Evidence That Two ATP-Dependent (Lon) Proteases in Borrelia burgdorferi Serve Different Functions

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Abstract

The canonical ATP-dependent protease Lon participates in an assortment of biological processes in bacteria, including the catalysis of damaged or senescent proteins and short-lived regulatory proteins. Borrelia spirochetes are unusual in that they code for two putative ATP-dependent Lon homologs, Lon-1 and Lon-2. Borrelia burgdorferi, the etiologic agent of Lyme disease, is transmitted through the blood feeding of Ixodes ticks. Previous work in our laboratory reported that B. burgdorferi lon-1 is upregulated transcriptionally by exposure to blood in vitro, while lon-2 is not. Because blood induction of Lon-1 may be of importance in the regulation of virulence factors critical for spirochete transmission, the clarification of functional roles for these two proteases in B. burgdorferi was the object of this study. On the chromosome, lon-2 is immediately downstream of ATP-dependent proteases clpP and clpX, an arrangement identical to that of lon of Escherichia coli. Phylogenetic analysis revealed that Lon-1 and Lon-2 cluster separately due to differences in the NH₂-terminal substrate binding domains that may reflect differences in substrate specificity. Recombinant Lon-1 manifested properties of an ATP-dependent chaperone-protease in vitro but did not complement an E. coli Lon mutant, while Lon-2 corrected two characteristic Lon-mutant phenotypes. We conclude that B. burgdorferi Lons -1 and -2 have distinct functional roles. Lon-2 functions in a manner consistent with canonical Lon, engaged in cellular homeostasis. Lon-1, by virtue of its blood induction, and as a unique feature of the Borreliae, may be important in host adaptation from the arthropod to a warm-blooded host.

Introduction

In nature, Borrelia burgdorferi, the agent of Lyme disease [1,2], must adapt to disparate hosts, alternating between Ixodes ticks and various small rodent species. It is thought that this adaptation is made possible through the remodeling of the spirochete outer surface in response to environmental cues such as temperature [3,4,5], pH [7,8,9,10], and microbial density [11,12,13]. A recognized example of this is the reciprocal expression of outer surface lipoprotein (Osp) AB and OspC. OspA and OspB are dominantly expressed when the spirochete is in culture or in the midgut of a flat unfed tick, then downregulated upon feeding and subsequent exposure to blood, increased temperature, and a drop in pH. OspC is concomitantly upregulated [3,4,13,14,15,16]. At this time the spirochetes experience a period of vigorous growth and migrate from the tick midgut to the salivary glands via the hemolymph [17]. This is followed by transmission to the mammalian host. The coordinated expression of OspAB and OspC has been proposed as an example of spirochete-vector interaction, with OspAB and OspC being implicated in spirochete adhesion to the tick midgut [18,19,20] and salivary gland respectively. At the time of feeding, abundant, surface exposed OspA and OspB need to be broken down to remodel the outer membrane. We have had a long-standing interest in the proteases of B. burgdorferi and their functions. In the absence of known secreted proteases in the genome of B. burgdorferi [21], we have documented the reliance of this organism on borrowed proteolytic activity. The plasminogen activation system is used by the spirochete to cross cellular and extracellular matrices by inducing the production of and/or incorporating enzymatically active plasmin, urokinase plasminogen activator, and metalloproteases onto its surface [15,22,23,24,25] in both vectors and vertebrate hosts. While plasmin was important in promoting the migration of B. burgdorferi through the tick, this mammalian protease did not have an effect on the remodeling the outer surface of the spirochete at this crucial time. However, OspC, which is the upregulated lipoprotein during tick feeding is a plasminogen receptor [26] providing further indication that this system is associated with migration of the spirochete in the vector.

In a previous study, our interest in the proteolytic remodeling of outer surface lipoproteins during the transition of the spirochete from tick to mammalian host led us to examine the B. burgdorferi transcriptome after exposure in vitro to increased temperature in the presence and absence of blood [6]. The changes that we
observed for the blood condition included the upregulation of OspC, other lipoproteins, and many genes of known or unknown function. One intriguing observation was the significant upregulation of a putative ATP-dependent protease La (Lon-1, BB0253) [21], a homolog of the Escherichia coli gene [27] and identical to the lon gene described for B. burgdorferi by Cloud et al. [28]. The B. burgdorferi genome also codes for a second putative ATP-dependent lon homolog, BB0613 (lon-2) [21], which was not differentially expressed in the array [6].

The canonical E. coli Lon (Lon-Ec), a conserved and much studied protease important for intracellular degradation of short-lived and abnormal proteins [27], has also been identified as a DNA-binding protein [29], and as a part of the heat shock regulon [30,31]. Besides bacteria, Lon homologs have also been found in other organisms including archaea [32], yeast [33,34,35], and animals [36,37]. It is an 87-kDa oligomeric serine protease with a structure consisting of three functional domains: the heterogenous NH2-terminal (LAN) domain, the ATPase domain, and the C-terminal proteolytic domain. Structural studies have demonstrated that Lon is a ring-shaped complex composed of multiple identical 87-kDa subunits, with Lon-Ec functioning as a hexamer [38]. Lon plays an important role in homeostasis by targeting abnormal proteins and unstable regulatory proteins such as the SulA division regulator [39] and RcsA, the positive regulator of capsular polysaccharide [40,41], and degrading tmRNA-tagged proteins during trans translation [42]. In addition, Lon has been shown to be a virulence factor for infection in mice [43,44]. Our interest in B. burgdorferi Lon-1 stemmed from the idea that as a protease, it could take part in the remodeling of the spirochete outer surface during the period following a blood meal in the tick. With this in mind, we sought to identify functional roles for Lon-1 and Lon-2 through phylogenetic analyses, and biochemical and genetic means. Our results support the idea that Lon-2 fulfills the role of canonical Lon and is involved in cellular quality control and homeostasis, while Lon-1 plays a role in host adaptation during the transition from tick to mammal.

Results/Discussion

Sporocetal Lon-1 and Lon-2 are only distantly related to canonical Lon

B. burgdorferi codes for two putative ATP-dependent Lon homologs designated Lon-1 and Lon-2 [21]. In a previous study, Lon-1 was upregulated in response to blood, while Lon-2 was not [6], though each is expressed by B. burgdorferi during culture in BS-KII medium (Figure 1A). The presence of more than one lon gene homolog is rare in the eu细菌ia, but appears to be a unique characteristic of the genus Borrelia, as the genomes for B. afzelii [45], B. garinii [46], B. recurrentis [47], B. duttonii [47], and B. hermsii [48] also code for two Lon homologs. Our interest in Lon-1 stemmed from its hypothetical function as a protease, and we hypothesized that it could be involved in the remodeling of spirochete outer surface lipoproteins during the transition from the tick to the mammal, thus warranting its investigation as a potential virulence factor. By extension, our hypothesis would imply that Lon-2 would likely function in a manner similar to that of Lon-Ec. The canonical Lon-Ec resides on the chromosome, adjacent to and downstream from two other ATP-dependent proteases, clpX and clpP, the proteolytic units comprising the ATP-dependent clpXP complex thought to share its role in cellular housekeeping. Examination of the chromosomal location of B. burgdorferi lon-2 reveals that it is arranged identically, immediately downstream of clpX and clpP, while Lon-1 is on a distant part of the chromosome (Figure 1). The lon-2 arrangement shown for B. burgdorferi in Figure 1 is maintained in other Borrelia, while Treponema and Leptospira code for single lon genes that are not arranged in the same fashion as canonical lon (data not shown).

Because the similarity of the chromosomal location of Lon-2 to canonical E. coli lon suggested that they could serve a similar function, we tested the premise that Lon-1 of B. burgdorferi may have other functions that could be unique to spirochetes, as transcription of this gene was upregulated on blood induction. Our assessment of the premise began with comparison of amino acid sequences (defined by Pfam, PF00004, PF02190, and PF05362) of Borrelia Lons with the Lon sequences of other spirochetes and more distantly related bacteria through the construction of phylogenetic trees (Figure 2). The trees we created included Lon amino acid sequences from spirochetes coding for two Lons (Lyme disease and relapsing fever Borreliae) and single Lons (Treponema, Leptospira, E. coli, and B. subtilis). In Figure 2A, a tree based on the entire amino acid sequences of Lon proteins is shown. One evident feature from the tree is that while all of the Lons are related to some extent, they began to diverge in the distant past. The Borrelia Lon-1s cluster together, indicating that they are closely related. Within the Lon-1 group, the relapsing fever Borreliae, consisting of B. recurrentis, B. duttonii, and B. hermsii form a subcluster, while the Lyme disease group, B. burgdorferi, B. afzelii, and B. garinii form another subcluster. Although distinct, Borrelia Lon-1 is also related to Lons of Treponema and Leptospira, suggesting a common ancestor. Lon-2 sequences from Lyme disease and relapsing fever Borreliae cluster separately from all the other bacteria, indicating a close relationship exclusive to the genus Borrelia, and form subclusters identical to those of Lon-1. We also performed the phylogenetic analyses using lon nucleotide sequences, with virtually identical results (data not shown).

The carboxy-terminal proteolytic domain and the ATPase domain of Lon are conserved across different bacterial species, whereas the NH2-terminal domain, which is responsible for substrate binding, is heterogeneous, and thus, the most useful for phylogenetic analysis. To determine a possible functional difference between Lon-1 and Lon-2, we tested the phylogenetic
Lon-1 and Lon-2 in Borrelia

Figure 1. Native Lon expression in *B. burgdorferi* and comparison of lon loci and flanking genes from *B. burgdorferi* and *E. coli*. (A) Native Lon-1 and Lon-2 expression in *B. burgdorferi* wild-type BB050.14 spirochetes. Lon-1 and Lon-2 were detected in whole cell lysates by use of rabbit and mouse antiserum, respectively. (B) *B. burgdorferi* lon-1 region. (C) *B. burgdorferi* lon-2 region. (D) *E. coli* lon region. Gene symbols and gene loci (The Institute for Genomic Research-TIGR) are given above and below each gene, respectively. CH, conserved hypothetical; leuS, leucyl-tRNA synthase; M23 pdp, M23 peptidase domain protein; tig, trigger factor; clpP, ATP-dependent proteolytic subunit of ClpA-ClpP serine protease; clpX, ATP-dependent specificity component of ClpP serine protease; rpsD, ribosomal protein S4; hupB, DNA-binding protein HU-beta; ybaU, putative protease maturation protein. Gene coordinates are indicated with arrows. One-kb size marker is shown at lower left.

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relatedness of the NH$_2$-terminal domains of the bacteria analyzed in Figure 2A (Figure 2B); Overall, the clustering pattern for the NH$_2$-terminal domain sequences closely resembles that of the whole molecule, implying that the clustering is driven by differences in the NH$_2$-terminal domain alone. Perhaps the most significant outcome of the phylogenetic analysis is that the NH$_2$-terminal domains of Lon-1 and Lon-2 are shown to be especially dissimilar, which may correlate with substrate specificity. Thus, Lon-1 and Lon-2 may each recognize different substrates. Another significant outcome of the phylogenetic analysis is that neither Lon-1 nor Lon-2 are closely related to the canonical Lons of *B. subtilis* and *E. coli*, preventing us from making sequence-driven inferences about the function of either *Borrelia* Lon.

Recombinant Lon-1 is enzymatically active

To further examine *B. burgdorferi* Lon function, we characterized the Lon-1 gene product to study its structure and test it for biological activity. For this purpose, we generated wild-type rLon-1 (strains and PCR primers are given in Tables 1 and 2). Proteolytic domains of Lons typically contain a lysine-serine dyad critical for catalysis with the lysine appearing 43 residues downstream of the catalytic serine [38]. Inspection of the deduced Lon-1 amino acid sequence shows that it contains a serine residue a position 714 (Swiss-Prot predicted active site S714) and a lysine at position 757 (Swiss-Prot predicted active site K757), therefore, we also generated a recombinant with the putative catalytic serine714 mutated to alanine (S714A) to abolish proteolytic activity and confirm its identity as part of the catalytic dyad. Recombinant wild-type (rLon-1) and mutant Lon-1 (rLon-1$^{S714A}$) was analyzed by SDS-PAGE and western blot (Figure 3) to assess their purity and confirm their size. Figure 3 panels A and B show that rLon-1 and rLon-1$^{S714A}$ were purified to homogeneity and their SDS-PAGE profiles are consistent with their predicted size (native Lon-1 has a predicted MW of 90,701 and a predicted MW of 92,864 with the attached His-tag).

To examine the catalytic activities of wild-type rLon-1 and rLon-1$^{S714A}$ we conducted ATPase and caseinolytic assays (Figure 4). Analysis of ATPase activity by a colorimetric dye-binding assay showed that both the rLon-1 and the rLon-1$^{S714A}$ exhibited similar levels of activity (Figure 4A). The ATPase activity of rLon-1$^{S714A}$ confirmed that the ATPase and the proteolytic domains function independently and that introduction of a mutation into the proteolytic domain does not affect the ability of Lon-1 to hydrolyze ATP. To test for rLon-1 and rLon-1$^{S714A}$ ATP-dependent proteolytic activity we measured the degradation of z-casein-fluorescein isothiocyanate (FITC-casein), in the presence of 10 mM ATP and 4 mM ATP [33,37,49]. In contrast to the ATPase assay, the rLon-1$^{S714A}$ did not show activity levels above that of the buffer control while the wild-type rLon-1 degraded FITC-casein in a time-dependent manner (Figure 4B). This confirms that through targeted mutagenesis of serine714, the catalytic serine residue was identified correctly. Omission of ATP from the assay verified that it is required for the degradation of FITC-casein by rLon-1, as expected for an ATP-dependent protease (Figure 4C, left panel).

The presence of Mg$^{2+}$ is critical for assembly of both *E. coli* and *Mycobacterium smegmatis* Lon subunits into proteolytic multimeric structures [50,51]. To investigate whether the caseinolytic activity of Lon-1 was Mg$^{2+}$-dependent we carried out the caseinolytic assay with wild-type rLon-1 in the presence and absence of Mg$^{2+}$ (for the absence of Mg condition, MgCl$_2$ was omitted from the reaction buffer). In the presence of Mg$^{2+}$ the catalytic activity was retained, however, the absence of Mg$^{2+}$ reduced the activity to background levels (Figure 4C, right panel).
Figure 2. Phylogenetic trees based on Lon amino acid sequences of spirochetes. (A) Phylogenetic tree of spirochetes derived from the amino acid sequences of Lon proteins from reference strains of *B. burgdorferi* sensu lato and relapsing fever *Borrelia*, *Leptospira* and *Treponema* species, with *E. coli* and *B. subtilis* as an external group. (B) Phylogenetic tree of spirochetes derived from the amino acid sequences of the amino-terminal NH2-terminal domains from reference strains of *B. burgdorferi* sensu lato and relapsing fever *Borrelia*, *Leptospira* and *Treponema* species, with *E. coli* and *B. subtilis* as outgroups. The neighbour joining trees were constructed with MEGA3 software. Bootstrap confidence levels above 50% are indicated to the left of each relevant cluster. Asterisks: * Lon-1; ** Lon-2. All of the sequences were obtained from Swiss-Prot Protein knowledgebase. Accession numbers are listed next to each species. Distance marker is shown at lower left for each panel.
This result implies that the assembly of Lon-1 monomers into multimeric structures is essential for proteolytic activity. We also examined the caseinolytic activity of wild-type rLon-1 over a gradient of pH conditions. The pH optimum for wild-type rLon-1 ranged from 9.0 to 9.5, which is similar to that of Lon-Ec [52] (Figure 4D). Exhaustive attempts to overexpress rLon-2 proved unsuccessful, as the detergent-soluble product was proteolytically inactive, precluding further use.

**Lon-1 does not degrade Borrelia-SsrA tagged reporter protein in vitro**

A variety of *in vivo* Lon substrates have been identified to date, including SulA, RcsA, and UmuD [40,53,54,55]. In addition, rLon-Ec was reported to degrade SsrA-tagged proteins, a characteristic that could be directly examined *in vitro* [42]. SsrA (also known as tmRNA) is a unique RNA molecule that is highly conserved in Eubacteria and plays a major role in dealing with stalled ribosomes caused by aberrant mRNAs. In this process, SsrA co-translationally places a peptide tag on the carboxyl termini of the incomplete polypeptides. These marked polypeptides are recognized by cellular proteases, including Lon, and readily degraded [56]. The ssrA sequence of *B. burgdorferi* has been identified and the presence of SsrA RNA product has been confirmed [57]. To test whether Lon-1 can degrade proteins marked with a *Borrelia*-SsrA tag, we constructed and purified a reporter protein, l-CI-N-SsrA Bb, which carries a *Borrelia*-SsrA tag.

### Table 1. Bacterial strains and plasmids used in this study.

| Strain or plasmid name | Genotype or description | Reference or source |
|------------------------|-------------------------|---------------------|
| *B. burgdorferi*       | B31MI MedImmune strain, wild-type | Reference [21] |
|                        | B31A3 cp9 – wild-type | Reference [74] |
| *E. coli*              | BL21 Star™(DE3)pLysS Expression host, F– ompT hsdSB (rB–, mB–) gal dcm met131 (DE3) pLysS (CamR) | Invitrogen, Carlsbad, CA |
|                        | Rosetta™ 2(DE3)pLysS Expression host, F– ompT hsdSB (rB–, mB–) gal dcm (DE3) pLysS RARE2 (CamR) | Novagen, Gibbstown, NJ |
|                        | HDB97 SG22622 mali+, wild-type lon | Reference [68] |
|                        | HDB98 HDB97 (lon<sup>110</sup>) | Reference [68] |
|                        | HDB98pBAD24 HDB98 containing pBAD24 | This study |
|                        | HDB98pBADlon-1 HDB98 containing pBAD24lon-1 | This study |
|                        | HDB98pBADlon-2 HDB98 containing pBAD24lon-2 | This study |
| **Plasmids**           | pET30a(+) Expression vector | Novagen |
|                        | pCK003.9 pET30a(+) expressing wt rLon-1 | This study |
|                        | pCK004.3 pET30a(+) expressing rLon-1<sup>I144A</sup> | This study |
|                        | pCK006.2 pET30a(+) expressing wt rLon-2 | This study |
|                        | pBAD24 Expression vector containing pBAD | Reference [75] |
|                        | pBADlon-1 pBAD24 with lon-1 cloned in | This study |
|                        | pBADlon-2 pBAD24 with lon-2 cloned in | This study |

All PCR primers shown here were designed for this study. *Restriction sites are underlined.

**Table 2. Oligonucleotide primers used in this study.**

| Primer Pair | RS<sup>a</sup> | Primer sequence (5′→3′) | Application |
|-------------|-----------------|--------------------------|-------------|
| P1F         | Ndel           | TTACATATGGAATCTAAAAAAGCTAGG | Amplification of lon-1 for cloning into pET30a |
| P2R         | Xhol           | TTACTCCGAAGAACATATTTAAGGCTCG | Amplification of lon-1 for cloning into pET30a |
| P21F        | Ndel           | CTCCCCATAGAAATCTATCTAAAAATAGA | Amplification of lon-1 for site-directed mutagenesis |
| P22R        | Xhol           | AACCCTCAGAAATATATCTAAAAATAAAACACTTC | Amplification of lon-1 for site-directed mutagenesis |
| P3F         | —               | CCCCCAAGATGCGCGTGGACGGAATTAC | Amplification of lon-1 for cloning into pET30a |
| P4R         | —               | AGCTTTGATATCTTACGAGCAAGGCCCAT | Amplification of lon-1 for cloning into pET30a |
| P23F        | —               | CCAAAAGATGCGCGTGGACGGAATTAC | Amplification of lon-1 for site-directed mutagenesis |
| P24R        | —               | GTTGGCAATGGAATACGACGAGCCGACCTTTTG | Amplification of lon-1 for site-directed mutagenesis |
| P50F        | Ncol           | CGCGGAGCATGAGATGATCTAAAAAACAGTCAG | Amplification of lon-1 for cloning into pBAD24 |
| P51R        | Ncol           | CGCGGAGCATGAGATGATCTAAAAAACAGTCAG | Amplification of lon-1 for cloning into pBAD24 |
| P52F        | Nhel           | CGCGGAGCATGAGATGATCTAAAAAACAGTCAG | Amplification of lon-1 for cloning into pBAD24 |
| P53R        | KpnI           | CGCGGAGCATGAGATGATCTAAAAAACAGTCAG | Amplification of lon-1 for cloning into pBAD24 |

<sup>a</sup>Restriction sites are underlined.

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temperature-sensitive phenotype could be a novel chaperone-like activity conferred to lon-1 through the absence of the proteolytic domain from the construct, thus allowing stabilization of the mutant ipx4 gene. To further characterize Lon-1 we investigated its capacity to act as a chaperone by use of an in vitro assay employing bovine insulin. Insulin is a 6-kDa polypeptide consisting of two chains, A and B, connected by two disulfide bridges. Reduction of the molecule results in aggregation of the B-chain, while the A-chain remains soluble [63]. The chaperone assay is based upon the prevention of B-chain aggregation through interaction with an added chaperone. Assay results are measured via light scattering caused by insulin aggregates [59,61]. Therefore, to determine if rLon-1 possessed chaperone-like activity, we tested the ability of rLon1 and rLon-1^{S714A} to inhibit the aggregation of insulin B-chain under reducing conditions. Kinetic spectrophotometric analysis revealed that in the absence of added Lon protein (buffer only), an irreversible formation of B-chain aggregates was the result. Addition of rLon-1^{S714A} without ATP did not significantly affect aggregate formation (Figure 6A, top panel). However, addition of rLon-1^{S714A} in the presence of increasing amounts of ATP resulted in a dose-dependent inhibition of aggregate formation (Figure 6A, lower panels). Wild-type rLon-1 enzymatically degraded the insulin and thus was not suitable for use in the assay (Figure 6B).

The preservation of chaperone-like activity despite mutagenesis of the proteolytic site in Lon-1 is consistent with that found in yeast and mammalian Lon [58,60]. The critical requirement for ATP in our Lon-1 chaperone assay results is in agreement with that reported for chaperones in general [64] and for yeast Lon [58,60], but differs with one study in which the chaperone-like activity of a bacterial Lon was investigated. In that report, Lon from Bacillus subtilis prevented insulin aggregation in an ATP-independent manner [59].

Protein stabilization and catalysis would seem to be opposing actions; nevertheless, ATP-dependent chaperone-proteases of the Clp and Lon families are well documented [62]. In the case of Lon-1, the demonstration of a chaperone-like activity in vitro, while interesting, does not definitively establish a role as a chaperone in vivo. On the other hand, differential recognition of potential substrates -CI-N-SsrA^Bb and insulin or casein by rLon-1 demonstrates an ability by the enzyme to discriminate, implying that it could act as a chaperone for one protein and degrade another.

**Lon-2 complements an *E. coli* lon mutant**

The implied redundancy of retaining two Lon genes necessarily raises the question of whether they are in actuality functionally equivalent, complementary, distinct, or overlapping, which may be reflected by similarities or differences in composition or expression pattern. *B. burgdorferi* Lons-1 and -2 are comparable in size (~90 kDa) and domain structure. However, Lon-2 may be more chemically similar to the archetypal Lon-Ec, as suggested by the similarity of their isoelectric points (Lon-2: pH = 6.04/Lon-Ec: pH = 6.32), and the similarity in its chromosomal arrangement to Lon-Ec. Lon-1, on the other hand, is an exceedingly basic protein (pI = 9.85). In our previous report, _lon-1_ was transcriptionally upregulated in _in vitro_ when exposed to blood and temperature shift in comparison to temperature shift alone. _Lon-1_ upregulation in response to temperature shift alone was not determined in this study [6]. However, in other studies, _lon-1_ was not activated by temperature [65,66], pH shift [66], or by cultivation in dialysis membrane chambers in rats [66,67]. In line with our own findings, none of the above microarray studies reported the differential expression of _lon-2_ as a result of any condition [65,66,67].
We further investigated the respective roles of *lon-1* and *lon-2* by testing experimentally whether either gene is functionally equivalent to *lon-Ec*. Use of a complementation strategy allowed us to investigate and compare the role(s) of *lon-1* and *lon-2* in an *in vivo* context and circumvent the inability to produce rLon-2. Lon-Ec specifically degrades RcsA, a positive activator for transcription of capsular polysaccharide (*cps*) genes [40,41]. Lon-deficiency in *E. coli* causes an accumulation of RcsA and a concommitant excess of CPS, resulting in a mucoid phenotype.

*Lon-1* or *lon-2* was inserted into the multiple cloning site of plasmid pBAD24, to produce plasmids pBADlon-1 and pBADlon-2. Each plasmid was transformed into *lon*-deficient strain HDB98 (*lon510*), which contains a *cpsB::lacZ* fusion reporter [68]. Measurement of β-galactosidase activity allowed us to assess the ability of Lon-1 or Lon-2 to correct the defect in *cpsB* regulation by RscA. As shown in Figure 7A, after culture at 30°C in the presence of 0.1% arabinose, HDB98 containing pBADlon-1 (HDB98pBADlon-1) exhibited β-galactosidase activity at levels similar to parental HDB98 and HDB98 containing vector alone (HDB98pBAD24). In HDB98pBADlon-2, however, β-galactosidase activity levels were significantly reduced in comparison to HDB98, HDB98pBAD24, and HDB98pBADlon-1 (P<0.001). The reduction in β-galactosidase levels did not occur in the absence of arabinose (Figure 7B). Expression of soluble Lon-1 and Lon-2 by *E. coli* containing plasmids pBADlon-1 and pBADlon-2 was verified by western blot (Figure 7C, D, E).

Another substrate for Lon is cell division inhibitor, SulA, which functions along with FtsZ protein to prevent disadvantageous cell division [39,69]. Accumulation of SulA due to a Lon deficiency in *E. coli* causes impaired cell division resulting in a filamentous morphology, a phenotype exhibited in culture by *lon510* mutant HDB98 (data not shown). In the presence of 0.2% arabinose, HDB98pBADlon-1 produced long filamentous forms similar to the bacteria grown without arabinose and to HDB98, while in HDB98pBADlon-2, arabinose induction of Lon-2 expression resulted in greatly reduced filament formation (Figure 8A). Expression of Lon-1 and Lon-2 was verified by western blot (Figure 8B). The ability of Lon-2 and not Lon-1 to complement HDB98 for filamentation and dysregulation of *cps* implies a differential capacity for these proteases to degrade RscA and SulA and supports a Lon-Ec-like role for Lon-2 in Borreliae.
We have shown that B. burgdorferi Lon-1 and -2 have characteristics in common with canonical Lon and with each other. Lon-1 protein displayed features of an ATP and Mg\(^{2+}\)-dependent chaperone-protease, but \textit{lon-1} did not complement an \textit{E. coli} \textit{lon} mutant, while \textit{lon-2} corrected two distinct \textit{lon} mutant phenotypes. Primary structural analysis showed that their respective ATPase and proteolytic domains are similar and characteristically Lon-like, but their substrate-binding domains are very different, suggesting that they may recognize divergent substrates. Taken together, this suggests that despite the structural similarities of Lon-1 and Lon-2, they appear to have distinct functional roles. The presence of Lon-1 as a unique feature of \textit{Borrelia} would indicate that it has functions relevant to this genus as arthropod-transmitted pathogens. Specifically to \textit{B. burgdorferi}, Lon-1 may function in host adaptation where it could be important as a protease during periods of rapid spirochete proliferation during a bloodmeal with its associated increases in protein biogenesis. It is also possible that during this period, Lon-1 could act as a chaperone for molecules that are not enzymatic substrates. Lon-2, on the other hand, functions as the canonical Lon, engaged in cellular homeostasis.

**Materials and Methods**

**Ethics statement**

All animal procedures were performed in compliance with the guidelines and with the approval of the Institutional Animal Care and Use Committee (IACUC) of Stony Brook University.
Generation of recombinant proteins

Purified Lon-Ec was a gift of Dr. A. Wali Karzai Department of Biochemistry and Cell Biology, Center for Infectious Diseases, Stony Brook University, Stony Brook, NY [42]. To generate recombinant Lon-1, the open reading frame of lon-1 [BB0253] was PCR amplified from B31MI [21] and ligation into the space between the Ndel and XhoI restriction sites of predigested vector pET30a (+) [Novagen, Gibbstown, NJ] using primers P1F and P2R (Table 2) to produce a construct that incorporated a 6-histidine tag at the C-terminal end. The resulting plasmid, pCK003.9 (Table 1), was introduced into BL21 Star™ DE3pLysS (Invitrogen). E. coli were grown at 37°C in Tryptose Phosphate Glucose medium until an OD600 of ~0.8 was reached, then induced at 37°C for 3–4 hours with 1.0 mM IPTG. Cell pellets were resuspended and sonicated for 30 seconds on ice in HisBind buffer (20 mM Tris HCl, pH 8.0, 0.5M NaCl, 20 mM imidazole) (Novagen) containing 0.2% NP40 (Pierce, Rockford, IL). Benzoase nuclease, 0.01%, (EMD Chemicals, Inc., Gibbstown, NJ) was added and the lysate was sonicated for an additional two minutes on ice. Clarified lysate was diluted in buffer A (20 mM Tris HCl, pH 8.0, 0.5M NaCl, 20 mM imidazole, 0.02% NP40) and applied to a column of HisTrap HP resin (GE Healthcare, Piscataway, NJ). Elution was carried out in three steps: 15%, 50%, and 100% of buffer B (20 mM Tris HCl pH 8.0, 0.5M NaCl, 1M imidazole, 0.02% NP40). The final buffer conditions were 20 mM Tris HCl pH 8.0, 0.5M NaCl, 0.02% NP40, 0.5M imidazole, 10% glycerol. Exhaustive attempts to produce functional recombinant Lon-2 were unsuccessful. All constructs were confirmed by DNA sequencing. Recombinant protein expression and purification was done at the NBC Protein Expression Core, Wadsworth Center, Albany, NY.

Site-directed mutagenesis of the Lon proteolytic domain

The QuickChange II Site-Directed Mutagenesis Kit (Stratagene) and primers P3F and P4R (Table 2) were used to introduce a point mutation in the proteolytic domain of the lon-1 open reading frame, converting the catalytically active serine residue to alanine (S714A). The resulting construct in pET30a (+), pCK004.3 (Table 1) was confirmed by sequencing and used to transform expression host BL21 Star™ DE3pLysS. Soluble mutant Lon-1 (rLon-1S714A) was expressed and purified as described above for wild-type rLon-1.

In vitro proteolysis of Borrelia-SsrA tagged reporter protein

The purification of λ-CI-N reporter protein was performed as described by Choy et al [42] with some modifications. Briefly, plasmid pPW500 [70], which encodes the λ-λ-N-tpAl gene with no stop codon, was first modified by site directed mutagenesis. In this process, the ttpAl terminator sequence of the reporter gene was replaced by the B. burgdorferi srrA-tag sequence that was obtained from the tmRNA website (http://www.indiana.edu/~tmrna/). Thus, the final plasmid construct carried the λ-λ-N with the B. burgdorferi srrA-tag sequence (λ-λ-N-srrA) and was transformed into an E. coli clpE clpX lon triple mutant. Bacterial cells were cultured in LB broth at 37°C and reporter expression was induced for 3 h with 1 mM IPTG when cultures reached mid-log phase. Finally, the λ-λ-N-SsrA39b reporter
protein was purified by affinity chromatography with the aid of an internal His6 epitope as described [42]. The quality of the purified product was determined by SDS-PAGE followed by Coomassie blue staining, and the size of each protein band was verified by MALDI-TOF mass spectroscopy.

**In vitro** proteolysis assays were carried out with a final concentration of 1.5 mM of either rLon-1 or rLon-Ec, 10 μM Δ-Cl-N-SsrA Bb or 0.3 mg/ml insulin as substrates, an ATP regeneration system (50 mM creatine phosphate, 80 μg/ml creatine kinase [56], 4 mM ATP), and a minimal activity buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 1 mM dithiothreitol). An additional 20 mM of dithiothreitol was included in assays with insulin. The reaction mixtures were incubated at 30°C and a fraction of each sample was collected at various time points for analysis by 15% Tris-tricine SDS-PAGE. Protein bands were quantified by using an Odyssey infrared imaging system (LiCor Biosciences, Lincoln NE).

**Phylogenetic amino acid sequence analysis**

Sequences and their annotations were obtained from the SwissProt Protein knowledgebase. The Clustal W algorithm [71] was used for sequence alignments and Mega 3 software version 3.1 [72] for phylogenetic analyses. Neighbor joining methods [73] were used to build phylogenetic trees. Percentage support values
were obtained through a bootstrap procedure. Analyses were also carried out using nucleotide sequences.

Complementation of E. coli lon mutant HDB98

Wild-type E. coli HDB97 and lon mutant HDB98 (lon510) were the gift of Dr. Harris D. Bernstein, Genetics and Biochemistry Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. B. burgdorferi lon-1 was amplified from B. burgdorferi strain B31A3 [74] by PCR using primers P50F and P51R and cloned into the NcoI restriction site of plasmid pBAD24 [75] which confers ampicillin resistance (American Type Culture Collection, Manassas, VA). The insert was placed eight nucleotides downstream from the optimized ribosomal binding site to form plasmid pBADlon-1. Lon-2 was amplified by PCR using primers P52R F and P53R and cloned into a region between the NheI and KpnI restriction sites of pBAD24, also leaving eight nucleotides between the pBAD24 ribosomal binding site and the lon-2 ATG start site to yield plasmid pBADlon-2. HDB98 was subsequently transformed with pBADlon-1 and pBADlon-2 to form strains HDB98pBADlon-1 and HDB98pBADlon-2. Both constructs were confirmed by nucleotide sequencing.

β-galactosidase assay

Measurement of β-galactosidase activity of E. coli strains was done as previously described [76,77]. HDB97 and HDB98, which contain a cpsB: lacZ reporter fusion, were cultured in LB medium. HDB98pBAD24 (containing empty pBAD24 vector), HDB98pBADlon-1 and HDB98pBADlon-2 were cultured in LB medium with ampicillin and 0.1% L-arabinose to induce expression of B. burgdorferi Lons. Activity units were calculated as follows:

\[
\text{Units} = 1000 \times \frac{A_{600}}{OD_{600} \times V \times RT}
\]

where \(OD_{600}\) represents the optical density of the culture, \(V\), the culture volume analyzed (ml), \(RT\), the reaction time (min), and \(A_{570}\), the absorbance reading after color development. Each strain was analyzed in triplicate and the experiment was repeated a total of five times. Data was tested for statistical relevance using InStat 3.0 Software (GraphPad Software, Inc., San Diego, CA).

Figure 8. lon-2, but not lon-1, complements the filamentous phenotype of an E. coli lon mutant. (A) Brightfield microscopy of E.coli cultures grown at 37 °C for 3.5 hours with or without 0.2% arabinose. Cells were applied to slides, heat fixed, and visualized by Gram stain. (B) Western blot showing arabinose-induced Lon-1 and Lon-2 expression after culture at 37 °C for 3.5 hours.

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Assays of enzymatic activity

Hydrolysis of casein by *B. burgdorferi* rLon-1 was used as a measure of proteolytic activity and was based on the method of Twining et al. [78]. Preparations of rLon-1 and rLon-1$^{8714A}$ contained 20 mM Tris-HCl, 0.5M NaCl, 0.5M imidazole, 0.02% NP40, and 10% glycerol. To assay for proteolytic activity, 5 μg of enzyme was incubated in 1.5 ml microcentrifuge tubes for 3 hours at 37°C with 100 μg of FITC-labeled casein, type I (Sigma). Final reaction conditions after addition of Lon proteins were: 50 mM Tris-HCl, pH 8, 10 mM MgCl$_2$, 4 mM ATP, 25 mM NaCl, 25 mM imidazole, 0.001% NP40, and 0.5% glycerol in a total volume of 100 μl. In assays designed to test for the influence of Mg$^{2+}$ on rLon-1 proteolytic activity, 10 mM MgCl$_2$ was omitted from the reaction buffer. Proteolysis was terminated by the addition of 10 μl of bovine serum albumin (10 mg/ml stock solution) immediately followed by 100 μl of 10% trichloroacetic acid (TCA), and 10 minutes of incubation on ice. The reaction contents were then centrifuged at 16 000 × g to separate TCA insoluble material. For each sample replicate, 125 μl of the TCA soluble supernatant fraction was transferred to the well of a 96-well plate (Falcon - Becton Dickenson) and neutralized by the addition of 100 μl of 10% trichloroacetic acid (TCA), and 10 minutes of incubation on ice. The reaction contents were then centrifuged at 16 000 × g to separate TCA insoluble material. For each sample replicate, 125 μl of the TCA soluble supernatant fraction was transferred to the well of a 96-well plate (Falcon - Becton Dickenson) and neutralized by the addition of 125 μl of 0.5 M CHES-Na, pH 12. Fluorescence was measured in a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) at wavelengths of 490 nm (excitation) and 525 nm (emission). The assay for measurement of optimum pH for casein degradation was carried out at 26°C for 16 hours and the following buffers were used to achieve the desired assay pH: 50 mM MES (pH 6.0, 6.5, 7.0), 50 mM Tris-HCl (pH 7.5, 8.0, 8.5), 50 mM 2-aminoethanepropanediol-HCl (AMPD) (pH 9.0, 9.5, 10.0), 50 mM CAPS (pH 10.5) [52].

The ATPase activity of rLon-1 and rLon-1$^{8714A}$ was measured colorimetrically by use of a kit-based assay which utilized the dye malachite green to detect the liberation of inorganic phosphate resulting from enzymatic ATP hydrolysis (Innova Biosciences, Cambridge, UK). The manufacturer’s protocol was followed for all assays and utilized 5 μg of enzyme per assay reaction. Components of the enzyme purification buffer resulted in final concentrations of 12.5 mM NaCl, 12.5 mM imidazole, 0.0005% NP40, and 0.25% glycerol in the reaction. Absorbances were measured in a SpectraMax M2 microplate reader at a wavelength of 655 nm.

**Assay of chaperone-like activity**

Reduction of its disulfide bonds induces the separation of insulin A and B chains with the attendant aggregation of the B chain [63]. The assay for chaperone-like activity is based upon the protection of reduced B-chain from aggregation by an added chaperone-like molecule. The degree of protection conferred is monitored by the measurement of resultant light scattering. In our assay, a modification of Farahbakhsh et al [79], 15 μg of bovine insulin was added to 25 μg of wild-type rLon-1 or mutant rLon-1$^{8714A}$ protein in the presence of 10 mM sodium phosphate, pH 7.2, 20 mM dithiothreitol, 500 mM NaCl, 50 mM imidazole, 1% glycerol, 20 mM MgCl$_2$, and either 4 mM or 10 mM ATP (total volume of 500 μl) in a 1-cm plastic cuvette. Light scattering was measured over time at 20°C in an Ultraspec 4000 UV/Vis spectrophotometer (Pharmacia Biotech, Piscataway, NJ) at a wavelength of 360 nm. A water-jacketed cell changer in conjunction with a heater/circulator (Neslab Instruments, Newington, NH) was used to ensure that the temperature was maintained. The data were collected and analyzed by SWIFT software in the kinetics mode (Pharmacia Biotech). Wild-type rLon-1 and mutant rLon-1$^{8714A}$ were also analyzed for their ability to degrade reduced insulin under chaperone assay conditions. These digestions were carried out in 1.5 ml microcentrifuge tubes under the conditions described for the chaperone assay, with the exceptions that 150 μg of insulin was used. Incubation was done for 2 hours at 37°C.

**SDS-PAGE and western blotting**

Tris-glycine SDS-PAGE of recombinant *B. burgdorferi* Lon proteins (1 μg) was conducted as described previously [80] in gels of 12.5% polyacrylamide. For *E. coli* used in complementation experiments, 1 ml of culture was centrifuged and resuspended in 100 μl 1× SDS-PAGE sample buffer, and boiled. Samples (10 μl) were electrophoresed by 12.5% Tris-glycine SDS-PAGE. In all cases, electrophoretic transfer of protein was carried out over a period of 16 hours in 0.01M CAPS buffer, pH 11. For analysis of Lon-1 insulin degradation in the chaperone assay, 100 μl of reaction mixture was added to 50 μl of 3× SDS-PAGE sample buffer and boiled. For each condition, 10 μl was electrophoresed by Tris-tricine SDS-PAGE using a 16.5% separating gel, a 10% spacer gel, and a 4% stacking gel. Equivalent volumes of degraded tagged reporter protein (2.2 μg) and insulin (4.5 μg) from SsrA experiments were analyzed using a 15% Tris-tricine separating gel alone. For direct staining, the gels were fixed in 50% methanol-12% acetic acid then stained with 0.1% Coomassie blue R-250 in 50% methanol-12% acetic acid. For western blotting, Lon-1 and Lon-2 in lysates were detected using rabbit and mouse polyclonal antibodies to Lon. Bands were visualized by use of an infrared scanner (Odyssey Infrared Imaging System, LiCor Biosciences).

**Accession/ID numbers for proteins sequences mentioned in the text**

Swiss-Prot Protein knowledgebase: *Borrelia burgdorferi* Lon-1 (Q9185), *Borrelia burgdorferi* Lon-2 (O51558), *Borrelia recurrentis* Lon-1 (B3RR69), *Borrelia recurrentis* Lon-2 (B3RPV9), *Borrelia duttoni* Lon-1 (B3RL78), *Borrelia duttoni* Lon-2 (B3RMG3), *Borrelia hermsii* Lon-1 (B2RZW3), *Borrelia hermsii* Lon-2 (B2SW00), *Borrelia garinii* Lon-1 (Q662B2), *Borrelia garinii* Lon-2 (Q660R0), *Borrelia afzelii* Lon-1 (Q8SNQ8), *Borrelia afzelii* Lon-2 (Q8SMP4), *Treponema pallidum* Lon (O83536), *Treponema denticola* Lon (Q7PX3), *Leptospira biflexa* (Q0LSL1), *Leptospira borgpetersenii* (Q04VA7), *Leptospira interrogans* (Q72UP9), *Escherichia coli* Lon (P0A9M0), *Bacillus subtilis* Lon (P37945).

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**Author Contributions**

Conceived and designed the experiments: JLC LIK AT NAO JLB. Performed the experiments: JLC LIK CK AT NAO. Analyzed the data: JLC LIK CK AT NAO RT. Contributed reagents/materials/analysis tools: RT. Wrote the paper: JLC LIK JLB.
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