Evolution of the ssrA degradation tag in *Mycoplasma*: Specificity switch to a different protease

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Stalled ribosomes in bacteria are rescued by the tmRNA system. In this process, the nascent polypeptide is modified by the addition of a short C-terminal sequence called the ssrA tag, which is encoded by tmRNA and allows normal termination and release of ribosomal subunits. In most bacteria, ssrA-tagged proteins are degraded by the AAA+ protease, ClpXP. However, in bacterial species of the genus *Mycoplasma*, genes for ClpXP and many other proteins were lost through reductive evolution. Interestingly, *Mycoplasma* ssrA tag sequences are very different from the tags in other bacteria. We report that ssrA-tagged proteins in *Mesoplasma florum*, a *Mycoplasma* species, are efficiently recognized and degraded by the AAA+ Lon protease. Thus, retaining degradation of ssrA-tagged translation products was apparently important enough during speciation of *Mycoplasma* to drive adaptation of the ssrA tag to a different protease. These results emphasize the importance of coupling proteolysis with tmRNA-mediated tagging and ribosome rescue.

AAA+ protease | Lon | reductive evolution | ClpX

Stalled ribosomes in bacteria are rescued by tmRNA, the product of the *ssrA* gene, in a process sometimes called *trans*-translation (1–4). When ribosomes reach the end of a mRNA lacking a stop codon, protein synthesis ceases, and release factors cannot be recruited to allow disassembly. These ribosomes are eventually rescued by tmRNA, which functions initially as an alanyl-tRNA and next as a surrogate messenger RNA to allow resumption of translation. A stop codon at the end of the tmRNA open reading frame allows normal termination of translation, release of the polypeptide, and recycling of the ribosomal subunits for new rounds of protein synthesis.

As a result of the tagging and ribosome rescue process, polypeptides liberated by the tmRNA system have an ssrA tag at the C terminus. In *Escherichia coli*, this tag consists of 11 residues with a C-terminal LAA-coo$^-$ that targets attached proteins for degradation by ClpXP, an AAA+ protease (1, 5–7). AAA+ proteases are responsible for most intracellular proteolysis and can harness the energy of ATP hydrolysis to degrade native and denatured proteins (for review, see ref. 8). Indeed, ClpXP is able to degrade substrates with high mechanical stability, ensuring elimination of ssrA-tagged proteins, regardless of their folding state. Other AAA+ proteases (ClpAP, FtsH, and Lon) and a non-AAA+ periplasmic protease Tsp also degrade ssrA-tagged proteins under some conditions in *E. coli*, but ClpXP is responsible for most degradation of ssrA-tagged substrates and is likely to serve the same role in most bacteria (6, 9–14).

Bacteria of the genus *Mycoplasma* (class Mollicutes) comprise a large group of nonmotile bacteria, characterized by the lack of a cell wall and by small genomes (15). *Mycoplasma* branched from Gram-positive bacteria by multiple rounds of reductive evolution to reach a genome size of 0.45–1.35 Mbp and are thought to be the smallest self-replicating organisms (16). During genome minimization, *Mycoplasma* discarded many genes and became largely parasitic organisms that rely on their hosts for many nutrients. Nevertheless, *Mycoplasma* retained the tmRNA tagging and ribosome rescue system. Interestingly, however, the ssrA tags encoded by the tmRNA molecules in most *Mycoplasma* are very different from those found in other bacteria (Table 1). Moreover, most *Mycoplasma* genomes encode only two AAA+ proteases, Lon and FtsH, and have lost the genes for ClpXP, ClpAP, HslUV, and Tsp. In the absence of ClpXP, it is possible that the tmRNA system in *Mycoplasma* is uncoupled from proteolysis. Alternatively, the unusual *Mycoplasma* ssrA tag could serve as a degradation signal for the endogenous Lon or FtsH proteases.

Because Lon is cytoplasmic, whereas FtsH is membrane-bound, it seemed most likely to us that Lon plays a major role in degrading ssrA-tagged proteins in *Mycoplasma*. To test this idea, we focused on the ssrA tag sequence and the Lon protease from *Mesoplasma florum*, a nonpathogenic and nonparasitic *Mycoplasma* with a genome size of 793 kb. *M. florum* shares many characteristics with its pathogenic cousins, but it can be cultured without special safety precautions. Here, we show that the ssrA tag sequence of *M. florum* (*mf*-ssrA) is efficiently recognized by the *M. florum* Lon protease (*mf*-Lon). Appending this tag to the C terminus of native or denatured proteins resulted in their rapid proteolysis by *mf*-Lon. Furthermore, *mf*-Lon did not degrade proteins bearing the *E. coli* ssrA tag (*ec*-ssrA), and *E. coli* Lon (*ec*-Lon) did not efficiently degrade proteins bearing the *mf*-ssrA tag. These results indicate that gene loss in *M. florum* forced coevolution of both the ssrA tag and Lon protease to permit efficient and specific degradation, supporting an important role for degradation of ssrA-tagged proteins in the evolutionary fitness of bacteria.

Results

Unusual Properties of ssrA Tags in Mycoplasma. The ssrA tag sequences of diverse bacterial species usually exhibit similarity, especially at their C terminus, which is important for interaction with ClpXP (Table 1; refs. 6 and 7). This observation, together with experiments in Gram-negative *E. coli* and Gram-positive *Bacillus subtilis* (6, 10, 11, 14), suggests that ClpXP is the key protease responsible for degradation of ssrA-tagged substrates in most bacteria. Interestingly, the ssrA tags in most *Mycoplasma* species are much longer than in other groups of bacteria and do not terminate with the normal LAA-coo$^-$ ClpX recognition motif (Table 1). For example, in *E. coli* and *B. subtilis*, the ssrA tag sequences are 11 and 14 residues long and end with NYA-LAA-coo$^-$ and NVALAA-coo$^-$, respectively. By contrast, the *M. florum* ssrA tag consists of 27 aa and terminates with ANYAFA-coo$^-$. The presence of several aromatic residues in this region of the *M. florum* tag is reminiscent of sequences that target certain substrates to *ec*-Lon (13, 17).

Activity of mf-Lon in *E. coli*. We cloned the *lon* gene from genomic *M. florum* DNA, replaced its TGA codons, which encode tryptophans in *M. florum* but are stop codons in *E. coli*, with

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Table 1. ssrA-tag sequences and AAA+ proteases in different bacteria

Type	Bacterium	ClpXP	ClpAP	HslUV	Lon	FtsH	ssrA-tag sequence
Gram-negative							
α-proteobacteria	*C. crescentus*	●	●	●	●	●	AANDNFAEEFAVAA
β-proteobacteria	*N. gonorrhoeae*	●	●	–	●	●	AANDETYALAA
γ-proteobacteria	*E. coli*	●	●	●	●	●	AANDENYALAA
δ-proteobacteria	*M. xanthus*	●	●	●	●	●	AANDNVELALAA
ε-proteobacteria	*H. pylori*	●	●	●	●	●	AVNNTDYAPAYAKAA
Gram-positive							
Actinobacteria	*M. tuberculosis*	●	C	–	–	●	AADSHQRDYALAA
Firmcutes/ Clostridia	*C. botulinum*	●	C	—	●	●	AANDNFALAA
Firmcutes/Bacilli	*B. subtilis*	●	C	●	●	●	AGKTNSFNQNVALAA
Firmcutes/ Lactobacilli	*S. pyogenes*	●	C	–	●	●	AAKNTNSYALAA
Mollicutes							
Mycoplasma	*M. florum*	–	–	–	●	●	AANKNEENTNEVPTFMLNAGQA**NYAFA**
Mycoplasma	*U. parvum*	–	–	–	●	●	AAENKKSSEVELNPAFMASATNA**NYAFA**Y
Mycoplasma	*M. genitalium*	–	–	–	●	●	ADKENNEVLVDPNLIINQQASV**NFAFA**
Mycoplasma	*M. pneumoniae*	–	–	–	●	●	ADKNNDEVLVDPMLIANQQASI**NYAFA**
Mycoplasma	*P. asteris*	–	–	–	●	●	AGNNKQTVTNTQDFAGQTPVYQMNFANSFSS**QLAFA**
Mycoplasma	*E. dolichum*	–	C	–	●	●	AGKTKFANIFGANQ**SVAFA**A
Other							
Cyanobacteria	*P. marinus*	●	C	–	–	●	AANKIVSFSRQTAPVAA
Aquificae	*A. aeolicus*	●	C	●	●	●	AAPEAELALAA
Thermotogae	*T. maritima*	●	C	●	●	●	AANEPVAVAA
Deinococcus	*D. radiodurans*	●	●	–	●	●	AGNQNYALAA

●, bacterium contains enzyme; –, bacterium does not contain enzyme; C, bacterium contains ClpCP, a relative of ClpAP. ClpCP does not appear to degrade ssrA-tagged proteins (10).

TGG codons, and found that *mf*-Lon could be expressed in *E. coli* (data not shown). We then coexpressed plasmid-encoded *mf*-Lon with a variant of β-galactosidase bearing a C-terminal *mf*-ssrA tag in an *E. coli* strain MC4100, which also contained the normal set of AAA+ proteases. Almost no β-galactosidase activity was detected in these cells (Fig. 1*A*). By contrast, high β-galactosidase activity was observed when *ec*-Lon was substituted for *mf*-Lon in the plasmid vector. β-Galactosidase activity was highest when a mock vector was used (Fig. 1*A*). These results

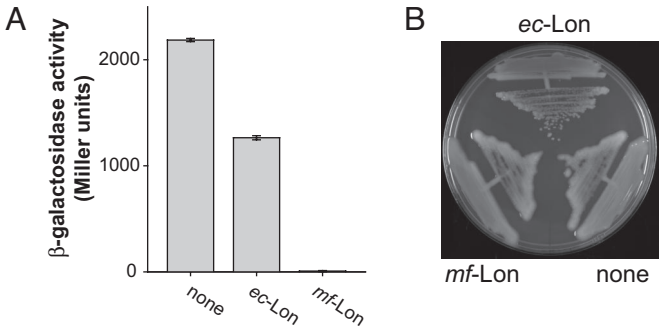

Fig. 1. Phenotypes of *mf*-Lon expression in *E. coli*. (*A*) *E. coli* strain MC4100 was transformed with a plasmid expressing an IPTG-inducible β-galactosidase-*mf*-ssrA fusion protein and a second plasmid expressing either *ec*-Lon, *mf*-Lon, or neither enzyme. Intracellular β-galactosidase-*mf*-ssrA was assayed by enzymatic cleavage of X-Gal as described in *Materials and Methods*. Values are averages (±1 SD; $n = 5$). (*B*) Complementation of the mucoid phenotype of a Δ*lon* strain by *ec*-Lon but not *mf*-Lon. *E. coli* strain JT4000 (MC4100 Δ*lon*) was transformed with plasmids expressing *ec*-Lon, *mf*-Lon, or a mock vector, as indicated. Cells were plated on minimal medium (45) agar plates with glycerol (0.4%) as a carbon source and chloramphenicol (10 μg/ml). In both panels, *ec*-Lon and *mf*-Lon were expressed at basal levels under control of an L-arabinose promoter (P_{BAD}) without added L-arabinose. Arabinose induction of the expression of either Lon enzyme resulted in growth arrest.

suggested that the *mf*-Lon protease recognizes and degrades the *mf*-ssrA-tagged protein much better than do the *ec*-Lon, ClpXP, ClpAP, HslU, or FtsH enzymes.

To test whether the *mf*-Lon enzyme could recognize a substrate normally degraded by *ec*-Lon, we performed a complementation assay by using an *E. coli lon*-null mutant, which exhibits a mucoid phenotype because *ec*-Lon normally degrades RcsA, a transcriptional activator of capsule synthesis genes (18). As expected, expression of *ec*-Lon complemented the mucoid phenotype of the *lon*-null strain (Fig. 1*B*). Importantly, however, expression of *mf*-Lon failed to suppress the mucoid phenotype (Fig. 1*B*). This result provides additional evidence that the specificities of the *ec*-Lon and *mf*-Lon proteases differ.

Degradation *in Vitro*. To provide evidence for direct recognition, we purified *mf*-Lon and assayed degradation *in vitro* of model proteins bearing the *mf*-ssrA tag. As potential substrates, we used *mf*-ssrA-tagged variants of the I27 domain of human titin, which has been used in previous studies of degradation by AAA+ proteases (13, 19, 20). In initial studies, we denatured titin-I27-*mf*-ssrA by carboxylmethylation of its two buried cysteine residues (19) to ensure that degradation would not depend on the unfolding power of the protease. As assayed by SDS/PAGE, this substrate was degraded efficiently by *mf*-Lon but poorly by *ec*-Lon (Fig. 2*A*). As additional specificity controls, we assayed degradation of carboxylmethylated titin-I27 with no degradation tag or with an *E. coli* ssrA tag. Neither protein was degraded efficiently by *mf*-Lon. Thus, degradation is highly specific and requires both the *mf*-Lon protease and the *mf*-ssrA tag of the substrate.

The degradation assays shown in Fig. 2*A* contained ATP. To test whether *mf*-Lon degradation required this nucleotide, we used a fluorescent unfolded variant of titin-I27-*mf*-ssrA, which allowed continuous assay of the degradation rate. As shown in Fig. 2*B*, *mf*-Lon degraded this fluorescent substrate in the

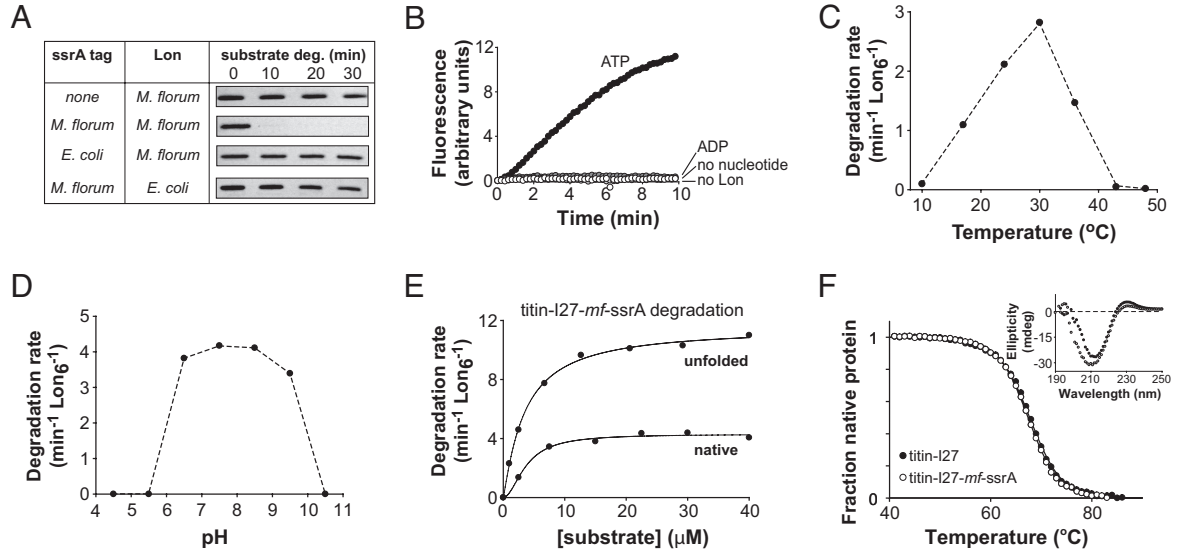

Fig. 2. Degradation activity and properties of *mf*-Lon. (*A*) SDS/PAGE assays of degradation of carboxylmethylated titin-I27 (5 μM) with different tags by *mf*-Lon or *ec*-Lon (150 nM hexamer). (*B*) Degradation of fluorescein-labeled titin-I27-*mf*-ssrA (5 μM) by *mf*-Lon (150 nM hexamer) required ATP and was not observed with ADP, without nucleotide, or in the absence of enzyme. Proteolysis resulted in an increase in fluorescence. (*C*) Rates at different temperatures for degradation of carboxylmethylated ^{35}S-labeled titin-I27-*mf*-ssrA (3 μM) by *mf*-Lon (30 nM hexamer) were determined at pH 7.5 by release of acid-soluble peptides. (*D*) Rates at different pH values for degradation of carboxylmethylated ^{35}S-labeled titin-I27-*mf*-ssrA (3 μM) by *mf*-Lon (30 nM hexamer) were determined at 30°C. The maximal degradation rate is higher than in *C* because the sodium phosphate buffer (50 mM) used in the temperature experiments was slightly inhibitory. (*E*) Michaelis–Menten plots. Steady-state rates of degradation of an unfolded substrate (carboxylmethylated ^{35}S-titin-*mf*-ssrA) or a native substrate (^{35}S-titin-*mf*-ssrA) by *mf*-Lon (10 nM hexamer) were assayed by release of acid-soluble peptides and plotted as a function of substrate concentration. The solid curves are fits ($R^2 \geq 0.99$) to the Hill form of the Michaelis–Menten equation ($V = V_{max}\cdot[S]^n/(K_m^n + [S]^n)$). (*F*) The titin-I27 protein (4 μM; filled circles) and titin-I27-*mf*-ssrA protein (4 μM; open circles) had the same thermal stability, as assayed by changes in CD ellipticity at 228 nm. Ellipticity data were fitted to a two-state model by nonlinear regression (46). (*Inset*) CD spectra at 25°C show that both titin variants (40 μM) are natively folded. The differences in the two spectra result from the contribution of the long unstructured *mf*-ssrA tag to the CD signal.

presence of ATP but not in the presence of ADP or without nucleotide. *E. coli* ClpXP did not degrade the unfolded fluorescent variant of titin-I27-*mf*-ssrA under any conditions (data not shown). To establish the effect of environmental conditions on *mf*-Lon activity, we measured degradation of carboxylmethylated titin-I27-*mf*-ssrA at different temperatures and pH values. Maximal activity was observed at 30°C between pH 6.5 and 8.5 (Fig. 2 *C* and *D*). *M. florum*, which was isolated from a lemon tree flower, grows at temperatures that rarely exceed 30°C, and the observed temperature dependence is therefore consistent with its normal physiology.

***mf*-Lon Is a Fast and Powerful Enzyme.** To measure kinetic parameters for degradation by *mf*-Lon, steady-state rates were determined at different concentrations of protein substrates. For the unfolded substrate, carboxylmethylated titin-I27-*mf*-ssrA, Michaelis–Menten analysis gave a K_m of 3.5 $\pm$ 0.3 μM (Fig. 2*E*). This value is similar to the K_m of ClpXP for substrates bearing the *E. coli* ssrA tag (19, 21–23). V_{max} for the *mf*-Lon degradation reaction was surprisingly fast (11.5 $\pm$ 0.3 min^{-1} Lon$_6^{-1}$), approximately three times faster than either *ec*-Lon or *ec*-ClpXP degrades unfolded titin (13, 19).

To determine whether *mf*-Lon could denature and degrade a stably folded substrate, we assayed proteolysis of native titin-I27-*mf*-ssrA. The titin-I27 protein is remarkably resistant to mechanical unfolding and has been used as a stringent test of the unfolding power of AAA+ proteases (13, 19, 20, 24, 25). The *mf*-Lon enzyme degraded native titin-I27-*mf*-ssrA efficiently, with a K_m of 3.7 $\pm$ 0.4 μM and a V_{max} of 4.3 $\pm$ 0.1 min^{-1} enz^{-1} (Fig. 2*E*). V_{max} for degradation of the native substrate was lower than for the unfolded variant, indicating that unfolding is the rate-limiting step in degradation.

Interestingly, *mf*-Lon degraded native titin-I27-*mf*-ssrA ≈8-fold faster than *ec*-Lon degraded a similar titin-I27 substrate with a different degradation tag (13). This result was not caused by a destabilizing effect of the *mf*-ssrA tag on the thermodynamic stability of native titin. As measured by changes in CD ellipticity, untagged titin-I27 and titin-I27-*mf*-ssrA had almost identical thermal stabilities (Fig. 2*F*). These results suggest that *mf*-Lon is a more powerful unfoldase than *ec*-Lon. Moreover, *mf*-Lon degraded native titin-I27-*mf*-ssrA ≈16-fold faster than *ec*-ClpXP degraded titin-I27-*ec*-ssrA (19).

Peptide Degradation and Recognition Determinants. To test whether *mf*-Lon recognized the *mf*-ssrA tag in the absence of an attached protein substrate, we synthesized a peptide corresponding approximately to the C-terminal half of the tag sequence (the full-length tag could not be synthesized easily). In this peptide, a *para*-aminobenzoic acid (PABA) fluorophore was placed at the N terminus, and a nitrotyrosine (nY), which serves as a quencher, was inserted at position 5. The peptide sequence was PABA-TFML-nY-NAGQANYAFA-coo$^-$ and is hereafter called FQ14-*mf*-ssrA. Proteolytic cleavage between the fluorophore and quencher results in enhanced fluorescence. By this assay, the FQ14-*mf*-ssrA peptide was degraded rapidly by *mf*-Lon in the presence of ATP (Fig. 3*A*). Degradation did not occur in the presence of ADP and was very slow without nucleotide (Fig. 3*A*). We conclude that the C-terminal half of the *mf*-ssrA tag is sufficient for recognition by *mf*-Lon.

In *Mycoplasma* ssrA tags, the region of highest information content is near the C terminus, where most sequences share the consensus NΦAΦA (Φ = F/Y/L) (Fig. 3*B*). The C terminus of the *M. florum* ssrA tag is NYAFA-coo$^-$. When we replaced this C-terminal sequence with N*D*A*D*A-coo$^-$ in an FQ14-*mf*-ssrA variant, the resulting peptide was degraded poorly by *mf*-Lon (Fig. 3*C*). This result suggested that the substituted aromatic

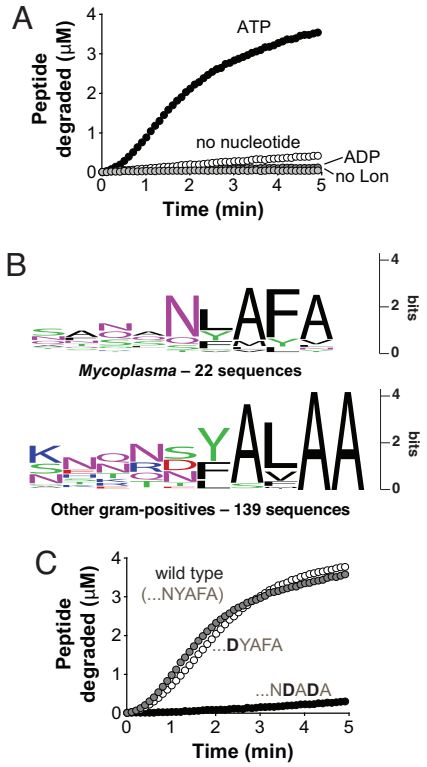

Fig. 3. Degradation of an *mf*-ssrA peptide by *mf*-Lon. (*A*) Degradation of the FQ14-*mf*-ssrA peptide (5 μM) by *mf*-Lon (0.15 μM hexamer) was assayed by increased fluorescence in the presence of 2 mM ATP, 2 mM ADP, without nucleotide, or without enzyme in the presence of 2 mM ATP. (*B*) WebLogo representation (http://weblogo.berkeley.edu/logo.cgi; ref. 47) of ssrA tag sequences from *Mycoplasma* and from other Gram-positive bacteria. Sequences of ssrA tags were obtained from the tmRNA website (http://www.indiana.edu/~tmrna/; ref. 48) or from genomic sequences by using the program ARAGORN (49). (*C*) The Y→D/F→D substitutions of the aromatic residues in the C-terminal pentapeptide of FQ14-*mf*-ssrA (5 μM) slowed degradation by *mf*-Lon (0.15 μM hexamer) dramatically. The N→D substitution in the C-terminal FQ14-*mf*-ssrA pentapeptide had little effect on degradation under the same conditions.

residues play important roles in recognition of the *mf*-ssrA tag by *mf*-Lon. Aromatic residues have also been implicated in peptide recognition by *ec*-Lon (13). Changing the NYAFA sequence of FQ14-*mf*-ssrA to *D*YAFA-coo$^-$ did not slow degradation by *mf*-Lon significantly (Fig. 3*B*).

Discussion

Evolutionary Implications of the Proteolytic Specificity Switch. The tmRNA system is encoded in all fully sequenced bacterial genomes, which currently number >700. The apparently universal conservation of this system points to an important biological role. However, the entire tmRNA system is dispensable for viability in many bacteria (for review, see refs. 2–4). Moreover, although tmRNA is genetically essential in *Neisseria gonorrhoeae*, degradation of the protein products of tmRNA tagging is not required for viability (26). Nevertheless, our results indicate that degradation of ssrA-tagged proteins is important for the fitness of bacteria. First, ssrA tagging was retained in *M. florum*, despite extreme evolutionary events involving massive gene loss. Second, to maintain degradation in the absence of ClpXP, the ssrA tag of *M. florum* had to adapt to allow recognition by a different protease. This switch in proteolytic specificity presumably required changes both in the tmRNA-encoded degradation tag and in the Lon protease.

The switch from ClpXP to Lon degradation of ssrA-tagged proteins represents a case of nonorthologous gene displacement (27). It is plausible that the initial event in the proteolytic specificity switch in *M. florum* was loss of ClpXP, which is principally responsible for degrading ssrA-tagged proteins in most other bacteria. However, the selective force driving coevolution of Lon and the ssrA tag to allow efficient degradation must have been a fitness advantage for mutants with improved ability to degrade the protein products of tmRNA tagging. Because ssrA-tagged proteins are generally incomplete proteins, they are typically unfolded and nonfunctional (28, 29). Thus, degrading these tagged proteins should allow productive recycling of their constituent amino acids to prevent deleterious effects caused by aggregation or other factors. Presumably, the same fitness advantages led to the initial evolution of ClpXP degradation of ssrA-tagged proteins. Indeed, many bacteria contain adaptor proteins that enhance ClpXP degradation of ssrA-tagged proteins and/or that redirect degradation of ssrA-tagged proteins from ClpAP to ClpXP (7, 30–33). Some of these adaptors recognize a portion of the ssrA tag, and it is possible that the long ssrA tags of *Mycoplasma* also serve as docking sites for adaptor proteins.

How complicated was the switch from ClpXP to Lon in proteolysis of ssrA-tagged substrates in the ancestor of *Mycoplasma*? *E. coli* Lon recognizes substrates with *ec*-ssrA tags weakly (12, 13). If the same were true for Lon in the bacteria from which *M. florum* evolved, then this would provide a low level of starting activity after loss of ClpXP. Moreover, the C-terminal sequences of *Mycoplasma* tags are sufficiently similar to those in other Gram-positive bacteria (Table 1; Fig. 3*B*) that only a few mutations would be required to convert a tag from the major Gram-positive group into a tag with the *Mycoplasma* consensus and thus presumably to improve Lon affinity. Our results show that some residues in the C-terminal consensus region of the *M. florum* ssrA tag are important determinants of *mf*-Lon recognition.

Lon Is a Powerful Protein Unfoldase. Lon plays a major role in the degradation of misfolded and damaged proteins in bacteria and in the organelles of eukaryotes (34). As a consequence, its ability to act as a robust protein unfoldase has generally been underappreciated. Using a tagged native substrate (titin-I27) that is very stable to mechanical denaturation, we found that *mf*-Lon appears to be a more powerful enzyme than either *ec*-Lon or *ec*-ClpXP. Under conditions of substrate saturation, each of these AAA+ proteases degraded tagged variants of native titin-I27 more slowly than the tagged unfolded protein. Thus, in each instance, enzymatic unfolding of the native substrate appears to be the rate-limiting step in the degradation reaction. However, *mf*-Lon degraded a tagged variant of titin-I27 ≈8-fold more rapidly than *ec*-Lon and nearly 16-fold faster than *ec*-ClpXP. Moreover, *mf*-Lon also degraded unfolded titin-I27 substantially faster than either *ec*-Lon or *ec*-ClpXP. The *mf*-Lon enzyme may have evolved into a faster and more powerful enzyme to compensate for the absence of ClpAP, ClpXP, and HslUV in *M. florum*. The only remaining AAA+ protease in this organism is FtsH, and its *E. coli* ortholog has been shown to have a weak unfoldase activity (35).

We anticipate that *mf*-Lon and other *Mycoplasma* Lon enzymes will play numerous biological roles both in regulation and in maintaining protein quality control. In *E. coli* and *Salmonella*, for example, *lon* mutants exhibit multiple defects, including difficulties in cell division, excessive capsule synthesis, poor survival after DNA damage, failure to degrade damaged proteins, and reduced virulence (34, 36–38).

An Experimental Windfall. Although Lon was one of the first ATP-dependent proteases to be purified and studied, mechanistic studies of other AAA+ proteases subsequently progressed

far more rapidly. A scarcity of soluble well behaved Lon substrates for biochemical experiments and structural studies has contributed to sluggish progress in understanding how this key enzyme recognizes, unfolds, translocates, and degrades substrates. By contrast, mechanistic studies of other AAA+ proteases, like ClpXP and ClpAP, were greatly accelerated because fusion of the *ec*-ssrA tag to any well behaved protein resulted in a good substrate, allowing steady-state kinetic constants to be determined, the effects of substrate stability and dynamics to be probed, and the design of novel substrates to address specific experimental questions (6, 19, 21–23, 35, 39–42). Our finding of a specific and tight interaction between *mf*-Lon and the *mf*-ssrA tag provides an exceptional opportunity to deepen understanding of Lon structure, function, and mechanism.

Materials and Methods

Plasmids. The LacZ-*mf*-ssrA protein contained the entire *mf*-ssrA tag fused to the C terminus of the *E. coli* LacZ protein and was cloned under control of the *lacZ* promoter into pSH21, which constitutively expresses the LacI repressor. After changing all of the TGA codons to TGG codons, the gene encoding *mf*-Lon was cloned into pBAD33. The *ec*-Lon enzyme was expressed from pBAD33-*lon* (43). Variants of the human titin-I27 domain were expressed from pSH21, under transcriptional control of a T7 promoter.

Proteins and Peptides. The *ec*-Lon protease was purified as described in ref. 13. *E. coli* ClpXP was a gift from Mary Lee (Massachusetts Institute of Technology). For purification of *mf*-Lon, a 2-liter culture of *E. coli* strain ER2566 (New England Biolabs) carrying plasmid pBAD33-*mf-lon* was grown at 37°C in 2XYT medium supplemented with chloramphenicol (20 μg/ml). At an A_{600} of 1.0, L-arabinose was added (0.2%, wt/vol), and the culture was grown for an additional 3 h before harvesting cells and freezing them at −80°C. After thawing cells in 25 ml of cold MF buffer [25 mM Hepes (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT], the cell suspension was lysed by using a French press. The lysate was centrifuged (18,000 × *g*, 30 min), and streptomycin sulfate was added to the supernatant to precipitate nucleic acids. After gentile agitation of the solution at 4 °C for 1 h, the solution was recentrifuged (18,000 × *g*, 30 min), and the supernatant was decanted and loaded onto a hydroxylapatite column equilibrated with MF buffer at room temperature. The column was washed with MF buffer and developed with a linear potassium phosphate (pH 7.5) gradient from 0 to 0.5 M. Fractions containing *mf*-Lon were pooled, concentrated with an Amicon concentrator with a 100-kDa cutoff, and loaded onto a 26/60 Sephacryl S300 gel filtration column (GE Healthcare) preequilibrated with MF buffer. Fractions containing *mf*-Lon at purity >90% were concentrated as described above, aliquoted, and kept frozen at −80°C.

The titin-I27-*mf*-ssrA protein was purified by using the Impact-CN system (New England Biolabs). The gene encoding titin-I27-*mf*-ssrA with an N-terminal His_6 tag was cloned into pTYB1 and transformed into an *E. coli* strain ER2566. A culture was grown at 37°C in 2XYT medium supplemented with ampicillin (100 μg/ml). At an A_{600} of 1.0, IPTG was added (0.5 mM), and the culture was grown for an additional 2.5 h before harvesting cells and freezing them at −80°C. After thawing cells in 25 ml of cold Ni buffer [25 mM Hepes (pH 7.5), 500 mM NaCl, 20 mM imidazole] containing 0.02 mg/ml lysozyme, the cell suspension was lysed by using a French press. The lysate was centrifuged, and the supernatant was mixed in a 50-ml tube with 2 ml of Ni-nitrilotriacetic acid resin (Qiagen; prewashed with Ni buffer) and incubated at 4°C for 15 min. The resin was washed three times with 20 ml of Ni buffer, resuspended in 5 ml of buffer, transferred to a gravity column, and washed once with 10 ml of buffer. The protein was eluted with 3 ml of Ni buffer containing 250 mM imidazole, loaded onto a 10-ml chitin–agarose column, and cleavage, and purification was performed according to the Impact-CN system protocol. Carboxylmethylation and purification of all other titin-I27 variants were performed as described in ref. 19.

FQ14-*mf*-ssrA peptides were synthesized by the Massachusetts Institute of Technology Biopolymers Laboratory. Peptide concentrations were determined from absorbance at 381 nm (ε = 2,200 M^{-1} cm^{-1}).

Biochemical and Biophysical Assays. Unless noted, degradation assays using *mf*-Lon were performed at 30°C in MF buffer. Degradation assays using *ec*-Lon were carried out as described in ref. 13. Unless noted, degradation reactions contained ATP (2 mM) and an ATP-regeneration system composed of 20 mM phosphoenolpyruvate and 10 units/ml pyruvate kinase. For degradation of radioactive substrates, trichloroacetic acid precipitation was carried out as described in ref. 6.

β-Galactosidase assays were carried out based on described procedures (44). Cells were grown at 30°C to midlog phase in LB broth supplemented with ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), and IPTG (1 mM), and absorbance at 600 nm was measured. Cells (20 μl) were lysed by mixing with 80 μl of B-PER protein extraction reagents (Pierce) containing PMSF (1 mM) and incubating the solution for 10 min at room temperature. To start the β-galactosidase reaction, 670 μl of 100 mM sodium phosphate (pH 7.4), 10 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and 1 mg/ml *o*-nitrophenyl β-D-galactoside were added. After incubation for 10 min at room temperature, 330 μl of 1 M Na_2CO_3 was added to stop the reaction, and the absorbance at 420 nm was measured. Miller units were calculated as: $(1{,}000 \cdot A_{420})/(A_{600} \cdot \text{culture volume} \cdot \text{incubation time} \cdot 1.61)$.

CD spectra were taken at 1-nm intervals in a 1-mm path length cuvette on an AVIV 400 instrument [40 μM protein in 10 mM potassium phosphate (pH 7.6), 20 mM KCl]. For thermal denaturation monitored by CD, the protein concentration was 4 μM [10 mM potassium phosphate (pH 7.6), 100 mM KCl]. A 10-mm path length cuvette was used, the heating rate was 1°C/min, and ellipticity at 228 nM was averaged for 10 s after temperature equilibration.

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1. Keiler KC, Waller PR, Sauer RT (1996) Role of a peptide-tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science* 271:990–993.
2. Withey JH, Friedman DI (2003) A salvage pathway for protein structures: tmRNA and *trans*-translation. *Annu Rev Microbiol* 57:101–123.
3. Moore SD, Sauer RT (2007) The tmRNA system for translational surveillance and ribosome rescue. *Annu Rev Biochem* 76:101–124.
4. Keiler KC (2008) Biology of *trans*-translation. *Annu Rev Microbiol* 62:133–151.
5. Tu GF, Reid GE, Zhang JG, Moritz RL, Simpson RJ (1995) C-terminal extension of truncated recombinant proteins in *Escherichia coli* with a 10Sa RNA decapeptide. *J Biol Chem* 270:9322–9326.
6. Gottesman S, Roche E, Zhou Y, Sauer RT (1998) The ClpXP and ClpAP proteases degrade proteins with carboxyl-terminal peptide tails added by the SsrA-tagging system. *Genes Dev* 12:1338–1347.
7. Flynn JM, *et al*. (2001) Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis. *Proc Natl Acad Sci USA* 98:10584–10589.
8. Sauer RT, *et al*. (2004) Sculpting the proteome with AAA(+) proteases and disassembly machines. *Cell* 119:9–18.
9. Herman C, Prakash S, Lu CZ, Matouschek A, Gross CA (2003) Lack of a robust unfoldase activity confers a unique level of substrate specificity to the universal AAA protease FtsH. *Mol Cell* 11:659–669.
10. Wiegert T, Schumann W (2001) SsrA-mediated tagging in *Bacillus subtilis*. *J Bacteriol* 183:3885–3889.
11. Farrell CM, Grossman AD, Sauer RT (2005) Cytoplasmic degradation of ssrA-tagged proteins. *Mol Microbiol* 57:1750–1761.
12. Choy JS, Aung LL, Karzai AW (2007) Lon protease degrades transfer-messenger RNA-tagged proteins. *J Bacteriol* 189:6564–6571.
13. Gur E, Sauer RT (2008) Recognition of misfolded proteins by Lon, an AAA+ protease. *Genes Dev* 22:2267–2277.
14. Lies MA, Maurizi MR (2008) Turnover of endogenous SsrA-tagged proteins mediated by ATP-dependent proteases in *Escherichia coli*. *J Biol Chem* 283:22918–22929.
15. Razin S, Yogev D, Naot Y (1998) Molecular biology and pathogenicity of mycoplasmas. *Microbiol Mol Biol Rev* 62:1094–1156.
16. Fadiel A, Eichenbaum KD, El Semary N, Epperson B (2007) *Front Biosci* 12:2020–2028.
17. Gonzalez M, Frank EG, Levine AS, Woodgate R (1998) Lon-mediated proteolysis of the *Escherichia coli* UmuD mutagenesis protein: *In vitro* degradation and identification of residues required for proteolysis. *Genes Dev* 12:3889–3899.
18. Torres-Cabassa AS, Gottesman S (1987) Capsule synthesis in *Escherichia coli* K-12 is regulated by proteolysis. *J Bacteriol* 169:981–989.
19. Kenniston JA, Baker TA, Fernandez JM, Sauer RT (2003) Linkage between ATP consumption and mechanical unfolding during the protein processing reactions of an AAA+ degradation machine. *Cell* 114:511–520.
20. Burton RE, Baker TA, Sauer RT (2005) Nucleotide-dependent substrate recognition by the AAA+ HslUV protease. *Nat Struct Mol Biol* 12:245–251.
21. Kim YI, Burton RE, Burton BM, Sauer RT, Baker TA (2000) Dynamics of substrate denaturation and translocation by the ClpXP degradation machine. *Mol Cell* 5:639–648.
22. Kenniston JA, Burton RE, Siddiqui SM, Baker TA, Sauer RT (2004) Effects of local protein stability and the geometric position of the substrate degradation tag on the efficiency of ClpXP denaturation and degradation. *J Struct Biol* 146:130–140.
23. Kenniston JA, Baker TA, Sauer RT (2005) Partitioning between unfolding and release of native domains during ClpXP degradation determines substrate selectivity and partial processing. *Proc Natl Acad Sci USA* 102:1390–1395.

24. Politou AS, Thomas DJ, Pastore A (1995) The folding and stability of titin immunoglobulin-like modules, with implications for the mechanism of elasticity. *Biophys J* 69:2601–2610.
25. Carrion-Vazquez M, *et al*. (1999) Mechanical and chemical unfolding of a single protein: A comparison. *Proc Natl Acad Sci USA* 96:3694–3699.
26. Huang C, Wolfgang MC, Withey J, Koomey M, Friedman DI (2000) Charged tmRNA but not tmRNA-mediated proteolysis is essential for *Neisseria gonorrhoeae* viability. *EMBO J* 19:1098–1107.
27. Koonin EV (2000) How many genes can make a cell: The minimal-gene-set concept. *Annu Rev Genomics Hum Genet* 1:99–116.
28. Roche ED, Sauer RT (2001) Identification of endogenous SsrA-tagged proteins reveals tagging at positions corresponding to stop codons. *J Biol Chem* 276:28509–28515.
29. Hong SJ, Lessner FH, Mahen EM, Keiler KC (2007) Proteomic identification of tmRNA substrates. *Proc Natl Acad Sci USA* 104:17128–17133.
30. Levchenko I, Seidel M, Sauer RT, Baker TA (2000) A specificity-enhancing factor for the ClpXP degradation machine. *Science* 289:2354–2356.
31. Dougan DA, Reid BG, Horwich AL, Bukau B (2002) ClpS, a substrate modulator of the ClpAP machine. *Mol Cell* 9:673–683.
32. Chien P, Perchuk BS, Laub MT, Sauer RT, Baker TA (2007) Direct and adaptor-mediated substrate recognition by an essential AAA+ protease. *Proc Natl Acad Sci USA* 104:6590–6595.
33. Lessner FH, Venters BJ, Keiler KC (2007) Proteolytic adaptor for transfer-messenger RNA-tagged proteins from α-proteobacteria. *J Bacteriol* 189:272–275.
34. Tsilibaris V, Maenhaut-Michel G, Van Melderen L (2006) Biological roles of the Lon ATP-dependent protease. *Res Microbiol* 157:701–713.
35. Herman C, Thévenet D, Bouloc P, Walker GC, D'Ari R (1998) Degradation of carboxyl-terminal-tagged cytoplasmic proteins by the *Escherichia coli* protease HflB (FtsH). *Genes Dev* 12:1348–1355.
36. Mizusawa S, Gottesman S (1983) Protein degradation in *Escherichia coli*: The *lon* gene controls the stability of SulA protein. *Proc Natl Acad Sci USA* 80:358–362.
37. Goff SA, Goldberg AL (1985) Production of abnormal proteins in *E. coli* stimulates transcription of *lon* and other heat shock genes. *Cell* 41:587–595.
38. Boddicker JD, Jones BD (2004) Lon protease activity causes down-regulation of *Salmonella* pathogenicity island 1 invasion gene expression after infection of epithelial cells. *Infect Immun* 72:2002–2013.
39. Weber-BanEU, Reid BG, Miranker AD, Horwich AL (1999) Global unfolding of a substrate protein by the Hsp100 chaperone ClpA. *Nature* 401:90–93.
40. Singh SK, Grimaud R, Hoskins JR, Wickner S, Maurizi MR (2000) Unfolding and internalization of proteins by the ATP-dependent proteases ClpXP and ClpAP. *Proc Natl Acad Sci USA* 97:8898–8903.
41. Lee C, Schwartz MP, Prakash S, Iwakura M, Matouschek A (2001) ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. *Mol Cell* 7:627–637.
42. Reid BG, Fenton WA, Horwich AL, Weber-BanEU (2001) ClpA mediates directional translocation of substrate proteins into the ClpP protease. *Proc Natl Acad Sci USA* 98:3768–3772.
43. Christensen SK, *et al*. (2004) Overproduction of the Lon protease triggers inhibition of translation in *Escherichia coli*: Involvement of the *yefM–yoeB* toxin-antitoxin system. *Mol Microbiol* 51:1705–1717.
44. Miller JH (1972) in *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
45. Davis BD, Mingioli ES (1950) Mutants of *Escherichia coli* requiring methionine or vitamin B12. *J Bacteriol* 60:17–28.
46. Mayr LM, Landt O, Hahn U, Schmid FX (1993) Stability and folding kinetics of ribonuclease T1 are strongly altered by the replacement of *cis*-proline 39 with alanine. *J Mol Biol* 231:897–912.
47. Crooks GE, Hon G, Chandonia JM, Brenner SE (2004) WebLogo: A sequence logo generator. *Genome Res* 14:1188–1190.
48. Gueneau de Novoa P, Williams KP (2004) The tmRNA website: Reductive evolution of tmRNA in plastids and other endosymbionts. *Nucleic Acids Res* 32:104–108.
49. Laslett D, Canback B (2004) ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences. *Nucleic Acids Res* 32:11–16.