evolved with tmRNA to detect an expanded set of recognition elements within the extended *MP*-tmRNA peptide tag sequence for efficient and rapid clearance of tagged proteins. Our findings also have implications for how Lon recognizes both its normal and tagged substrates.

RESULTS

Construction of a reporter protein harboring the *Mycoplasma pneumoniae* tmRNA tag

To investigate the relationship between *Mycoplasma pneumoniae* (*MP*) tmRNA tagged proteins and Lon protease, we fused the *MP*-tmRNA tag nucleotide sequence to the 3' end of the N-terminal domain of the γ -*cl* gene to generate a reporter substrate for *MP*-Lon. We cloned, expressed, and purified the resulting λ -*cl*-N-ssrA_{MP} protein product. However, the purified λ -*cl*-N-ssrA_{MP} protein resolved into two distinct bands, suggesting a mixture of two related proteins with a molecular weight difference of 1–2 kDa (data not shown). In order to determine the identity of the two proteins, we subjected the purified proteins to MALDI-TOF mass spectrometry analysis. This analysis showed the higher molecular weight band (15102 Da) to be the full-length λ -*cl*-N-ssrA_{MP} reporter and the lower molecular weight band (13893 Da) to be a truncated reporter protein carrying the N-terminal part of the *MP*-tmRNA tag but missing the C-terminal 11 amino acid residues (Fig. 1B, and Fig. S1). These results suggested that part of the *MP*-tmRNA tag sequence served as a recognition site for one or more *E. coli* peptidases, leading to cleavage of the C-terminal 11 amino acids of the tag. Analysis of this reporter in ClpXP and Lon deficient strains suggested that these proteases were not responsible for generating the shorter product. The cleavage product is henceforth referred to as λ -*cl*-N-ssrA_{MP-11}.

Purified *MP*-Lon degrades *MP*-tmRNA tagged proteins selectively and with high efficiency

The *E. coli* tmRNA tag (*EC*-tag) is rich in information content, with binding determinants for ClpXP, SspB, ClpAP, and Lon proteases (Farrell *et al.*, 2007, Flynn *et al.*, 2004, Flynn *et al.*, 2001, Levchenko *et al.*, 2000, Martin *et al.*, 2008, McGinness *et al.*, 2007, Dulebohn *et al.*, 2007, Choy *et al.*, 2007, Karzai & Sauer, 2001, Gottesman *et al.*, 1998). Since our purified λ -*cl*-N-ssrA_{MP} reporter protein contained a mixture of both full-length λ -*cl*-N-ssrA_{MP} and truncated λ -*cl*-N-ssrA_{MP-11}, we reasoned that perhaps the shorter reporter might serve as an internal control for our *in vitro* proteolysis assay, as it might be missing some key Lon recognition determinants. We, therefore, tested the ability of purified *MP*-Lon to degrade the *MP*-tmRNA tagged reporter proteins. As shown in Figure 1C, *MP*-Lon was fully capable of selectively degrading full-length λ -*cl*-N-ssrA_{MP}. The *MP*-Lon mediated degradation process was dependent on the presence of two key elements. First, the reaction was absolutely dependent on the presence of ATP, suggesting that ATP binding and hydrolysis was required for substrate binding and unfolding. Second, reporter protein degradation was dependent on the presence of full-length *MP*-tmRNA tag. This result was confirmed by the finding that *MP*-Lon was unable to degrade either the truncated λ -*cl*-N-

ssrA_{MP-11} (internal control) or the untagged λ -cI-N reporter protein (Fig. 1). Thus, the addition of *MP*-tmRNA tag converted a non-substrate protein into an efficient substrate for degradation by the *MP*-Lon protease. In addition, the fact that *MP*-Lon was unable to efficiently degrade the truncated λ -cI-N-ssrA_{MP-11} reporter suggested that the C-terminal 11 amino acids of the *MP*-tmRNA tag contain key elements for recognition and degradation by *MP*-Lon. These findings also provided a convenient internal control (λ -cI-N-ssrA_{MP-11}) for future proteolysis experiments.

***MP*-Lon is a more efficient protease**

Despite extensive length and amino acid sequence differences between the *EC*- and *MP*-tmRNA tags, they still share some common attributes. For instance, the C-termini of both tags end with a mixture of hydrophobic and small amino acids (Fig. 1A). Therefore, we endeavored to determine whether the *MP*-Lon protease could recognize *EC*-tmRNA tag, and whether the *EC*-Lon protease could recognize *MP*-tmRNA tag. To this end, we expressed and purified *MP*-Lon, *EC*-Lon, and two tagged reporter proteins: one carrying the *EC*-tmRNA tag (λ -cI-N-ssrA_{EC}) and the other carrying the *MP*-tmRNA tag (λ -cI-N-ssrA_{MP}). In *in vitro* proteolysis assays, using 100 nM *MP*-Lon hexamer, we observed degradation of λ -cI-N-ssrA_{EC} (Fig. 1D, top panel), suggesting that *EC*-tmRNA tag was capable of targeting substrates to proteolysis by *MP*-Lon. In a similar analysis of the *EC*-Lon protease, however, we noticed a compelling difference between the two proteases. When we used an equivalent concentration (100 nM hexamer) of *EC*-Lon protease and a reporter protein with *MP*-tmRNA tag (λ -cI-N-ssrA_{MP}), we observed only modest degradation of the tagged protein. However, when we increased the concentration of *EC*-Lon to 400 nM hexamer, efficient degradation of λ -cI-N-ssrA_{MP} was observed (Fig. 1D, lower panel). These data indicated that *EC*-Lon has lower affinity for the *MP*-tmRNA tag. The observed lower affinity of *EC*-Lon was not due to loss of enzymatic activity during purification or storage. We observed similar proteolytic activities with several independent preparations of both *MP*- and *EC*-Lon proteases, and both enzymes were equally active against denatured Casein (data not shown). These results suggested that in the absence of the Clp system the *Mycoplasma* Lon protease has evolved to become a much more efficient protease capable of specific recognition and selective degradation of *MP*-tmRNA tagged proteins.

***MP*-Lon recognizes multiple sequence motifs within the expanded *MP*-tmRNA tag**

Having established that *MP*-Lon was active and fully capable of selectively recognizing and degrading *MP*-tmRNA tagged proteins with high efficiency, we sought to gain insights into the extended nature of the *MP*-tmRNA tag. We reasoned that perhaps in the absence of *clp* genes in *Mycoplasma*, the extended *MP*-tmRNA tag sequence had evolved to provide enhanced recognition of tagged proteins by the *MP*-Lon protease. Such an enhancing effect on Lon recognition could be achieved through expansion of tag sequence elements for direct Lon recognition, acquisition of mediatory sequences for cofactor binding, or a combination of both. To date, a protein cofactor for Lon protease has not been identified. Additionally, it is thought that evolutionary adaptations have resulted in massive gene loss in *Mycoplasma*. Therefore, it is less likely that a new cofactor that facilitates substrate delivery to Lon would have emerged. We thus hypothesized that in addition to the C-terminal sequence elements, the expanded *MP*-tmRNA tag might have evolved added recognition elements for efficient recognition by *MP*-Lon. To directly test this idea, we divided the 27-amino-acid long tmRNA tag into 9 regions and substituted, where possible, the native tag residues with aspartic acids (Fig. 2A). The reporter proteins, carrying *MP*-tmRNA tag variants with aspartic acids substitutions at each of the 9 regions, were cloned, expressed, and purified. An interesting and immediate outcome of this analysis was that upon substitution of aspartic acid residues in regions 5 through 9 we observed a major change in the pattern of the purified reporter proteins (Fig. 2A). More specifically, we observed a total absence of the

shorter λ -cI-N-ssrA_{MP-11} tagged protein, suggesting that the *E. coli* peptidases responsible for this cleavage were sensitive to introduction of aspartic acid residues in this region of the *MP*-tmRNA tag.

To ensure that all proteolysis assays had the same ratio of full-length λ -cI-N-ssrA_{MP} reporter and the degradation resistant λ -cI-N-ssrA_{MP-11} internal control, we constructed a reporter gene variant, by introducing a stop codon after codon 15 of the tag, which produced a reporter that was identical to λ -cI-N-ssrA_{MP-11} control (Fig. 1B). We expressed and purified the λ -cI-N-ssrA_{MP-11} control protein and added it to each purified λ -cI-N-ssrA_{MP} tag variant at a concentration that yielded equimolar ratios of the two proteins (at 1:1 molar ratio of each full-length λ -cI-N-ssrA_{MP} tag variant and the λ -cI-N-ssrA_{MP-11} control). We assessed the effect of aspartic acid substitutions on *MP*-Lon recognition of *MP*-tmRNA tagged reporters by subjecting each λ -cI-N-ssrA_{MP} variant to *in vitro* proteolysis (Fig. 2B). In order to better illustrate the effect of aspartic acid substitutions in each of the nine regions of the *MP*-tmRNA tag, we quantified the remaining amount of full-length reporter at each time point and have presented the 15-minute time point values in bar graph format as an indicator of their apparent degradation efficiency. This analysis demonstrated that aspartic acid substitutions in the extreme C-terminal segment of the *MP*-tmRNA tag (regions 8 and 9) were very effective in preventing directed degradation of the λ -cI-N-ssrA_{MP} reporter protein by *MP*-Lon (Fig. 2B). These results were consistent with our earlier observation that *MP*-Lon is unable to efficiently degrade the λ -cI-N-ssrA_{MP-11} control protein, and provided strong support for the conclusion that some key *MP*-Lon recognition elements were located within the last 11 amino acid residues of *MP*-tmRNA tag. Aspartic acid substitutions in region 6 and region 7, although located within the last 11 amino acids of the *MP*-tag, produced only a weak effect on degradation of the λ -cI-N-ssrA_{MP} reporter by *MP*-Lon.

Most significantly, aspartic acid substitutions in region 5 of the reporter rendered it highly resistant to proteolysis by *MP*-Lon, despite the fact that this reporter variant had the full complement of the C-terminal recognition elements (Fig. 2B). These results suggested that amino acid residues in region 5 of the *MP*-tmRNA tag constitute an additional critical recognition element for binding and degradation by *MP*-Lon. Furthermore, aspartic acid substitutions in region 4, located just upstream of region 5, resulted in close to 50% reduction in the degradation of the reporter substrate, suggesting an intermediate level of contributions by this region in recognition by *MP*-Lon. In contrast, aspartic acid substitutions in regions 1, 2, or 3 did not have any substantial effect on the ability of *MP*-Lon to degrade the λ -cI-N-ssrA_{MP} reporter protein (Fig. 2B). Taken together, these results lend strong support to the conclusion that *MP*-Lon requires multiple signaling elements in the *MP*-tmRNA tag for efficient recognition and degradation of tagged proteins. Key signaling elements for these multipartite interactions appear to be located in discrete sites, including the extreme C-terminus (regions 8 and 9), and in the middle of the tag (regions 4 and 5).

Cumulative contributions of key signal residues to the efficient recognition of *MP*-tmRNA tag

Having established that discrete *MP*-tmRNA tag sequence elements were required for Lon recognition, we set out to assess the relative contributions of individual amino acids in each of the key signaling sites to recognition by *MP*-Lon. To this end, we made single aspartic acid substitutions in regions 5, 8 and 9 to verify their importance to recognition by *MP*-Lon (Fig. 3A). Single aspartic acid substitutions were also made in a control region (region 7) to confirm that it did not contain important Lon recognition elements. Hydrophobic amino acid residues in each region of the tag were targeted, as it has been reported that *E. coli* Lon prefers these residues in some of its proteolytic substrates (Gur & Sauer, 2008b). Targeted residues included L14, I15, A16 in region 5, I22 in region 7, Y24 in region 8, and F26 in

region 9. Site directed mutagenesis was used to introduce the desired substitutions. Each λ -cI-N-ssrA_{MP} reporter protein variant was expressed and purified to near homogeneity. We assessed the effect of each single aspartic acid substitution on recognition and degradation by *MP*-Lon in the *in vitro* proteolysis assay (Fig. 3A). Quantification of the reporter substrate variants showed a range of modest effects on degradation by *MP*-Lon but none of the single Asp substitutions resulted in complete inhibition of *MP*-Lon activity. The two aromatic residues in regions 8 and 9 (Y24 and F26) made larger contributions than hydrophobic residues in region 5. Among the residues in region 5, the relatively bigger hydrophobic residues (L14 and I15) contributed more than the smaller A16. Amino acid I22 in region 7, despite being hydrophobic, did not have a substantial effect on substrate degradation by *MP*-Lon (Fig. 3A). This finding was consistent with the observation that region 7 was not a primary recognition determinant. These results support the conclusion that *MP*-Lon recognizes multiple sequence elements within the extended tmRNA tag, and that individual residues within these region make small but important contribution to recognition by *MP*-Lon. Therefore, the additive input of the individual signal elements contributes to the overall recognition of the *MP*-tmRNA tag by *MP*-Lon.

The tmRNA tag was initially identified as a potential proteolysis signal based on similarity of its last 5 amino acids (YALAA) with recognition signal of C-terminal specific proteases (Keiler et al., 1996). Mutational analysis of the tmRNA tag in a number of bacterial species, including *E. coli* and *Yersinia pseudotuberculosis* (Flynn et al., 2001, Gottesman et al., 1998, Okan et al., 2006, Roche & Sauer, 1999, Roche & Sauer, 2001, Sundermeier et al., 2005, Keiler et al., 1996), has confirmed the importance of these residues for recognition by cellular proteases. For instance, the last three amino acid residues (LAA-COO⁻) of the *EC*-tmRNA tag are of particular importance for recognition by the ClpXP system. Conversion of the ultimate and penultimate alanines of the tag to aspartic acid residues (from LAA to LDD) renders tagged proteins highly resistant to proteolysis by ClpXP. The last four residues of the *MP*-tmRNA tag (YAFA) have a similar mix of large hydrophobic and small amino acids (Fig. 1A). We have already demonstrated that the two aromatic amino acids are required for recognition by *MP*-Lon (Fig. 2). Our analysis also demonstrated that A16 in region 5 of the *MP*-tmRNA tag made no substantial contributions to recognition by *MP*-Lon (Fig. 3A). We were curious to know why alanines were conserved at the C-terminus of the tag and whether they also served as signals for recognition by *MP*-Lon. To assess the importance of these residues, we constructed reporter proteins where the last four amino acids of the tag were independently changed from YAFA-COO⁻ to either -DADA-COO⁻ or -YDFD-COO⁻ and evaluated the degradation of these substrates by *MP*-Lon (Fig. 3B). Interestingly, both tag variants were highly resistant to proteolysis, suggesting that the two alanines at the C-terminus of the tag were as critical to recognition by *MP*-Lon as the two aromatic residues.

***EC*-Lon is also sensitive to aspartic acid substitutions in multiple sequence elements within the expanded *MP*-tmRNA tag**

We have shown that *EC*-Lon is capable of recognizing and degrading *MP*-tmRNA tagged proteins (Fig. 1D). However, efficient degradation requires higher concentrations of *EC*-Lon, suggesting lower affinity for proteins carrying the *MP*-tmRNA tag. Gur and Sauer (Gur & Sauer, 2008b) have recently shown that *EC*-Lon prefers large hydrophobic residues in its binding site and that *EC*-Lon can unfold and degrade stably folded proteins provided they have accessible recognition signals. Since the last four amino acid residues of *MP*-tmRNA tag (-YAFA-COO⁻) constitute an ideal binding site for *EC*-Lon, we wondered whether the presence of these residues was necessary and sufficient for degradation of a substrate carrying the *MP*-tmRNA tag, or whether *EC*-Lon was also sensitive to changes that affect internal signals within the *MP*-tmRNA tag.

To address these questions, we evaluated the ability of *EC*-Lon to degrade single aspartic acid substitution variants of the λ -cI-N-ssrA_{MP} reporter protein (Fig. 3C). We found that *EC*-Lon was not affected by aspartic acid substitutions in regions 1 or 2, and was only weakly sensitive to substitutions in region 3 of the tmRNA tag (data not shown). *E. coli* Lon was more sensitive to a single aspartic acid substitution in region 7 (I22D) of the *MP*-tag (Fig. 3C). However, *EC*-Lon was highly sensitive to single aspartic acid substitutions in region 5 (L14D, and I15D) and in regions 8–9 (Y24D and F26D) of the *MP*-tag. Taken together, these data suggested that the low affinity interactions between the *EC*-Lon and *MP*-tmRNA tag were similarly distributed over multiple regions of the tag peptide and disrupting these interactions had a negative impact on the propensity of *EC*-Lon to bind and degrade its target substrate. These results also suggested that the presence of large hydrophobic residues at the C-terminus of a substrate was necessary but not sufficient for degradation and that *EC*-Lon made similar, albeit lower affinity, contacts with additional internal elements of the *MP*-tmRNA tag.

Steady-state kinetic analysis of tagged protein degradation by *MP*-Lon and *EC*-Lon

The finding that *MP*-Lon and *EC*-Lon proteases degraded tmRNA tagged proteins to different extents suggested that these proteases had different affinities for tagged proteins. Similarly, the fact that aspartic acid substitutions in multiple key regions of the *MP*-tag affected degradation of the reporter protein by both Lon proteases suggested that these alterations had lowered the affinity of these enzymes for tagged substrates. To gain further insights into these enzymatic processes we carried out steady-state kinetic analysis of the degradation of tagged proteins by the *MP*-Lon and *EC*-Lon proteases. However, to perform the steady-state analysis we needed to purify [³⁵S]-labeled λ -cI-N-ssrA_{MP} reporter protein from a strain that did not produce the truncated λ -cI-N-ssrA_{MP-11} product. Although this truncated reporter had served as an extremely useful internal control in our initial proteolysis assays, its presence as a [³⁵S]-labeled protein would adversely affect the accuracy of our kinetic measurements. Therefore, we needed to identify cellular proteases responsible for the cleavage activity. Since we expressed the reporter protein in a *clpX⁻ clpP⁻ lon⁻* strain, we knew that the cleavage activity was not due to Clp or Lon proteases (Fig. S1). In subsequent studies we determined that two proteases, DegP and Tsp, were responsible for the cleavage activity and that the truncated reporter product was not generated in a strain lacking both *prc*, which encodes for Tsp, and *degP* genes (Fig. S2). The identification of the proteases responsible for production of the truncated reporter protein enabled us to use the *degP⁻ prc⁻* strain for the expression and purification of homogeneous full-length λ -cI-N-ssrA_{MP} reporter proteins needed for our steady-state kinetics analysis.

To determine directly whether *MP*-Lon and *EC*-Lon had different affinities for tagged reporter proteins, we purified full-length [³⁵S]-labeled λ -cI-N-ssrA_{MP} reporter protein, and its aspartic acid substitution variants, and measured the rates at which proteolysis by Lon generated acid-soluble radioactive peptides. We determined steady-state rates of substrate degradation by *MP*-Lon and *EC*-Lon at a range of reporter protein concentrations and fitted the data to the Michaelis-Menten equation (Fig. 4). This analysis showed *MP*-Lon to have high affinity, as reflected by the Michaelis constant (K_M), for the λ -cI-N-ssrA_{MP} reporter, with a K_M value of $0.50 \pm 0.04 \mu\text{M}$ and $V_{\max}$ of $5.1 \pm 0.1 \text{ min}^{-1} \text{ Lon}_6^{-1}$ (Fig. 4A, and Table I). *EC*-Lon exhibited greater than 30-fold lower affinity for the same λ -cI-N-ssrA_{MP} substrate, with K_M value of $17.4 \pm 2.0 \mu\text{M}$ and $V_{\max}$ of $6.4 \pm 0.2 \text{ min}^{-1} \text{ Lon}_6^{-1}$ (Fig. 4B). A similar analysis of the degradation of a substrate carrying the *E. coli* tmRNA tag, λ -cI-N-ssrA_{EC}, showed *MP*-Lon to have close to 10-fold lower affinity for this substrate as compared to the λ -cI-N-ssrA_{MP} reporter, with a K_M value of $4.9 \pm 0.4 \mu\text{M}$ and $V_{\max}$ of $5.1 \pm 0.1 \text{ min}^{-1} \text{ Lon}_6^{-1}$ (Fig. 4C, and Table I). These data were fully consistent with our finding

that *MP*-Lon had higher affinity for *MP*-tmRNA tagged proteins and degraded these substrates with high selectivity and efficiency.

Next, we examined the effect of aspartic acid substitutions in key Lon recognition determinants of the *MP*-tmRNA tag on degradation by *MP*-Lon. We chose three λ -cI-N-ssrA_{MP} aspartic acid substitution variants for this analysis: two with alterations in regions of the tmRNA tag sequence (region 5 and region 8) that constituted major determinants for Lon recognition, and one with alterations in a region of the tmRNA tag (region 6) that had only a small effect on degradation by Lon (Fig. 5). Steady-state kinetic analysis showed that aspartic acid substitutions in region 8 of the *MP*-tmRNA tag had the largest effect on the Michaelis constant, increasing K_M from $0.50 \pm 0.04 \mu\text{M}$ to $29.8 \pm 2.7 \mu\text{M}$ (Fig. 5A, and Table I). The next largest effect on the Michaelis constant was seen with aspartic acid substitutions in the internal region 5 of the tmRNA tag, increasing K_M by 18-fold to $9.0 \pm 0.6 \mu\text{M}$ (Fig. 5B, and Table I). Aspartic acid substitution in region 6 of the tmRNA tag had only a modest effect on the Michaelis constant, increasing K_M to $1.3 \pm 0.1 \mu\text{M}$ (Fig. 5C, and Table I). We also attempted to measure steady-state kinetic parameters for the degradation of region 5 variant by *EC*-Lon. However, due to the dramatic affect of aspartic acid substitutions in this region on the rate of degradation by *EC*-Lon, we were unable to obtain initial velocity data, even at very high (100 μM) substrate concentrations. These results suggested that aspartic acid substitutions in the internal segment of the *MP*-tmRNA tag had lowered the already weak affinity of *EC*-Lon for the tagged substrate to such an extent that made the tagged protein virtually unrecognizable by the *E. coli* protease. Taken together, these data are consistent with the conclusion that *MP*-Lon recognizes multiple sequence elements in the extended *MP*-tmRNA tag and that key recognition signals, which play a critical role in substrate binding and degradation by Lon, are not limited to the extreme C-terminus of the tag peptide. Furthermore, *EC*-Lon also recognizes the same key elements, albeit with much lower affinity, and is dramatically impacted by substitutions that alter these determinants.

***MP*-Lon can partially complement *E. coli lon*⁻ phenotypes**

Next, we wished to ascertain whether *MP*-Lon was capable of complementing *E. coli lon*⁻ phenotypes. The basic rationale for this analysis was to determine whether *MP*-Lon had retained the capacity to recognize well-known substrates of *EC*-Lon, or had its substrate specificity evolved away from gene products not present in *Mycoplasma*. We chose two well-known substrates of *EC*-Lon, Sula and RcsA, as they are not substrates of the tmRNA system and facile assays for their distinct *in vivo* activities are available. Sula is a cell division inhibitor induced by DNA damage that is rapidly degraded by Lon upon completion of DNA repair. Accumulation of Sula in *E. coli lon*⁻ strains renders cells sensitive to UV light and methylmethane sulfonate (MMS), a DNA damaging agent (Howard-Flanders *et al.*, 1964). To evaluate Sula stability, we assessed the capability of *MP*-Lon to complement the sensitivity of *E. coli lon*⁻ cells to MMS. First, we assayed the sensitivity of *E. coli* HDB98 *lon*⁻ mutant and its otherwise isogenic parental HDB97 strain to MMS. As shown in Figure 6A, the parental HDB97 strain was able to grow on plates irrespective of the presence or absence of MMS. In contrast, the *lon*⁻ HDB98 strain was unable to grow in the presence of MMS (Fig. 6A). To complement the phenotype of the HDB98 *lon*⁻ strain, we provided either *EC*-Lon or *MP*-Lon function on an arabinose-inducible complementation plasmid. Introduction of a plasmid harboring the *EC*-Lon gene, when induced with 0.01% arabinose, fully complemented the MMS sensitive phenotype of the *lon* strain (Fig. 6B). A plasmid carrying the *MP*-Lon gene provided partial complementation of the MMS sensitive phenotype, but only when induced to a higher level with 0.2% arabinose (Fig. 6C). These results suggested that *MP*-Lon recognized Sula weakly and degraded it with much lower efficiency, thus providing only partial complementation of the MMS sensitive phenotype.

Another *EC*-Lon substrate that we evaluated was RcsA, a positive regulator of capsular-polysaccharide biosynthesis genes (Markovitz, 1964). Both the parental HDB97 strain and its *lon*⁻ HDB98 derivative contain a chromosomal *cpsB::lacZ* fusion insert (Bernstein & Hyndman, 2001), which affords a convenient assay for monitoring RcsA function via β -galactosidase expression and activity. As shown in Figure 7, we observed high levels of β -galactosidase activity in the Lon deficient HDB98 strain, suggesting RcsA protein accumulated in these cells due to the absence of Lon function. Introduction of a plasmid carrying *EC*-Lon, when induced with 0.01% arabinose, resulted in full complementation of this phenotype and reduced the β -galactosidase activity to background levels (Fig. 7). Expression of plasmid-borne *MP*-Lon reduced β -galactosidase activity to approximately 25% of *lon*⁻ cells. Higher-level expression, with 0.1% arabinose, of *MP*-Lon did not result in improved complementation of the RcsA phenotype (Fig. 6). These results indicated that *MP*-Lon degraded RcsA, like Sula, with much lower efficiency. Taken together, these data supported the notion that although *mp-Lon* has become a robust protease, its substrate specificity has evolved away from those substrates that are not present in *Mycoplasma*, thus it is unable to efficiently bind and degrade proteins that are normally degraded by its *E. coli* counterpart.

DISCUSSION

Timely and efficient removal of unwanted, damaged or unfolded proteins via proteolysis is vital for the cell. This is highlighted by the fact that interference with proteolysis severely impairs the cell's ability to survive under adverse environmental conditions. Signals that target a protein for proteolysis are either intrinsic to the primary structure of the protein or are extrinsically appended to convert it into a substrate for proteolysis (Baker & Sauer, 2006, Gottesman, 2003). The SmpB-tmRNA quality control system, which rescues ribosomes stalled on defective mRNAs, is the only known biological process that co-translationally appends a peptide tag to proteins associated with stalled ribosomes (Dulebohn et al., 2007, Karzai et al., 2000, Keiler, 2008, Withey & Friedman, 2003). The small peptide tag, encoded by the mRNA-like domain of tmRNA, contains signals that directs tagged proteins to proteolysis by various cellular proteases (Choy et al., 2007, Gottesman et al., 1998, Herman et al., 1998, Keiler et al., 1996). While the specific peptide sequence encoded by tmRNA varies among bacterial species, studies carried out with *E. coli* tmRNA have confirmed the ability of the tmRNA peptide tag to promote targeted proteolysis by the Tsp, ClpXP, ClpAP, FtsH, and Lon proteases (Gottesman et al., 1998, Herman et al., 1998, Keiler et al., 1996, Choy et al., 2007). The tmRNA mediated translation quality control system is thus intimately linked, by providing substrates for directed proteolysis, to protease-mediated protein quality control pathways.

The genes encoding SmpB and tmRNA are represented in all species of bacteria with available genomic sequence data. The preservation of SmpB and tmRNA thus reflects the extent of conservation and the evolutionary significance of this unique quality control system. Genome wide mutagenesis results suggest that genes encoding SmpB and tmRNA are essential in *Mycoplasma genitalium* and *Mycoplasma pneumoniae* (Hutchison et al., 1999). Interestingly, of all the soluble cytoplasmic AAA+ proteases that recognize tmRNA tagged proteins, only Lon protease is present in *Mycoplasma*, and is presumably essential for its survival (Glass et al., 2006, Hutchison et al., 1999).

A case for co-evolution of the tmRNA tag and Lon protease in *Mycoplasma*

In this study, we have demonstrated that the *Mycoplasma pneumoniae* Lon protease recognizes and degrades *MP*-tmRNA tagged proteins efficiently and selectively. Most significantly, our data demonstrate that recognition of the tmRNA tag by *MP*-Lon is not limited to the extreme C-terminal residues of the tag. Rather, multiple sequence elements

within the extended *MP*-tmRNA tag provide critical signaling cues for recognition and selective proteolysis by *MP*-Lon. Our findings thus support a model wherein *MP*-Lon, as the sole soluble cytoplasmic AAA+ protease, has evolved to become a more robust and efficient enzyme for selective recognition and proteolytic turnover of tmRNA tagged proteins. We propose that the extended *Mycoplasma* tmRNA tag has correspondingly evolved to present multiple recognition signals for Lon, and perhaps other proteases.

A recent study showed that proteins carrying tmRNA tag from *Mesoplasma florum* (*mf*) are efficiently recognized and degraded by the *mf*-Lon protease (Gur & Sauer, 2008a). Through substitution analysis of the C-terminal end of the *mf*-tmRNA tag, the authors concluded that the two aromatic residues (Y and F of the -YAFA-COO⁻ motif) were responsible for recognition of the tag peptide by *mf*-Lon. Their analysis was limited to the C-terminal part of the *mf*-tag, particularly to the two aromatic residues, yet their conclusion agrees, in part, with our findings. Our analysis of the equivalent region of *MP*-tmRNA tag demonstrates that all four residues of the -YAFA-COO⁻ motif, including the two alanines, are essential for recognition and degradation by *MP*-Lon. Recognition of the C-terminal proximal alanine residues by *MP*-Lon is thus reminiscent of the role played by the ultimate and penultimate alanines of the *E. coli* tag in recognition and degradation by the ClpXP system (Flynn et al., 2001).

The issue of the length of *Mycoplasma* tmRNA tag and its utility, i.e. whether additional recognition signals are present within the extended *mf*-tag, were not addressed in the Gur and Sauer report (Gur & Sauer, 2008a). Our analysis of the entire *MP*-tmRNA tag clearly demonstrates that additional distinct parts of the *MP*-tag harbor signals that are critical for degradation by *MP*-Lon, as changes in these determinants render tagged substrates highly resistant to proteolysis despite the presence of the intact C-terminal -YAFA-COO⁻ motif (Fig. 2 and 5). These results suggest that interactions between Lon and tmRNA tag are multipartite in nature, and that the tag-recognition pocket of *MP*-Lon is likely larger, encompassing both the C-terminal and internal signal elements of the tag. Therefore, these findings provide a much more plausible explanation for the extended nature of the *Mycoplasma* tmRNA tag and hint at co-evolution of the tag sequence and Lon, the sole cytoplasmic AAA+ protease in *Mycoplasma*. This conclusion is further supported by our analysis of the degradation by *EC*-Lon of a protein carrying the *MP*-tmRNA tag. As noted earlier, the *MP*-tmRNA tag has large hydrophobic residues at its C-terminus (Fig. 1A), which should make it an ideal substrate for *EC*-Lon. Indeed, *EC*-Lon does recognize and degrade an *MP*-tmRNA tagged substrate (Fig. 1D). However, if the C-terminal -YAFA-COO⁻ determinant constituted the sole recognition signal for *EC*-Lon then it should not have been affected by aspartic acid substitutions in regions 4 and 5 of the *MP*-tmRNA tag. The fact that *EC*-Lon is affected by distal aspartic acid substitutions, despite the presence of the C-terminal recognition motif, suggests that the tag recognition pocket of *EC*-Lon is correspondingly larger and encompasses more than the last four amino acids of the *MP*-tmRNA tag. We propose that *EC*-Lon also recognizes additional parts of the *MP*-tmRNA tag. We submit that the reason for the substantial affect of aspartic acid substitutions in the internal part of the *MP*-tmRNA tag is that interactions of *EC*-Lon with tmRNA tag are comparatively weaker and therefore more sensitive to disrupting alterations. Indeed, this conclusion is supported by our analysis of the steady-state parameters of the degradation of tmRNA tagged proteins by *EC*-Lon and *MP*-Lon (Fig. 4 and Table I).

The new picture that emerges from our analysis suggests that Lon-tmRNA tag interactions have co-evolved in *Mycoplasma*, such that the *MP*-tmRNA tag has become longer to present more contact sites for an existing recognition pocket in the substrate binding domain of Lon, and *MP*-Lon has co-evolved to recognize these signals better, primarily through optimization of pre-existing low affinity sites (Fig. 8A). Although we favor this simpler

model, we cannot rule out the alternative possibility that Lon only recognizes the C-terminal –YAFA-COO[−] motif, but binding of the internal –MLIA– signaling motif of the tag to a distal allosteric-site on Lon modulates its activity (Fig. 8B). We also wish to make clear that the aforementioned analysis do not exclude the possible acquisition of new contact sites or an expansion of the binding pocket in *MP*-Lon.

Although we have identified distinct regions of the *MP*-tmRNA tag that are critical for Lon recognition, there are approximately 10 amino acids at the N-terminus of the *MP*-tag that are not directly involved in recognition by Lon. It is plausible that these sequences serve as the binding sites for auxiliary cofactors in a manner analogous to the role of SspB in degradation by ClpXP, or that of ClpS in degradation by ClpAP (Bolon *et al.*, 2004, Chien *et al.*, 2007a, Chien *et al.*, 2007b, Farrell *et al.*, 2005, Levchenko *et al.*, 2005, Levchenko *et al.*, 2000, Lies & Maurizi, 2008, McGinness *et al.*, 2007, Park *et al.*, 2007, Thibault *et al.*, 2006, Erbse *et al.*, 2006, Hou *et al.*, 2008, Mogk *et al.*, 2007, Wang *et al.*, 2008a, Wang *et al.*, 2008b). To date, however, adaptor proteins have not been reported for Lon protease analogs. Nonetheless, it is conceivable that Lon, like other AAA⁺ proteases, possesses a cofactor(s) that regulates its substrate range and specificity. Alternatively, the N-terminal part of the *MP*-tmRNA tag might carry signals for recognition by the membrane-associated FtsH protease. Future studies are required to explore these possibilities.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli strains BL21 (DE3)/pLysS and CH1019 were used to express *MP*-Lon and *EC*-Lon proteases, respectively. λ -*cI*-N reporter derivatives were expressed in either the W3110 *clp[−]XclpP[−]lon[−]* protease deficient strain to minimize proteolysis or the *degP[−]prc[−]* strain (lacking the DegP and Tsp proteases) (Chen *et al.*, 2004) to produce an intact version of the λ -*cI*-N-ssrA_{MP} protein (Fig. S2). *In vivo* complementation assays were carried out in a *lon[−]* HDB98 strain or its otherwise isogenetic HDB97 parental strain (Bernstein & Hyndman, 2001). The gene encoding *EC*-Lon protease was cloned into pET21b vector. The gene encoding *MP*-Lon protease was cloned into pET28b vector. An internal TGA codon in the *MP*-*lon* gene, which encodes for tryptophan in *Mycoplasma*, was changed to TGG to prevent premature termination of translation during expression in *E. coli*. Both *EC*-Lon and *MP*-Lon genes were also cloned into pBAD18cm vector to generate the complementation constructs for *in vivo* studies. The pPW500 plasmid, harboring the λ -*cI*-N-trpAt reporter construct, was modified to express the untagged λ -*cI*-N protein, the hard coded *E. coli* tmRNA tagged (λ -*cI*-N-ssrA_{EC}) protein, or the hard coded *M. pneumonia* tmRNA tagged λ -*cI*-N-ssrA_{MP} protein. Reporter constructs expressing aspartic acids substitution tag variants of λ -*cI*-N-ssrA_{MP} were generated by standard site directed mutagenesis, using λ -*cI*-N-ssrA_{MP} DNA as a template.

Protein Expression and Purification

To purify *MP*-Lon, *E. coli* strain BL21 (DE3)/pLysS harboring pET28b-*MP*-Lon-His6 was grown in 3 liters of LB supplemented with 100 μ g/mL ampicillin and 30 μ g/mL chloramphenicol. *MP*-Lon expression was induced at OD₆₀₀ ~ 0.7 by addition of 1 mM IPTG and continued for 3 hours at 30° C. Cells were harvested by centrifugation at 4000 rpm, and the resulting cell pellets were stored at –80° C. The frozen cell pellets were resuspended in 100 mL of lysis buffer (50 mM KHPO₄ pH6.9, 1 mM EDTA, 1 mM DTT, and 10% glycerol) and lysed by sonication at 4° C. Cellular debris was removed by centrifugation at 30,000 $\times$ g for 2 hours. P11 cellulose phosphate resin (Whatman, GE Healthcare) was activated according to the manufacturer's instructions and pre-equilibrated in lysis buffer. The cleared cell lysate was loaded by gravity flow onto 20 mL of pre-

equilibrated P11 resin and washed with 200 mL of lysis buffer. Bound proteins were eluted with 200 mL of elution buffer (400 mM KHPO₄ pH6.9, 1 mM EDTA, 1 mM DTT, and 10% glycerol). The eluted proteins were concentrated down to 2 mL and loaded onto an AKTA-FPLC Sephacryl S300 column (GE Healthcare), pre-equilibrated in Lon storage buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 10 mM MgCl₂, 1 mM DTT and 20% glycerol). Fractions containing purified *MP*-Lon protease were pooled, concentrated, flash frozen in liquid nitrogen and stored at -80°C . Purification of *EC*-Lon, from *E. coli* strain CH1019 (DE3) harboring pET21b-*ecLon*, was carried out essentially as described above for *MP*-Lon.

The λ -cI-N protein and its various tmRNA tagged variants were expressed in *E. coli* strain W3110 *clpX⁻clpP⁻lon⁻*. Expression of each reporter protein was induced at OD₆₀₀ ~ 0.7 by addition of 1 mM IPTG and continued for 3 hours at 37°C. Cells were harvested by centrifugation at 4000 rpm, and the resulting cell pellets were stored at -80°C . Frozen cell pellets were resuspended in a Ni²⁺-NTA lysis buffer (50 mM KHPO₄ pH7.0, 100 mM KCl, 1 mM EDTA, 2 mM β -ME, and 10 mM Imidazole). Cells were lysed by sonication at 4°C and cell debris removed by centrifugation at 30,000 $\times$ g for 2 hours. The cleared cell lysates were mixed with 2 mL of Ni²⁺-NTA slurry equilibrated in the Ni²⁺-NTA lysis buffer and rocked for 2 hours at 4 °C. The resin was washed with 200 mL of the lysis buffer and the bound protein was eluted with 30 mL of elution buffer (50 mM KHPO₄ pH7.0, 100 mM KCl, 1 mM EDTA, 20 mM β -ME, and 250 mM Imidazole). The eluted protein was concentrated to 5 mL, buffer exchanged into Q-Sepharose buffer A (50 mM Tris-HCl pH7.5, 50 mM KCl, 1 mM EDTA and 2 mM β -ME) and loaded onto a pre-equilibrated AKTA FPLC Q-Sepharose 10/10 column. The column was washed with 20 CV of buffer A and the bound protein was eluted by the application of a linear KCl gradient from 0% buffer B to 100% Buffer B (50 mM Tris-HCl pH7.5, 1M KCl, 1 mM EDTA and 2 mM β -ME). Fractions containing the reporter protein were pooled, concentrated and dialyzed into storage buffer (50 mM Tris-HCl pH7.5, 50 mM KCl, 1 mM DTT and 10% glycerol). Protein aliquots were flash frozen in liquid nitrogen and stored at -80°C .

***In vitro* proteolysis assay**

Each *in vitro* proteolysis assay was carried out in an 80 μ L reaction mixture containing Lon activity buffer (50 mM Tris-HCl pH8.0, 10 mM MgCl₂, 1 mM DTT, and 10% glycerol), ATP regeneration system (50 mM creatine phosphate, 80 μ g/mL creatine kinase, and 4 mM ATP), 10 μ M substrate, and Lon protease (either 100 nM *MP*-Lon₆ or 400 nM *EC*-Lon₆). The reaction mixture was assembled and incubated at 30° C. Aliquots were taken at designated time points and the reaction stopped by adding equal volume of 2X SDS-PAGE sample buffer. The reaction products were resolved by electrophoresis in a 15% trisicine gel and quantified by Image-J software.

³⁵S-labeled substrate preparation and proteolysis

³⁵S-labeled λ -cI-N-ssrA_{MP} and its tag peptide variants were expressed and purified as follows. Cultures of 50 mL were grown in LB broth at 37 °C to an OD₆₀₀ of between 0.8 and 1.0. Cells were harvested by centrifugation at 4000 rpm and the cell pellets were resuspended in 50 mL of M9 media supplemented with DOC mix lacking methionine and cysteine (US Biological). Cultures were induced with 1.0 mM IPTG and grown for an additional 20 min. A ³⁵S-methionine/³⁵S-cysteine mixture (2.0mCi, Perkin-Elmer) was added to each culture and the cells were grown for an additional 2.0 hours at 37 °C. Cells were harvested and lysed in B-PER reagent (Pierce) containing 500 mM KCl, 2 mM β -ME, 0.1mg/mL lysozyme, 5U/mL DNaseI, and a protease inhibitor cocktail (Pierce). ³⁵S-labelled proteins were purified by chromatography on a Ni-NTA column, essentially as described above. The purified ³⁵S-labeled λ -cI-N-ssrA_{MP} reporter proteins were buffer exchanged into storage buffer (50 mM Tris-HCl pH7.5, 50 mM KCl, 1 mM DTT and 10% glycerol) using

an Econo-Pac-10DG desalting column (Biorad). Protein aliquots were flash frozen in liquid nitrogen and stored at -80°C .

Lon mediated proteolysis of ^{35}S -labeled λ -cI-N-ssrA_{MP} and its tag peptide variants was performed at 30°C using 100 nM of each Lon₆ in a degradation buffer (50 mM Tris-HCl pH8.0, 10 mM MgCl₂, 1 mM DTT, and 10% glycerol) that was supplemented with an ATP regeneration system (50 mM creatine phosphate, 80 $\mu\text{g}/\text{mL}$ creatine kinase, and 4 mM ATP). Proteolysis reaction samples, 10 μL , were taken at indicated time points and quenched immediately by the addition of 20 μL of 20% trichloroacetic acid (TCA) and 10 μL of 10% BSA. The TCA-insoluble material was removed by centrifugation at 14,000 rpm in a microcentrifuge. The amount of ^{35}S -labeled proteolytic peptides present in the supernatant was measured in a liquid scintillation counter. Initial rates of λ -cI-N-ssrA_{MP} proteolysis by EC-Lon or MP-Lon were measured at a range of substrate concentrations. Each assay was repeated at least three times and the data were fitted to the Michaelis–Menten equation to determine K_M and V_{max} values.

***In vivo* MMS sensitivity assay**

Strains assayed for MMS sensitivity were grown in LB to an OD₆₀₀ of ~ 1.5 . Serial dilutions of each culture were made in LB and 10 μL of the diluted culture was spotted on appropriate plates either with or without 0.025% MMS. The plates were then incubated at 30°C for 40 hours. The assays, performed with independent cultures, were repeated at least three times. A representative example is shown.

β -galactosidase assay

The production of β -galactosidase in each strain analyzed was quantified using a modified Miller Method (Griffith & Wolf, 2002). The assay was performed at least three times, each time in triplicates. A representative experiment is shown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Baker TA, Sauer RT. ATP-dependent proteases of bacteria: recognition logic and operating principles. *Trends in biochemical sciences*. 2006; 31:647–653. [PubMed: 17074491]
- Bernstein HD, Hyndman JB. Physiological basis for conservation of the signal recognition particle targeting pathway in Escherichia coli. *J Bacteriol*. 2001; 183:2187–2197. [PubMed: 11244056]
- Bolon DN, Grant RA, Baker TA, Sauer RT. Nucleotide-dependent substrate handoff from the SspB adaptor to the AAA+ ClpXP protease. *Mol Cell*. 2004; 16:343–350. [PubMed: 15525508]
- Chen C, Snedecor B, Nishihara JC, Joly JC, McFarland N, Andersen DC, Battersby JE, Champion KM. High-level accumulation of a recombinant antibody fragment in the periplasm of Escherichia coli requires a triple-mutant (degP prc spr) host strain. *Biotechnol Bioeng*. 2004; 85:463–474. [PubMed: 14760686]
- Chien P, Grant RA, Sauer RT, Baker TA. Structure and substrate specificity of an SspB ortholog: design implications for AAA+ adaptors. *Structure*. 2007a; 15:1296–1305. [PubMed: 17937918]

- Chien P, Perchuk BS, Laub MT, Sauer RT, Baker TA. Direct and adaptor-mediated substrate recognition by an essential AAA+ protease. *Proc Natl Acad Sci U S A*. 2007b; 104:6590–6595. [PubMed: 17420450]
- Choy JS, Aung LL, Karzai AW. Lon protease degrades transfer-messenger RNA-tagged proteins. *J Bacteriol*. 2007; 189:6564–6571. [PubMed: 17616591]
- Dulebohn D, Choy J, Sundermeier T, Okan N, Karzai AW. Trans-translation: the tmRNA-mediated surveillance mechanism for ribosome rescue, directed protein degradation, and nonstop mRNA decay. *Biochemistry*. 2007; 46:4681–4693. [PubMed: 17397189]
- Erbse A, Schmidt R, Bornemann T, Schneider-Mergener J, Mogk A, Zahn R, Dougan DA, Bukau B. ClpS is an essential component of the N-end rule pathway in *Escherichia coli*. *Nature*. 2006; 439:753–756. [PubMed: 16467841]
- Farrell CM, Baker TA, Sauer RT. Altered specificity of a AAA+ protease. *Mol Cell*. 2007; 25:161–166. [PubMed: 17218279]
- Farrell CM, Grossman AD, Sauer RT. Cytoplasmic degradation of ssrA-tagged proteins. *Molecular microbiology*. 2005; 57:1750–1761. [PubMed: 16135238]
- Flynn JM, Levchenko I, Sauer RT, Baker TA. Modulating substrate choice: the SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+ protease ClpXP for degradation. *Genes Dev*. 2004; 18:2292–2301. [PubMed: 15371343]
- Flynn JM, Levchenko I, Seidel M, Wickner SH, Sauer RT, Baker TA. Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis. *Proc Natl Acad Sci U S A*. 2001; 98:10584–10589. [PubMed: 11535833]
- Glass JI, Assad-Garcia N, Alperovich N, Yooseph S, Lewis MR, Maruf M, Hutchison CA 3rd, Smith HO, Venter JC. Essential genes of a minimal bacterium. *Proc Natl Acad Sci U S A*. 2006; 103:425–430. [PubMed: 16407165]
- Gottesman S. Proteases and their targets in *Escherichia coli*. *Annu Rev Genet*. 1996; 30:465–506. [PubMed: 8982462]
- Gottesman S. Proteolysis in bacterial regulatory circuits. *Annu Rev Cell Dev Biol*. 2003; 19:565–587. [PubMed: 14570582]
- Gottesman S, Roche E, Zhou Y, Sauer RT. The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev*. 1998; 12:1338–1347. [PubMed: 9573050]
- Griffith KL, Wolf RE Jr. Measuring beta-galactosidase activity in bacteria: cell growth, permeabilization, and enzyme assays in 96-well arrays. *Biochem Biophys Res Commun*. 2002; 290:397–402. [PubMed: 11779182]
- Gur E, Sauer RT. Evolution of the ssrA degradation tag in *Mycoplasma*: specificity switch to a different protease. *Proc Natl Acad Sci U S A*. 2008a; 105:16113–16118. [PubMed: 18852454]
- Gur E, Sauer RT. Recognition of misfolded proteins by Lon, a AAA (+)protease. *Genes Dev*. 2008b; 22:2267–2277. [PubMed: 18708584]
- Herman C, Prakash S, Lu CZ, Matouschek A, Gross CA. Lack of a robust unfoldase activity confers a unique level of substrate specificity to the universal AAA protease FtsH. *Mol Cell*. 2003; 11:659–669. [PubMed: 12667449]
- Herman C, Thevenet D, Boulloc P, Walker GC, D'Ari R. Degradation of carboxy-terminal-tagged cytoplasmic proteins by the *Escherichia coli* protease HflB (FtsH). *Genes Dev*. 1998; 12:1348–1355. [PubMed: 9573051]
- Hou JY, Sauer RT, Baker TA. Distinct structural elements of the adaptor ClpS are required for regulating degradation by ClpAP. *Nature structural & molecular biology*. 2008; 15:288–294.
- Howard-Flanders P, Simson E, Theriot L. A Locus That Controls Filament Formation and Sensitivity to Radiation in *Escherichia coli* K-12. *Genetics*. 1964; 49:237–246. [PubMed: 14124942]
- Hutchison CA, Peterson SN, Gill SR, Cline RT, White O, Fraser CM, Smith HO, Venter JC. Global transposon mutagenesis and a minimal *Mycoplasma* genome. *Science*. 1999; 286:2165–2169. [PubMed: 10591650]
- Karzai AW, Roche ED, Sauer RT. The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue. *Nature structural biology*. 2000; 7:449–455.

- Karzai AW, Sauer RT. Protein factors associated with the SsrA.SmpB tagging and ribosome rescue complex. *Proc Natl Acad Sci U S A*. 2001; 98:3040–3044. [PubMed: 11248028]
- Keiler KC. Biology of trans-Translation. *Annu Rev Microbiol*. 2008; 62:133–151. [PubMed: 18557701]
- Keiler KC, Silber KR, Downard KM, Papayannopoulos IA, Biemann K, Sauer RT. C-terminal specific protein degradation: activity and substrate specificity of the Tsp protease. *Protein Sci*. 1995; 4:1507–1515. [PubMed: 8520476]
- Keiler KC, Waller PR, Sauer RT. Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science*. 1996; 271:990–993. [PubMed: 8584937]
- Kihara A, Akiyama Y, Ito K. FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit. *Proc Natl Acad Sci U S A*. 1995; 92:4532–4536. [PubMed: 7753838]
- Kihara A, Akiyama Y, Ito K. Dislocation of membrane proteins in FtsH-mediated proteolysis. *EMBO J*. 1999; 18:2970–2981. [PubMed: 10357810]
- Levchenko I, Grant RA, Flynn JM, Sauer RT, Baker TA. Versatile modes of peptide recognition by the AAA+ adaptor protein SspB. *Nature structural & molecular biology*. 2005; 12:520–525.
- Levchenko I, Seidel M, Sauer RT, Baker TA. A specificity-enhancing factor for the ClpXP degradation machine. *Science*. 2000; 289:2354–2356. [PubMed: 11009422]
- Lies M, Maurizi MR. Turnover of endogenous SsrA-tagged proteins mediated by ATP-dependent proteases in *Escherichia coli*. *J Biol Chem*. 2008; 283:22918–22929. [PubMed: 18550539]
- Markovitz A. Regulatory Mechanisms for Synthesis of Capsular Polysaccharide in Mucoid Mutants of *Escherichia coli* K12. *Proc Natl Acad Sci U S A*. 1964; 51:239–246. [PubMed: 14124321]
- Martin A, Baker TA, Sauer RT. Diverse pore loops of the AAA+ ClpX machine mediate unassisted and adaptor-dependent recognition of ssrA-tagged substrates. *Mol Cell*. 2008; 29:441–450. [PubMed: 18313382]
- McGinness KE, Bolon DN, Kaganovich M, Baker TA, Sauer RT. Altered tethering of the SspB adaptor to the ClpXP protease causes changes in substrate delivery. *J Biol Chem*. 2007; 282:11465–11473. [PubMed: 17317664]
- Mogk A, Schmidt R, Bukau B. The N-end rule pathway for regulated proteolysis: prokaryotic and eukaryotic strategies. *Trends Cell Biol*. 2007; 17:165–172. [PubMed: 17306546]
- Okan NA, Bliska JB, Karzai AW. A Role for the SmpB-SsrA system in *Yersinia pseudotuberculosis* pathogenesis. *PLoS Pathog*. 2006; 2:e6. [PubMed: 16450010]
- Park EY, Lee BG, Hong SB, Kim HW, Jeon H, Song HK. Structural basis of SspB-tail recognition by the zinc binding domain of ClpX. *J Mol Biol*. 2007; 367:514–526. [PubMed: 17258768]
- Parsell DA, Silber KR, Sauer RT. Carboxy-terminal determinants of intracellular protein degradation. *Genes Dev*. 1990; 4:277–286. [PubMed: 2186965]
- Roche ED, Sauer RT. SsrA-mediated peptide tagging caused by rare codons and tRNA scarcity. *EMBO J*. 1999; 18:4579–4589. [PubMed: 10449423]
- Roche ED, Sauer RT. Identification of endogenous SsrA-tagged proteins reveals tagging at positions corresponding to stop codons. *J Biol Chem*. 2001; 276:28509–28515. [PubMed: 11373298]
- Silber KR, Keiler KC, Sauer RT. Tsp: a tail-specific protease that selectively degrades proteins with nonpolar C termini. *Proc Natl Acad Sci U S A*. 1992; 89:295–299. [PubMed: 1729701]
- Silber KR, Sauer RT. Deletion of the *prc* (*tsp*) gene provides evidence for additional tail-specific proteolytic activity in *Escherichia coli* K-12. *Mol Gen Genet*. 1994; 242:237–240. [PubMed: 8159175]
- Sundermeier TR, Dulebohn DP, Cho HJ, Karzai AW. A previously uncharacterized role for small protein B (SmpB) in transfer messenger RNA-mediated trans-translation. *Proc Natl Acad Sci U S A*. 2005; 102:2316–2321. [PubMed: 15699355]
- Thibault G, Yudin J, Wong P, Tsitrin V, Sprangers R, Zhao R, Houry WA. Specificity in substrate and cofactor recognition by the N-terminal domain of the chaperone ClpX. *Proc Natl Acad Sci U S A*. 2006; 103:17724–17729. [PubMed: 17090685]

- Tripathi LP, Sowdhamini R. Genome-wide survey of prokaryotic serine proteases: analysis of distribution and domain architectures of five serine protease families in prokaryotes. *BMC Genomics*. 2008; 9:549. [PubMed: 19019219]
- Wang KH, Oakes ES, Sauer RT, Baker TA. Tuning the strength of a bacterial N-end rule degradation signal. *J Biol Chem*. 2008a; 283:24600–24607. [PubMed: 18550545]
- Wang KH, Roman-Hernandez G, Grant RA, Sauer RT, Baker TA. The molecular basis of N-end rule recognition. *Mol Cell*. 2008b; 32:406–414. [PubMed: 18995838]
- Withy JH, Friedman DI. A salvage pathway for protein structures: tmRNA and trans-translation. *Annu Rev Microbiol*. 2003; 57:101–123. [PubMed: 12730326]

Figure 1.

MP-Lon selectively degrades a protein carrying the *MP*-tmRNA tag. (A) The amino acid sequences of the *MP*-tmRNA and *EC*-tmRNA tag are shown, with the C-terminal hydrophobic residues displayed in larger-size font for comparison. (B) Schematic representation of the λ-cl-N-ssrA_{MP} reporter, the λ-cl-N-ssrA_{MP-11} internal control and the λ-cl-N untagged reporter. (C) *In vitro* proteolysis assays were carried out at 30°C in minimum Lon activity buffer, which contained 50 mM Tris-HCl pH8.0, 10 mM MgCl₂, 1 mM DTT, and 10% glycerol. The reactions also contained 100 nM *MP*-Lon₆, 10 μM substrate, 4 mM ATP (when indicated) and an ATP regeneration system. Aliquots were taken at designated time points, quenched with an equal volume of 2x SDS-sample buffer, resolved by electrophoresis on 15% Tris-tricine gel and stained with Coomassie Brilliant Blue^{R250}. (D) Reactions were carried out as described in (C) with the designated Lon₆ concentration. *MP*-Lon efficiently degrades λ-cl-N-ssrA_{EC} at lower concentration (100 nM) while *EC*-Lon requires higher concentration (400 nM) to effectively degrade λ-cl-N-ssrA_{MP}.

Figure 2.

Aspartic acid substitutions in various regions of the *MP*-tmRNA tag sequence result in alterations in its degradation pattern. (A) The *MP*-tmRNA tag sequence was divided into 9 regions. Amino acid residues within each region were substituted with aspartic acids. All λ -cI-N-ssrA_{MP} reporter substrates, carrying *MP*-tmRNA tag aspartic acid variants in each of the 9 regions, were individually purified, resolved by electrophoresis on a 15% Tris-tricine gel and stained with Coomassie Brilliant Blue^{R250}. Aspartic acid substitutions in region 5 through 9 affect proteolytic cleavage of the λ -cI-N-ssrA_{MP} reporter and production of the shorter λ -cI-N-ssrA_{MP-11} fragment. (B) The effect of aspartic acid substitutions, in each of the 9 regions of the *MP*-tmRNA tag, on degradation of λ -cI-N-ssrA_{MP} reporter by *MP*-Lon₆ (100 nM) were assessed in an *in vitro* proteolysis assay as described in Fig. 1. The amount of each full-length λ -cI-N-ssrA_{MP} variant left at the 15 min time point, as an indicator of the efficiency of *MP*-Lon in degradation, was quantified using ImageJ and represented in bar graph format. Each experiment was repeated at least three times and the graph represents mean $\pm$ standard deviation of three independent repeats.

Figure 3.

The contribution of amino acid residues in multiple regions of the *MP*-tmRNA tag to degradation by Lon. (A) Reporter substrates carrying single aspartic acid substitution in key signaling motifs in *MP*-tmRNA tag were subjected to proteolysis by *MP*-Lon₆ (100 nM) and analyzed as described in Fig. 2. Each experiment was repeated at least three times and the graph represents mean $\pm$ standard deviation of three independent repeats. (B) The role of the C-terminal four residues of *MP*-tmRNA tag in degradation by *MP*-Lon. λ -cl-N-ssrA_{MP} reporter variants, with the C-terminal residues of the *MP*-tmRNA tag altered to either –DADA or –YDFD, were analyzed in the *in vitro* proteolysis assay by *MP*-Lon₆ (100 nM), as described in Fig. 2. (C) The effect of single aspartic acid substitutions in key signaling motifs of *MP*-tmRNA tag on degradation of the λ -cl-N-ssrA_{MP} reporter by *EC*-Lon. Reporter substrates carrying single aspartic acid substitution in key signaling motifs in *MP*-tmRNA tag were subjected to proteolysis by *EC*-Lon₆ (400 nM) and analyzed as described

in Fig. 2. Each experiment was repeated at least three times and the graph represents mean $\pm$ standard deviation of three independent repeats.

Figure 4.

Steady-state kinetic analysis of the degradation of a [^{35}S]-labeled tmRNA tagged protein by *MP-Lon* and *EC-Lon*. Degradation velocities were measured at a range of substrate concentrations and the data were fitted to the Michaelis-Menton equation. Each experiment was repeated at least three times. Shown are representative Michaelis-Menton plots for the degradation $\lambda\text{-cl-N-ssrA}_{\text{MP}}$ by (A) 100 nM *MP-Lon*₆ ($K_M = 0.50 \pm 0.04 \mu\text{M}$ and $V_{\text{max}} = 5.1 \pm 0.1 \text{ min}^{-1} \text{Lon}_6^{-1}$), (B) 100 nM *EC-Lon*₆ ($K_M = 17.4 \pm 2.0 \mu\text{M}$ and $V_{\text{max}} = 6.4 \pm 0.2 \text{ min}^{-1} \text{Lon}_6^{-1}$) and (C) the degradation $\lambda\text{-cl-N-ssrA}_{\text{EC}}$ by 100 nM *MP-Lon*₆ ($K_M = 4.9 \pm 0.4 \mu\text{M}$ and $V_{\text{max}} = 4.8 \pm 0.1 \text{ min}^{-1} \text{Lon}_6^{-1}$).

Figure 5.

Steady-state kinetic analysis of the degradation of a [^{35}S]-labeled protein carrying *MP*-tmRNA tag sequence variants by *MP*-Lon. Degradation velocities were measured at a range of substrate concentrations and the data were fitted to the Michaelis-Menton equation. Each experiment was repeated at least three times. Shown are representative Michaelis-Menton plots for degradation by 100 nM *MP*-Lon₆ of λ -cl-N-ssrA_{MP} tag sequence variants, carrying aspartic acid substitutions in (A) region 5 ($K_M = 9.0 \pm 0.6 \mu\text{M}$ and $V_{\text{max}} = 3.1 \pm 0.1 \text{ min}^{-1} \text{ Lon}_6^{-1}$), (B) region 6 ($K_M = 1.3 \pm 0.1 \mu\text{M}$ and $V_{\text{max}} = 4.2 \pm 0.1 \text{ min}^{-1} \text{ Lon}_6^{-1}$) and (C) region 8 ($K_M = 29.8 \pm 2.7 \mu\text{M}$ and $V_{\text{max}} = 2.6 \pm 0.1 \text{ min}^{-1} \text{ Lon}_6^{-1}$) of the *MP*-tmRNA tag.

Figure 6.

MMS sensitivity assay for complementation of the *SulA* phenotype of *E. coli* *Ion*⁻ cells by *MP*-Lon. The ability of various *E. coli* strains to survive in the presence of 0.025% MMS was assessed by spotting 10μL of serially diluted cultures onto plates either in the presence or absence of MMS. Each complementation experiment was repeated at least three times, and a representative example of each assay is shown. Panel (A) shows the abilities of the HDB97 parental strain and its isogenic HDB98 *Ion*⁻ mutant to grow in the presence of MMS. (B) The HDB98 *Ion*⁻ strain was complemented with either the empty pBAD vector or pBAD-*EC*-Lon. The expression of plasmid borne *EC*-Lon was induced by 0.01% arabinose. (C) The HDB98 *Ion*⁻ strain was complemented with either the empty pBAD

vector or pBAD-*MP*-Lon. The expression of plasmid borne *MP*-Lon was induced by 0.2% arabinose.

Figure 7.

Beta-galactosidase assay for complementation of the RcsA phenotype of *E. coli* cells by *MP-Lon*. Beta-galactosidase activity was assayed, in each of the indicated HDB97 parental and HDB98 *lon*⁻ strains, using the ONPG substrate, the hydrolysis of which gives rise to an absorbance signal at 420 nm. Strains harboring the complementation plasmids were grown either in the presence or absence of the inducer (0.1% arabinose) to affect the expression of the plasmid borne *EC-Lon* or *MP-Lon* proteases. All beta-galactosidase activities reported are the means of three or more assays and the graph represents mean $\pm$ standard deviation of three independent repeats.

Figure 8.

Proposed model for co-evolution of multipartite interactions between Lon and tmRNA tag. Both *MP*-Lon and *EC*-Lon proteases contact multiple discrete signaling elements within the *MP*-tmRNA tag sequence. We propose that pre-existing multipartite weak interactions (thin dashed lines) between Lon and tmRNA tag have co-evolved in *Mycoplasma* to facilitate strong (bold dashed lines) and selective recognition of tagged proteins. To accommodate these multipartite interactions, either: (A) the binding pocket of Lon protease is large enough to encompass the last 16 residues of the *MP*-tmRNA tag, or (B) the binding pocket of Lon encompasses only the C-terminal 4 residues of the tag while the internal signaling motif interacts with an allosterically regulated site on the periphery of the peptide-binding pocket of Lon. See Discussion for details.

Table 1

Steady-state kinetic parameters for degradation by *MP*-Lon of a protein carrying either the wild type *MP*-tmRNA tag, representative aspartic acid substitution variants of the *MP*-tmRNA tag, or the wild type *EC*-tmRNA tag.

Substrate	K_M (μM)	$V_{\max}$ (Substrate degraded $\text{min}^{-1} \text{Lon}_6^{-1}$)
λ -cI-N-ssrA _{MP}	0.51 ± 0.04	5.1 ± 0.1
λ -cI-N-ssrA _{MP-region 5}	9.0 ± 0.6	3.1 ± 0.1
λ -cI-N-ssrA _{MP-region 6}	1.3 ± 0.1	4.2 ± 0.1
λ -cI-N-ssrA _{MP-region 8}	29.8 ± 2.7	2.6 ± 0.1
λ -cI-N-ssrA _{EC}	4.9 ± 0.4	4.8 ± 0.1