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The ATP-Dependent Lon Protease of *Salmonella enterica* Serovar Typhimurium Regulates Invasion and Expression of Genes Carried on *Salmonella* Pathogenicity Island 1

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An early step in the pathogenesis of *Salmonella enterica* serovar Typhimurium infection is bacterial penetration of the intestinal epithelium. Penetration requires the expression of invasion genes found in *Salmonella* pathogenicity island 1 (SPI1). These genes are controlled in a complex manner by regulators in SPI1, including HilA and InvF, and those outside SPI1, such as two-component regulatory systems and small DNA-binding proteins. We report here that the expression of invasion genes and the invasive phenotype of *S. enterica* serovar Typhimurium are negatively regulated by the ATP-dependent Lon protease, which is known to be a major contributor to proteolysis in *Escherichia coli*. A disrupted mutant of *lon* was able to efficiently invade cultured epithelial cells and showed increased production and secretion of three identified SPI1 proteins, SipA, SipC, and SipD. The *lon* mutant also showed a dramatic enhancement in transcription of the SPI1 genes *hilA*, *invF*, *sipA*, and *sipC*. The increases ranged from 10-fold to almost 40-fold. It is well known that the expression of SPI1 genes is also regulated in response to several environmental conditions. We found that the disruption of *lon* does not abolish the repression of *hilA* and *sipC* expression by high-oxygen or low-osmolarity conditions, suggesting that Lon represses SPI1 gene expression by a regulatory pathway independent of these environmental signals. Since HilA is thought to function as a central regulator of SPI1 gene expression, it is speculated that Lon may regulate SPI1 gene expression by proteolysis of putative factors required for activation of *hilA* expression.

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen that causes gastroenteritis in humans and systemic diseases similar to typhoid in mice. Following oral infection, bacteria colonize the intestinal tract, penetrate the intestinal epithelium, and access systemic sites through the lymphatic and blood circulation systems (8). Passage of *Salmonella* through the intestinal epithelium is thought to be initiated by bacterial invasion into M cells and enterocytes (26, 48). The process of epithelial invasion can be studied experimentally since *S. enterica* serovar Typhimurium invades cultured epithelial cells in vitro. Many of the genes required for epithelial invasion have been found within *Salmonella* pathogenicity island 1 (SPI1), which is a contiguous 40-kb region at centisome 63 of the chromosome (39; for reviews, see references 14 and 23). SPI1 invasion genes encode a bacterial type III secretion apparatus and several effectors which contribute to pathogenesis through an interaction with eukaryotic proteins. The type III apparatus is a multiprotein complex that is thought to build a contiguous channel across both the bacterial and epithelial cell membranes, resulting in efficient translocation of bacterial effectors directly into the cytosol of epithelial cells (for reviews, see references 14 and 23). The secreted effectors are thought to interact with eukaryotic proteins to activate signal transduction pathways and rearrange the actin cytoskeleton and lead to membrane ruffling and engulfment of bacterium.

The expression of SPI1 genes appears to be regulated at several stages in a complex manner by regulators within SPI1, including HilA and InvF, and those outside SPI1, such as the two-component regulators, the flagellum-associated genes, and the small DNA-binding proteins (for a review, see reference 14). HilA, a member of the OmpR/ToxR family, directly binds to and activates the promoter of SPI1 operons and functions as a central regulator of invasion gene expression (31). InvF, an AraC-like transcriptional regulator, promotes expression of HilA-activated effector genes by inducing their transcription from a second, HilA-independent promoter (13). Recently, it has been demonstrated that two transcriptional regulators of SPI1, HilC and HilD, allow the expression of *hilA* by counteracting the action of an unknown repressor (32). Furthermore, *Salmonella* invasion gene expression is known to be regulated by multiple environmental signals, including osmolarity, oxygen tension, pH, and the growth rate of bacteria (6, 17, 22, 30). All of these environmental and regulatory factors appear to regulate invasion gene expression by controlling *hilA* expression (33). Such complex regulations would ensure that invasion genes are appropriately expressed when *Salmonella* infects the host.

Proteolysis plays a key role in prokaryotic and eukaryotic

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties[a]	Reference or source
Strains		
S. enterica serovar Typhimurium		
χ3306	Virulent strain, *gyrA*	53
CS2022	*lon*::Cm in χ3306	This study
χ3306 *phoP*::Km	*phoP*::Km in χ3306	34
CS2099	CS2022 harboring pTKY530	This study
CS2110	*hilA*::Tn*5lacZY* in χ3306	This study
CS2114	*invF*::Tn*5lacZY* in χ3306	This study
CS2116	*sipA*::Tn*5lacZY* in χ3306	This study
CS2118	*sipC*::Tn*5lacZY* in χ3306	This study
CS2130	*hilA*::Tn*5lacZY* in CS2022	This study
CS2134	*sipA*::Tn*5lacZY* in CS2022	This study
CS2136	*sipC*::Tn*5lacZY* in CS2022	This study
CS2138	*invF*::Tn*5lacZY* in CS2022	This study
CS2152	*hilA*::Tn*5lacZY* in χ3306 *phoP*::Km	This study
CS2156	*invF*::Tn*5lacZY* in χ3306 *phoP*::Km	This study
CS2158	*sipA*::Tn*5lacZY* in χ3306 *phoP*::Km	This study
CS2160	*sipC*::Tn*5lacZY* in χ3306 *phoP*::Km	This study
CS2239	CS2022 harboring pMW119	This study
CS2347	χ3306 harboring pMW119	This study
E. coli		
SM10(λ*pir*)	*thri thr leu tonA lacY supE recA*::RP4-2Tc::MuKm λ*pir*	Our collection
DH5α	F$^-$ *recA endA gyrA thi hsdR supE relA* Δ(*lacZYA-argF*) *deoR* ϕ80*lac* (Δ*lacZ*)M15	Our collection
Plasmids		
pTKY229	Carrying *ori*γ of R6K and *oriT* of RP4	Our collection
pNK2884	Carrying a Cm gene	53
pT7Blue-2	Cloning vector	Our collection
pMW119	Low-copy-number plasmid	Our collection
pTKY501	pT7Blue-2 with a 0.5-kb fragment containing a part of *lon* amplified by PCR	This study
pTKY503	pTKY501 carrying *lon*::Cm	This study
pTKY513	pTKY229 with a 1.6-kb *Pst*I-*Hin*dIII fragment carrying *lon*::Cm	This study
pTKY530	pMW119 carrying the *lon* gene	This study

[a] Abbreviations: Cm, chloramphenicol resistance; Km, kanamycin resistance, Tc, tetracycline resistance.

cells by controlling the availability of critical regulatory proteins and removing abnormal and misfolded proteins. In bacteria, most intracellular proteolysis is initiated by four energy-dependent proteases, including Lon, the Clp family (ClpAP and ClpXP), HslUV, and FtsH, which are also known to be stress-induced proteins (for reviews, see references 11, 21, and 41) Among them, Lon is responsible for more than half of all energy-dependent proteolysis in *Escherichia coli* (29, 35). Lon, first isolated from *E. coli*, has been identified in every organism examined thus far, including gram-positive and gram-negative bacteria, yeast, plants, and humans (9, 12). Lon consists of four identical 87-kDa subunits, each having a highly charged N-terminal domain, a centrally located ATP binding domain, and a proteolytically active C-terminal domain (4, 10, 19, 20). In addition to eliminating the irreversibly damaged proteins, Lon appears to perform important functions in the bacterial cell through its ability to degrade proteins that regulate gene expression. For example, Lon participates in the regulation of the SOS response in *E. coli* through its capacity to degrade SulA, an inhibitor of cell division, thereby allowing cells to resume division after the physiological response to DNA damage (46). Furthermore, Lon is involved in the production of capsular polysaccharides by degrading RcsA, which is a transcriptional activator of the biosynthetic genes of capsular polysaccharides (50). Recent studies have also provided evidence that Lon is responsible for the regulation of various pathways in a number of other bacteria. For instance, Lon homologues participate in the morphological development of *Myxococcus xanthus* (49), sporulation in *Bacillus subtilis* (45), the regulation of cell cycle progression in *Caulobacter crescentus* (52), iron acquisition by *Azospirillum brasilense* (40), and lateral flagellar biosynthesis in *Vibrio parahaemolyticus* (47). The role of the Lon protease in connection with bacterial pathogenesis has been demonstrated by the recent report in which the *Brucella abortus lon* homologue was shown to be required for wild-type virulence during the initial stage of infection in the mouse, but it is not essential for the establishment and maintenance of chronic infection in the host (43).

In this study, we provide evidence that the Lon protease of *Salmonella* functions as a negative regulator for controlling the expression of invasion genes on SPI1 and the ability to invade cultured epithelial cells. We have identified a *lon* homologue of *S. enterica* serovar Typhimurium virulent strain χ3306 and constructed a *lon* insertion mutant and characterized it. Consequently, we have found that the disruption of the *lon* gene results in a dramatic increase in the ability to invade Intestine-407 cells, in the secretion of invasion-associated proteins, and in the expression of invasion genes encoded on the SPI1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and genetic techniques. Bacterial strains used in this study are shown in Table 1. To construct the strains carrying the invasion

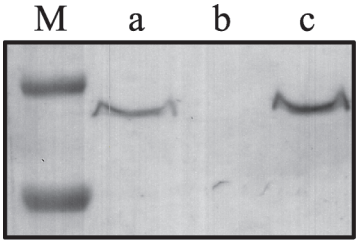

FIG. 1. Immunoblot analysis of proteins from strains CS2347 (wild type) (lane a), CS2239 (*lon*::Cm) (lane b), and CS2099(*lon*::Cm) (lane c) with an antiserum against *E. coli* Lon. Lane M contains molecular mass standards of 94.0 (upper band) and 51.1 (lower band) kDa.

gene-*lacZY* fusion on the chromosome, bacteriophage P22 was propagated on *Salmonella* strains containing *hilA*::Tn*5lacZY*, *invF*::Tn*5lacZY*, *sspA* (*sipA*)::Tn*5lacZY*, and *sspC* (*sipC*)::Tn*5lacZY*, which were kindly provided by C. A. Lee (6), and the resultant lysates were used for infection of strains χ3306, CS2022 (*lon*::Cm), and χ3306 *phoP*::Km. The transductants were selected for linked tetracycline resistance.

Growth conditions. Bacteria were routinely grown in L broth (1% Bacto tryptone [Difco, Detroit, Mich.]–0.5% Bacto yeast extract [Difco]–0.5% sodium chloride [pH 7.4]) and L agar at 37°C. When necessary, the medium was supplemented with chloramphenicol (25 μg/ml), tetracycline (10 μg/ml), ampicillin (25 μg/ml), and/or nalidixic acid (25 μg/ml). For growth under oxygen-limiting conditions, bacterial cells were incubated without agitation overnight until the cultures reached a density of 5×10^8 to 5×10^9 CFU/ml. For growth under aerobic conditions, the overnight culture was diluted 1:1,000 and then incubated with vigorous shaking until the culture reached an optical density at 600 nm (OD_{600}) of 0.5. For growth in high- and low-osmolarity conditions, bacterial cultures were grown in L broth with and without, respectively, 1% NaCl.

DNA techniques. DNA purification, ligation, restriction analysis, and gel electrophoresis were carried out as previously described (54). Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were products of TaKaRa Shuzo Co. (Ohtsu, Japan). PCR was performed with *Taq* polymerase from TaKaRa Shuzo Co.

Construction of a *lon* insertion mutant. To construct the *lon*::Cm mutant CS2022, the DNA fragment between nucleotides (nt) 2922 and 3395 in the *lon* coding region was initially amplified by PCR and cloned into pT7Blue-2. The resultant plasmid, pTKY501, was cleaved at the *Bss*HII sites (nt 3208 and 3210) in the cloned fragment and ligated to the chloramphenicol resistance gene cassette which was generated from *Bam*HI-digested pNK2884 and then filled. The resultant plasmid, pTKY503, was cleaved at the *Pst*I and *Hin*dIII sites in the vector, and the overhanging ends were filled. The generated *lon*::Cm fragment was ligated to the filled-in *Eco*RI site of pTKY229, which is a previously constructed transferable suicide vector (54). The resultant mutator plasmid, pTKY513, was introduced into strain SM10(λ*pir*), which provides the π protein required for the replication of the suicide vector by transformation. The chromosomal *lon* gene was replaced by the *lon*::Cm construct by conjugative crosses as previously described (54). The mutant was selected for resistance to chloramphenicol and nalidixic acid. A double-crossover event resulting in the *lon*::Cm mutant was assessed by its sensitivity to ampicillin. Disruption of the *lon* gene was checked by immunoblotting with anti-*E. coli* Lon serum (Fig. 1).

Cloning *lon* into a low-copy-number plasmid. Synthetic oligonucleotides 2716, which has an *Eco*RI recognition sequence (5′-GCGCAGAATTCTGGCGAATAA-3′), and 5327, which has a *Hin*dIII recognition sequence (5′-CCGAAAAAGCTTGCCAGCCCTG-3′), were used as primers in a PCR to amplify a DNA fragment between nt 2716 and 5327 in the *lon* coding region. The 2,612-bp PCR fragment was cloned into pMW119, which had been digested with *Eco*RI and *Hin*dIII, yielding pTKY530.

Tissue culture growth condition and invasion assay. Intestine-407 cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum. Cells (2×10^5/ml) were seeded into 24-well tissue culture plates to obtain about 90% confluent monolayers on the following day. For bacterial cultures, a single colony was inoculated into L broth and grown to an OD_{600} of 0.5 with shaking at 37°C. Bacterial cells were washed with Hanks' balanced salt solution (HBSS) (Sigma, St. Louis, Mo.) and used to inoculate monolayers previously washed with HBSS at multiplicities of infection ranging from 1 to 10. The monolayers were incubated for 2 h at 37°C, then washed thoroughly with HBSS, and lysed with 0.2% Triton X-100 in phosphate-buffered saline to determine the total number of bacteria associated with the culture cells. Alternatively, to assess the number of intracellular bacteria, the infected tissue culture cells were further incubated for 3 h in Dulbecco's modified Eagle's medium containing gentamicin (100 μg/ml) to eliminate extracellular bacteria before lysis with the Triton X-100 solution. Bacterial numbers were determined by plating the lysates on L agar plates after the appropriate dilution.

SDS-polyacrylamide gel electrophoresis of the secreted proteins. Bacterial cells were grown in 3 ml of L broth to an OD_{600} of 0.5 at 37°C and removed by centrifugation at $10,000 \times g$ for 10 min. The supernatant was filtrated using a Millex-HV filter (Millipore, Bedford, Mass.), then mixed with prechilled trichloroacetic acid (TCA) (final concentration, 10%), chilled on ice for 15 min, and centrifuged at $10,000 \times g$ for 10 min. The pellets were washed once with acetone and suspended in 30 μl of sodium dodecyl sulfate (SDS) sample buffer (28).

Two-dimensional gel electrophoresis of the secreted proteins. For preparation of the protein components secreted into medium, 20 ml of culture, incubated as described above, was centrifuged to remove bacterial cells. The filtrated supernatant was mixed with prechilled TCA (final concentration, 10%), chilled on ice for 15 min, and centrifuged at $10,000 \times g$ for 20 min. The pellets were washed once with 5% cold TCA and then with acetone. The acetone washing was repeated twice to completely remove TCA from the precipitate. The pellet was solubilized in sample buffer containing 8 M urea, 0.5% Nonidet P-40, 10 mM dithiothreitol, and 0.2% Bio-Lyte 3/10 (Bio-Rad, Hercules, Calif.). Isoelectric focusing in the first dimension was performed using a Protean IEF cell (Bio-Rad). Samples were focused in polyacrylamide gels within a pH range of 3 to 10 according to the manufacturer's instructions and resolved in the second dimension on SDS–10% polyacrylamide slab gels. Total proteins were stained with Coomassie brilliant blue.

Immunoblot analysis. Gel electrophoresis was carried out according to the method of Laemmli (28) using SDS–10% polyacrylamide gels. The separated proteins were transferred onto Immobilon-P (Millipore) and then incubated with anti-*E. coli* Lon antibody (1:25,000) followed by alkaline phosphatase-conjugated anti-rabbit immunoglobulin G. The enzymatic reactions were performed in the presence of 0.3 mg of nitroblue tetrazolium (Dojindo, Kumamoto, Japan)/ml and 0.15 mg of 5-bromo-4-chloro-3-indolylphosphate (Amresco, Solon, Ohio)/ml.

Identification of proteins. To determine the N-terminal protein sequence, proteins were separated by two-dimensional gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore). The proteins were stained with Coomassie brilliant blue, cut out, and analyzed with a protein sequencer (Shimadzu PPSQ-21 Protein Sequencer). Alternatively, protein spots of interest were excised from the gel, destained, and digested in situ with endopeptidase Lys-C. After digestion overnight at 37°C, samples were centrifuged and further purified using Zip-TipC18 pipette tips (Millipore). An aliquot of the sample was taken for analysis by matrix-assisted laser desorption ionization (mass spectrometry).

β-Galactosidase assay. β-Galactosidase activity was determined by the method of Miller (37). The enzyme units presented here are averages of at least three independent assays.

RESULTS

Construction of a genetically defined *Salmonella lon* mutant. The *Salmonella lon* homologue encoding a protein 99.0% identical to the Lon protein of *E. coli* (10) was identified downstream of the *clpP*/*clpX* operon in a clone that was previously isolated from the chromosome of *S. enterica* serovar Typhimurium χ3306 (53). The open reading frame for Lon is 2,355 bp long (nt 2921 to 5275) and encodes a peptide of 785 amino acid residues with a predicted molecular mass of 87 kDa. The deduced amino acid sequence of Lon contains a potential consensus serine protease domain DGPSAG (4) at positions 676 to 681 and a possible Walker ATP binding motif, GPPG VGKT and KNPLFLLD, at positions 356 to 363 and 416 to 423, respectively. These characteristics are consistent with the function of Lon as a serine protease which is dependent on ATP hydrolysis for its activity. The *lon* gene was also found to be followed by a homologue of *E. coli hupB* that is 271 bp long (nt 5484 to 5754). In *E. coli*, *hupB* encodes the β-subunit of the histone-like protein HU which binds to and condenses DNA (27).

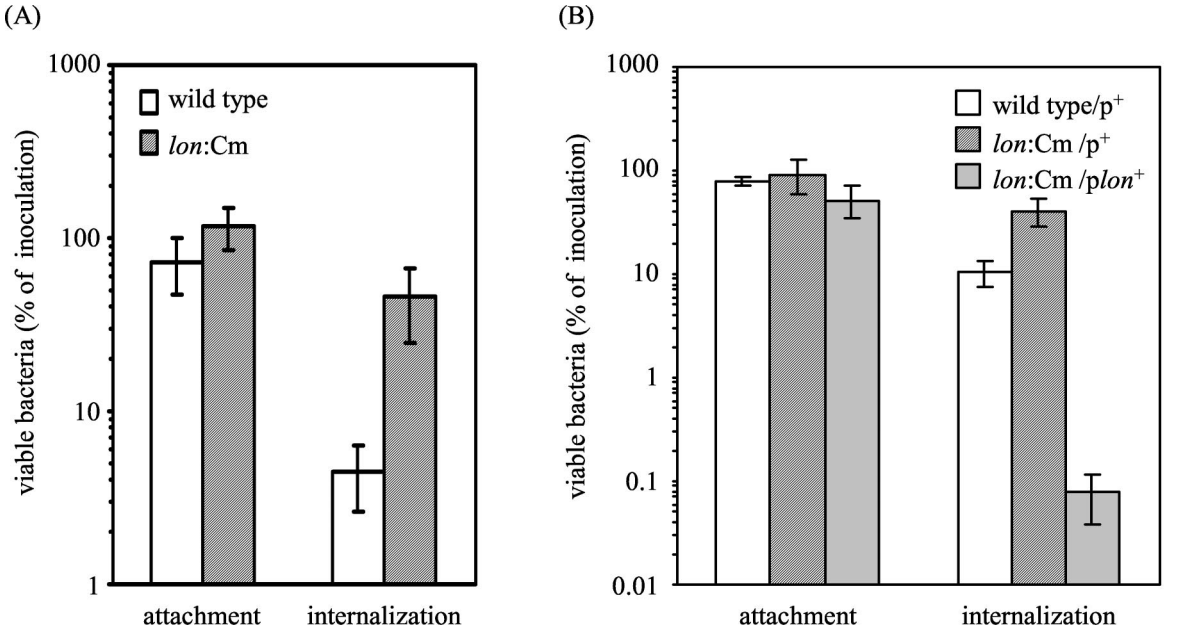

FIG. 2. Effect of *lon* disruption on the ability of *S. enterica* serovar Typhimurium to adhere to and enter cultured Intestine-407 cells (A) and on the suppression of the mutation for invasion by providing functional Lon (B). Bacterial cells of strain χ3306 (wild type), CS2022 (*lon*:Cm), CS2347 (wild type/p^+), CS2239 (*lon*:Cm/p^+), and CS2099 (*lon*:Cm/p*lon*$^+$) for the invasion assay were prepared as described in Materials and Methods. The values represent the means and standard deviations of strains tested in triplicate. p, pMW119; p*lon*, pTKY530.

A genetically defined *lon* mutant was constructed by inserting a chloramphenicol resistance gene into a *lon* gene. The disruption of the *lon* gene was confirmed by immunoblotting analysis in which antiserum specific for the *E. coli* Lon protein failed to recognize a Lon-specific protein in the cell lysate of the mutant strain, whereas a band corresponding to approximately 87 kDa was detected in cell lysates from the wild-type and *lon*::Cm mutant strains carrying the *lon*$^+$ plasmid (Fig. 1). The *lon*::Cm mutant demonstrated a phenotype indicative of a defective stress response. The mutant showed increased sensitivities to UV (similar to that described in a previous report [15]), killing by polymyxin B, and an acidic environment (e.g., pH 3.5) (data not shown). However, the *lon*::Cm mutant did not show temperature sensitivity for growth, that is, the mutant could grow as well as the wild type at 37 and 42°C.

Disruption of the *lon* gene in *S. enterica* serovar Typhimurium markedly stimulates invasion of Intestine-407 cells. Since Lon appears to perform important functions in bacterial cells through its ability to degrade proteins that regulate gene expression (14, 40, 45, 46, 47, 49, 50), we hypothesized that the Lon homologue may have an important role in the expression of pathogenesis of *S. enterica* serovar Typhimurium. To determine whether Lon functions in such a manner, we examined its ability to invade epithelial cells, which is an important initial step in the pathogenesis of *S. enterica* serovar Typhimurium. Cultured Intestine-407 cells were used to assess the ability of the *lon*::Cm mutant to invade in vitro (Fig. 2A). There was a 10-fold increase in the invasion level of the *lon*::Cm mutant compared to that of the parental strain χ3306. On the other hand, the *lon*::Cm mutant adhered to the monolayers of Intestine-407 cells at levels equivalent to those of wild-type bacteria, suggesting that Lon protease does not function at the step of adhesion of *S. enterica* serovar Typhimurium to the epithelial cells but is responsible for the subsequent invasion of the host cells.

Proteome analysis of *Salmonella* proteins secreted into medium. The bacterium's ability to enter nonphagocytic cells is known to be mediated by proteins that are secreted by the SPI1-encoded type III machinery. To know the effect of the *lon* disruption on the expression of these secreted proteins, we analyzed the protein profile secreted by the *lon*::Cm mutant. Compared to that of the wild-type strain, there were increased levels of several proteins in the culture supernatant of the *lon*::Cm mutant (Fig. 3A). To identify the proteins secreted into the medium, we performed amino-terminal sequence analysis and mass spectrometric analysis of the protein spots on the gel after two-dimensional gel electrophoresis. Proteome analysis (Fig. 3B) identified the SipA, SipC, and SipD proteins secreted by the SPI-encoded type III machinery and the FljB, HAP1, HAP2, and HAP3 proteins secreted by the type III machinery involved in flagellum synthesis. SipC (44-kDa protein) itself is known to translocate into cultured epithelial cells, and it directly bundles actin filaments (for reviews, see references 14 and 23). SipA is one of the largest known proteins (74 kDa) and is also an actin-binding protein. SipA and SipC collaborate to generate stable networks of F-actin bundles, leading to the remodeling of the cell actin cytoskeleton and internalization of *Salmonella*. SipD (38-kDa protein) is required for the translocation of effector proteins such as SipA and SipC into the host cells. Significantly higher levels of SipA, SipC, and SipD are secreted in the *lon*::Cm mutant, whereas FljB, HAP1, HAP2, and HAP3 were observed in the mutant cells at the same levels as in the wild-type cells. This result is consistent with the observation that *lon* disruption does not

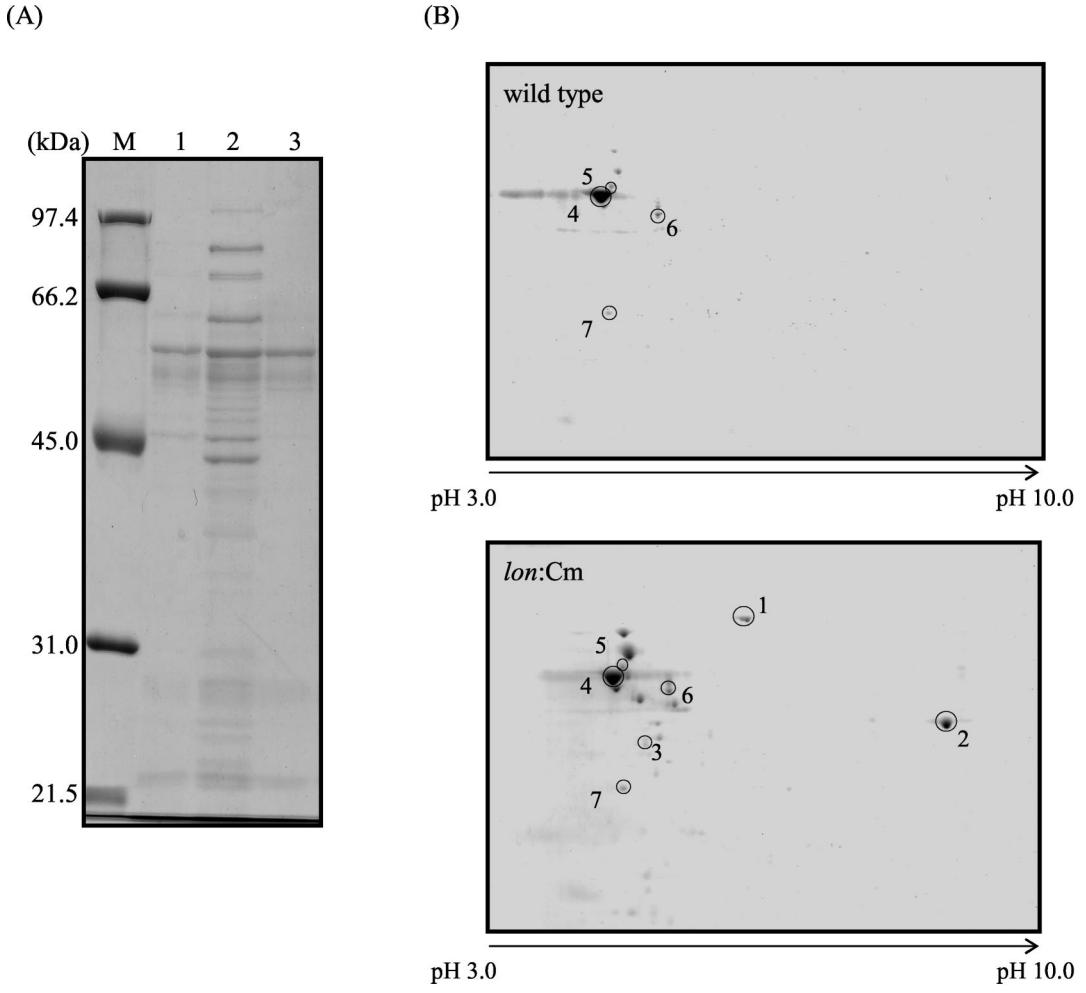

FIG. 3. (A) SDS-polyacrylamide gel electrophoresis patterns of secreted proteins from *S. enterica* serovar Typhimurium strains. The culture supernatants prepared from strains CS2347 (wild type) (lane 1), CS2239 (*lon*::Cm) (lane 2), and CS2099 (*lon*::Cm) (lane 3) were analyzed. Lane M contains molecular mass standards. (B) Two-dimensional gel electrophoresis patterns of the secreted proteins. Protein spots were excised for mass analysis or transferred to a polyvinylidene difluoride membrane for N-terminal amino acid sequence analysis and are discussed in the text. Spots: 1, SipA; 2, SipC; 3, SipD; 4, FljB; 5, HAP1; 6, HAP2; 7, HAP3.

affect flagellum formation or motility of *Salmonella* (our unpublished data). The increased amounts of the secreted effectors SipA, SipC, and SipD account for the enhanced efficiency of the *lon*::Cm mutant to invade Intestine-407 cells.

Effect of *lon* disruption on expression of SPI1 invasion genes in *S. enterica* serovar Typhimurium. The invasiveness of *Salmonella* is thought to be modulated by regulating invasion gene expression. This regulation is mediated by several transcriptional regulators of SPI1, including InvF and HilA (6, 13, 16). To examine whether the enhanced invasion ability of the *lon*::Cm mutant is due to increased expression of the virulence genes on SPI1, we tested the expression of four genes, *hilA*, *invF*, *sipA*, and *sipC*, using chromosomal *lacZY* fusions (6) in the *lon* disruption background. The products of *sipA* and *sipC* are effector proteins described above. The *invF* gene encodes a transcriptional regulator required for the expression of *sipA* and *sipC* (13). The *hilA* gene encodes a regulator that appears to directly activate *invF* expression, therefore activating expression of secreted effector genes *sipA* and *sipC* (6). The *sipA* and *sipC* genes appear to have two promoters, an InvF-dependent promoter and a HilA-dependent promoter (13). Thus, HilA directly and indirectly regulates the expression of the *sipA* and *sipC* genes. In all cases, expression of the fusions was dramatically increased in the *lon*::Cm mutant (Fig. 4A). The increases ranged from 10-fold (for *sipA*) to almost 40-fold (for *invF*, *sipC*, and *hilA*). To examine whether the absence of Lon resulted in a nonspecific effect to stabilize β-galactosidase in the cell, we measured the levels of expression of a gene of flagellar regulation, *fliA*, using *lacZY* fusions with both the *lon*::Cm mutant and wild-type cells. The level of β-galactosidase activity expressed by the *fliA-lacZY* fusion in the *lon*::Cm cells was almost identical to that expressed in the wild-type cells (data not shown), suggesting the specific effect of depletion of Lon on the expression of SPI1 genes demonstrated in the present study. The two-component regulator PhoP/PhoQ is known to repress *hilA* expression (7). We also examined the expression from the promoters of these SPI1 genes in the *phoP* disruption background as a reference (Fig. 4A). In this case, the increases

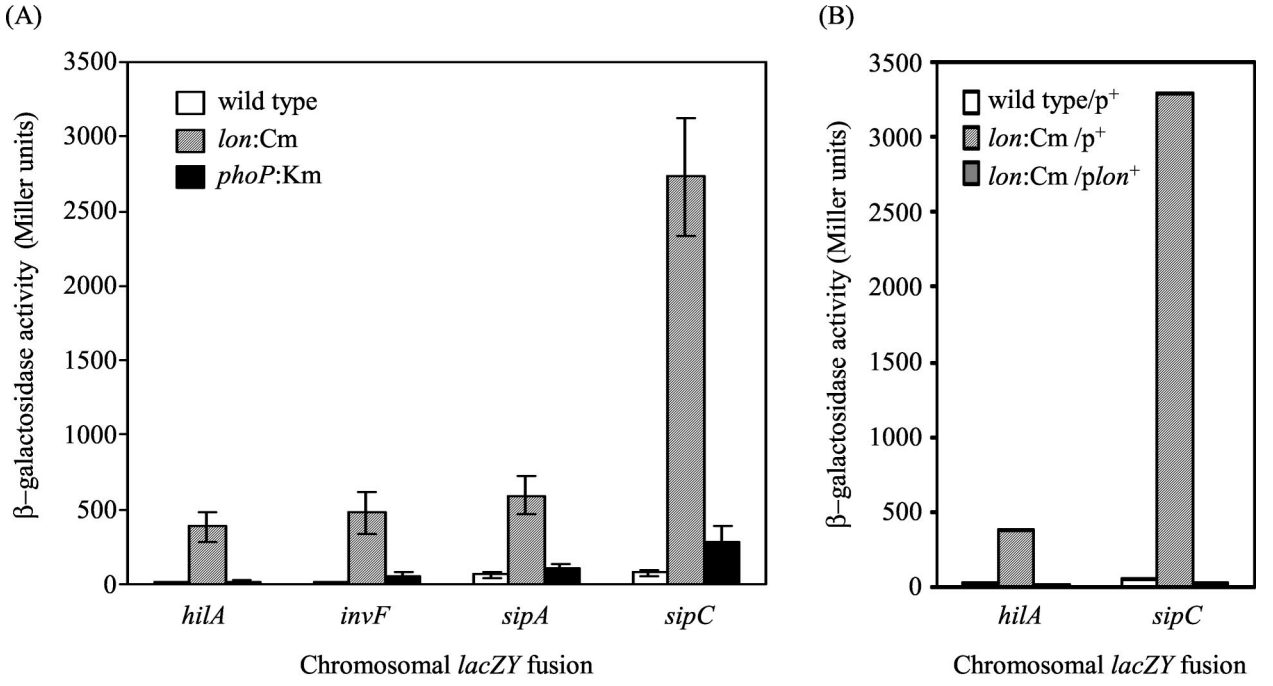

FIG. 4. (A) Effect of *lon* disruption on expression of SPI1 invasion genes. The levels of expression of invasion gene-*lacZY* fusions in either strain χ3306 (wild type) or CS2022 (*lon*:Cm) were assayed for β-galactosidase activity. The levels of activity in the *phoP* disruption background (*phoP*:Km) are also shown. (B) Effect of suppression of *lon* disruption on expression of invasion genes by providing a functional Lon. The levels of expression of *lacZY* fusion to *hilA* or *sipC* in strain CS2347 (wild type/p$^+$), CS2239 (*lon*:Cm/p$^+$), or CS2099 (*lon*:Cm/p*lon*$^+$) were assayed. The values represent the means and standard deviations of samples tested in triplicate. p, pMW119; p*lon*, pTKY530.

in the expression of the SPI1 genes ranged from 1.7-fold (for *hilA* and *sipA*) to 3.8-fold (for *invF* and *sipC*). These results suggest that the expression of SPI1 genes is strongly repressed by the Lon protease in *S. enterica* serovar Typhimurium. Therefore, the enhanced invasion of Intestine-407 cells observed with the *lon*::Cm mutant is due to the increased expression of the SPI1 invasion genes.

Transcomplementation of *lon* disruption. To confirm that the enhanced ability to invade epithelial cells and the increased expression of the SPI1 genes in the strain CS2022 are due to the *lon* disruption, a functional *lon* gene was cloned in a low-copy-number vector, and the resultant plasmid, pTKY530, was introduced into the *lon*::Cm mutant. The resultant strain CS2099 was tested for complementation of the *lon* disruption by measuring the level of invasion of Intestine-407 cells and the β-galactosidase activity expressed from the promoters of virulence genes fused to *lacZY*. The extent of complementation in the *lon*::Cm mutant cells is shown in Fig. 2B and 4B. The enhanced invasion by the *lon*::Cm mutant was fully suppressed by providing a functional copy of *lon* on the low-copy-number vector (Fig. 2B). The increased expression of the *hilA* and *sipC* genes in the *lon*::Cm mutant was also suppressed by providing a functional copy of *lon* (Fig. 4B). Interestingly, the levels for both invasion and expression of the virulence genes in the *lon*::Cm cells complemented in *trans* are lower than those in the wild-type cells. Since overproduction of Lon is generally harmful for bacterial cells, these low levels may be partly explained by the slightly higher amount of Lon protein in cells complemented in *trans* by the low-copy-number plasmid as shown by immunoblotting analysis (Fig. 1).

Disruption of the *lon* gene does not abolish environmental regulation of SPI1 gene expression. It is known that changes in environmental signals, such as oxygen tension, osmolarity, and pH, alter the expression of invasion genes on SPI1 (6, 25). This regulation is mediated by modulating *hilA* expression, although the regulatory pathways appear to act independently of each other (31). The expression of *hilA* is reduced under high-oxygen or low-osmolarity (no salt) conditions (6). To test whether Lon is responsible for the environmental regulation of SPI1 genes, we examined the effects of oxygen tension and osmolarity on the expression of *hilA-lacZY* and *sipC-lacZY* in the *lon*::Cm mutant. As expected, the *lon*::Cm mutant exhibited increased expression of the promoters of *hilA* and *sipC* under both activating (high salt and limited oxygen) and repressing (no salt and high oxygen) conditions compared to expression under the corresponding conditions by wild-type bacteria (Fig. 5). However, both the no-salt and high-oxygen conditions resulted in overall reduced expression of the *hilA* and *sipC* genes in the *lon*::Cm mutant. These results suggest that Lon is responsible for repressing the expression of the SPI1 genes by a regulatory pathway which is independent of the one responsible for repression by high-oxygen or low-osmolarity conditions.

DISCUSSION

Energy-dependent proteolysis plays a key role in prokaryotic and eukaryotic cells by controlling the availability of critical regulatory proteins, ensuring the proper stoichiometry for multiprotein complexes, and helping the rapid elimination of ab-

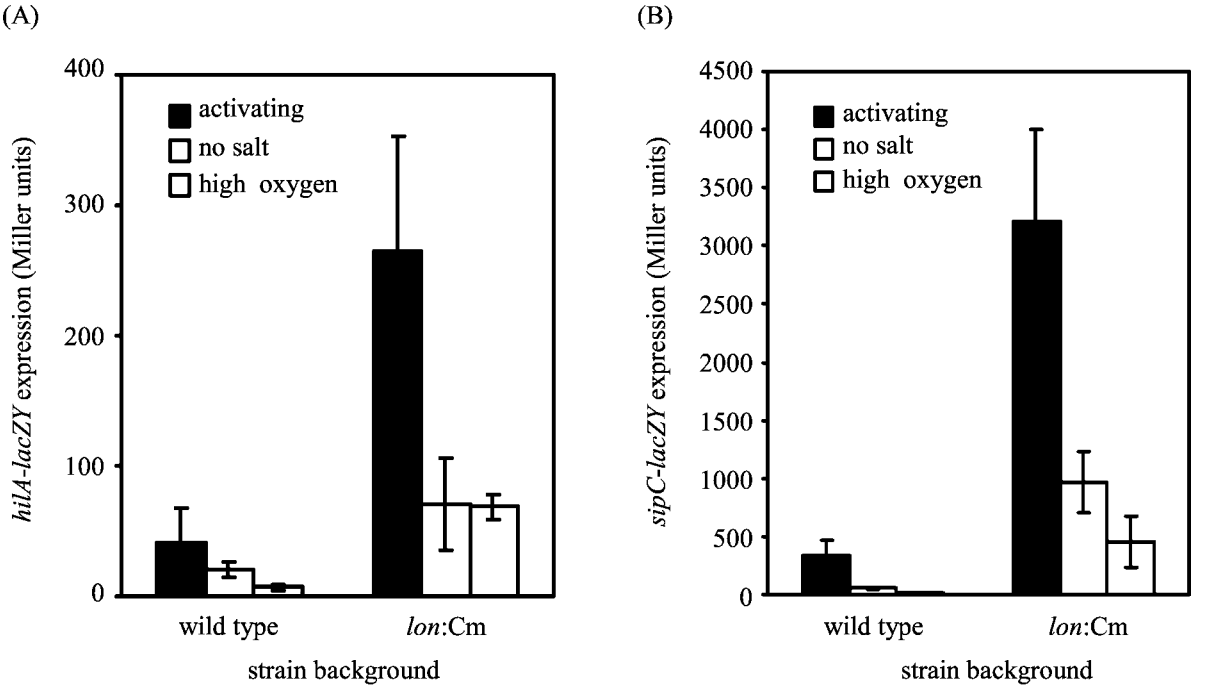

FIG. 5. *lon* disruption does not abolish the regulation of expression of *hilA-lacZY* (A) and *sipC-lacZY* (B) under various environmental conditions. Cultures exposed to activating conditions were grown in high-salt L broth (1% NaCl) under oxygen-limiting conditions. No-salt cultures were grown in L broth without NaCl under oxygen-limiting conditions. High-oxygen cultures were grown in high-salt L broth (1% NaCl) under oxygen conditions. The values are means and standard deviations of averages of at least three independent assays of β-galactosidase activity.

normal proteins (for a review, see reference 36). In *E. coli*, Lon is responsible for more than half of all energy-dependent proteolysis. In the present study, we have demonstrated that depletion of the Lon protease results in the enhanced ability of *S. enterica* serovar Typhimurium to invade cultured epithelial cells. Epithelial invasion by *Salmonella* is thought to be mediated by several effectors secreted by the type III secretory apparatus. During invasion, the secreted effectors are thought to interact with host proteins to rearrange the actin cytoskeleton and cause membrane ruffling and macropinocytosis in the host cell, finally inducing the engulfment of bacteria (for reviews, see references 14 and 23). Therefore, the increased amount of the effectors SipA, SipC, and SipD secreted into the medium by cells from which Lon had been depleted accounts for the enhanced ability of the *lon*-disrupted mutant to invade Intestine-407 cells. The genes of the invasion-associated type III secretion apparatus and the secreted effector proteins encoded by SPI1 are regulated in vitro by several transcriptional factors that may help limit invasion gene expression to the appropriate sites during infection. At the center of invasion gene regulation is *hilA*, which encodes a transcriptional activator homologous to the OmpR/ToxR family of transcriptional activators (5). HilA directly activates promoters of SPI1 operons encoding the type III apparatus and secreted effectors such as SipA, SipB, SipC, and SipD and the transcriptional regulator InvF (33). Since HilA directly modulates *invF* expression, InvF-dependent transcription of effector genes is also regulated indirectly by HilA. Thus, HilA directly and/or indirectly activates the expression of invasion genes on SPI1, thereby playing a central role in the regulatory hierarchy to control invasion-related gene expression.

Many global regulatory networks that are not encoded in SPI1 have been implicated in controlling *hilA* expression. They include several two-component systems such as BarA/SirA (1, 3), EnvZ/OmpR (33), and PhoP/PhoQ (22, 42). Furthermore, the small DNA-binding proteins H-NS, HU, and Fis have been demonstrated to repress *hilA* expression (18, 51), although it is unknown whether these binding proteins modulate *hilA* expression directly or indirectly. The expression of *hilA* also appears to be controlled by several other factors, e.g., CsrA (2) and RNase E (44), which are known to operate at the level of mRNA stability. Other factors activating *hilA* expression include a positive regulator for the class 2 flagellar operon, FliZ, and an acyl coenzyme A synthetase, FadD, although the mechanisms whereby these factors influence *hilA* expression remain unclear (33). Another potential repressor of *hilA* expression is a protein called HilE, which lacks homology to any known protein (18). Our result is the first evidence that *hilA* expression is strongly repressed by the ATP-dependent protease Lon.

The expression of SPI1 genes is also tightly regulated in *Salmonella* in response to environmental conditions such as oxygen tension, pH, osmolarity, and growth phase (6, 17, 30). Much of the response to these conditions is mediated by HilA. The expression of *hilA* and invasion genes is severely repressed by high oxygen or low osmolarity (6). In the *lon* insertion mutant, the expression of *hilA* and *sipC* is still reduced under high-oxygen or low-osmolarity (no salt) conditions (Fig. 5). Thus, the pathway by which Lon regulates *hilA* expression is

distinct from that mediating environmental regulation of *hilA* expression.

Recently, two SPI1-encoded factors, HilC and HilD, which derepress *hilA* expression, were identified and shown to be members of the AraC/XylS family of transcriptional regulators and to independently bind to these upstream repressing sequences to counteract the repression of *hilA* expression (32). It is not likely that Lon directly binds to the upstream repressing sequences in order to repress *hilA* expression, since it has not been shown to have DNA binding activity. It is likely that Lon may function through its proteolytic activity to regulate the availability of HilC and/or HilD. Furthermore, Lon may be involved in repressing the activity of positive transcriptional regulators encoded outside SPI1 such as BarA/SirA and/or EnvZ/OmpR. The possibility that Lon targets FliZ to repress *hilA* expression can be excluded because *lon* disruption does not affect the production of flagellum-related proteins whose expression is activated by FliZ (Fig. 3).

Two transcriptional regulators specifically targeted by the Lon protease, RcsA and StpA, have been identified. RcsA is a positive regulator of transcription of the *cps* genes necessary for capsule synthesis (50). StpA is a paralogue of the H-NS protein, which is a major component of the bacterial DNA-binding protein, and it stimulates the expression of stringently regulated genes by its effect on local DNA topology (24). Many studies have revealed several distinct pathways that can modulate the ability of *Salmonella* to express *hilA* and invade host cells. Integration of signals from these different pathways may help restrict invasion gene expression during infection. To understand how the regulation of *hilA* expression by Lon might be achieved, genetic and biochemical studies to determine molecules targeted by Lon are in progress.

Besides SPI1, which is required for efficient invasion of the intestinal epithelium, SPI2, which encodes the ability to interfere with bactericidal mechanisms in the macrophage after phagocytosis, is essential for *Salmonella* virulence. While most of the regulatory factors control expression of only one set of virulence factors (i.e., invasion genes), it has been demonstrated that some regulatory pathways modulate the expression of more than one set of virulence factors. One example of this is the model for the regulation of virulence genes by the PhoP/PhoQ system, in which two virulence properties of *Salmonella*, epithelial invasion and survival within macrophages, are oppositely modulated (7, 38). We recently found that the *lon* disruption attenuates the virulence of *S. enterica* serovar Typhimurium in mice after infection by both oral and peritoneal routes, suggesting that Lon is essentially required for systemic *Salmonella* infection in mice (unpublished data). Like the PhoP/PhoQ regulatory system, Lon may oppositely modulate two major contributors, epithelial invasion and survival within macrophages, for the expression of pathogenicity. Future studies must focus on determining how the regulation of invasion and the survival of bacteria to cause systemic infection are achieved in *Salmonella* by the Lon protease.

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