

Coordinated Regulation of Expression of *Salmonella* Pathogenicity Island 1 and Flagellar Type III Secretion Systems by ATP-Dependent ClpXP Protease[∇]

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Received 25 August 2007/Accepted 11 January 2008

Salmonella enterica serovar Typhimurium delivers a variety of proteins via the *Salmonella* pathogenicity island 1 (SPI1)-encoded type III secretion system into host cells, where they elicit several physiological changes, including bacterial invasion, macrophage apoptosis, and enteropathogenesis. Once *Salmonella* has established a systemic infection, excess macrophage apoptosis would be detrimental to the pathogen, as it utilizes macrophages as vectors for systemic dissemination throughout the host. Therefore, SPI1 expression must be restricted to one or a few specific locations in the host. In the present study, we have demonstrated that the expression of this complex of genes is repressed by the ATP-dependent ClpXP protease, which therefore suppresses macrophage apoptosis. Depletion of ClpXP caused significant increases in the amounts of two SPI1-encoded transcriptional regulators, HilC and HilD, leading to the stimulation of *hilA* induction and therefore activation of SPI1 expression. Our evidence shows that ClpXP regulates cellular levels of HilC and HilD via the control of flagellar gene expression. Subsequent experiments demonstrated that the flagellum-related gene product FlhZ controls HilD posttranscriptionally, and this in turn activates HilC. These findings suggest that the ClpXP protease coregulates SPI1-related virulence phenotypes and motility. ClpXP is a member of the stress protein family induced in bacteria exposed to hostile environments such as macrophages.

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen that causes gastroenteritis in humans and systemic diseases similar to typhoid fever in mice. Specific virulence factors encoded within the *Salmonella* pathogenicity islands (SPIs) are required for the development of salmonellosis (20, 24, 37, 43). One SPI, SPI1, encodes a type III secretion system with a multiprotein secretion apparatus, termed a needle complex, as well as effector proteins that are injected into the host cell cytoplasm via this apparatus (19, 32, 33, 56, 67). Various functions have been attributed to the SPI1 type III secretion system, including actin rearrangement, which causes the bacterium to be engulfed (9, 18, 47); macrophage apoptosis (7, 26, 30); and enteropathogenesis (65). After invasion of intestinal epithelial cells, *Salmonella* is able to disseminate to any tissue. It propagates within macrophages, a process that requires a second type III secretion system encoded on SPI2. Once *Salmonella* has established a systemic infection, excess macrophage apoptosis would be detrimental. During this systemic phase, the organisms need to repress and delay the onset of apoptosis to allow sufficient time for them to replicate, escape, and invade new macrophages. Thus, SPI1 gene expression must be suppressed during the systemic phase of infection. We have previously reported that a member of the ATP-dependent protease family, Lon, negatively regulates SPI1 gene expression (58, 60) and that this down-regulation is quite important for suppressing SPI1-dependent apoptosis suf-

ficiently to allow time for *Salmonella* to replicate within the macrophages (57). It has been demonstrated that Lon is also important for down-regulating SPI1 gene expression in epithelial cells (5).

ATP-dependent proteolysis functions as a precise regulatory mechanism that limits the availability of key enzymes or critical regulatory proteins controlling gene expression. In addition to Lon, four ATP-dependent proteases, i.e., ClpAP, ClpXP, HslVU, and FtsH, have been found in gram-negative bacteria (for a review, see references 22 and 42). These are collectively called AAA⁺ (ATPases associated with diverse cellular activities plus) proteases (46). ClpXP is a bipartite protease responsible for degrading certain key regulatory proteins and aberrant translation products bearing the SsrA degradation tag, which is added cotranslationally to nascent polypeptides when ribosomes stall (16, 21, 23, 61). The ClpP component of ClpXP consists of two stacked heptameric rings, which enclose a central chamber containing the proteolytic active site. The ClpX component is a hexameric-ring ATPase that binds substrate proteins, denatures them, and translocates the unfolded polypeptides into the ClpP degradation chamber (48).

In the present study, we demonstrate that ClpXP negatively regulates the expression of SPI1 and hence suppresses macrophage apoptosis and the invasion of epithelial cells. The control of expression of the SPI1 type III secretion system is complex: several transcriptional regulators are present within the island. Among these, the master regulator is HilA, a member of the OmpR/ToxR family of transcriptional regulators, which can activate expression either directly or indirectly by increasing the expression of another regulator, InvF (2, 31, 37, 39). InvF, in a complex with the chaperone protein SicA, induces expression of the *sic/sip* operon (11). Two homologous proteins, HilC and HilD, both members of the AraC/XylS

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[∇] Published ahead of print on 1 February 2008.

family of transcriptional regulators, induce *hilA* expression (12, 51, 53). HilC and HilD can each individually bind to the DNA immediately upstream of *hilA*, and it is believed that this binding leads to *hilA* expression (12, 52, 53). It is further suggested that HilD acts as a direct activator of *hilA* (6), as opposed to a derepressor as previously assumed (52, 53). It has also been demonstrated that HilC and HilD each induce the expression of the other as well as inducing their own promoters (40, 47). Recent data have shown that loss of HilD decreases *hilC* transcription but that loss of HilC does not significantly alter *hilD* expression (13, 15), suggesting that HilD may be at the top of the hierarchy of regulation of SPI1 expression. In addition to these SPI1-encoded regulators, genetic studies have identified many regulatory proteins encoded outside the island. One such regulator, Lon, has been shown to degrade both HilC and HilD, thus down-regulating SPI1 gene expression (58).

The structure of the SPI1 type III secretion apparatus is similar to the basal structure of the flagellum (4). Assembly of a flagellum requires the export of protein subunits from the cytoplasm to the outer surface of the cell by a mechanism that resembles those of secretion systems (4, 27). Furthermore, many components of the flagellar export apparatus share a high degree of homology with those of the type III secretion machinery. These findings suggest that these two type III machineries have a common evolutionary origin. We have previously reported that ClpXP negatively regulates flagellum biogenesis by controlling the turnover of the FlhD/FlhC complex, which functions as a master regulator at the apex of the transcription hierarchy of the flagellar regulon (61, 62).

Here, we have studied the mechanism by which ClpXP negatively controls SPI1 gene expression. The present results, coupled with our previous results (61, 62), indicate that ClpXP coregulates the expression of two different type III secretion systems, SPI1 related and flagellum related.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All strains used in this study are derivatives of *S. enterica* serovar Typhimurium χ 3306 and are listed in Table 1. Plasmids pTKY559 and pTKY562 carry a *hilC* promoter-*lacZ* fusion and a *hilD* promoter-*lacZ* fusion, respectively (60). Plasmid pTKY651 carries a $P_{AllacO-1}$ promoter-*hilD* fusion (60).

To construct the *fliZ* disruption mutants, P22 phages were propagated on KK1397 (34) to transduce the *fliZ::Km* mutation. The lysate was used for infection of *S. enterica* serovar Typhimurium strains, and the transductants were selected by kanamycin resistance. Bacterial cells were routinely grown in L broth (1% Bacto tryptone [Difco, Detroit, MI], 0.5% Bacto yeast extract [Difco], 0.5% sodium chloride [pH 7.4]) or on L agar. The media were supplemented with chloramphenicol (25 μ g ml⁻¹), ampicillin (25 μ g ml⁻¹), kanamycin (25 μ g ml⁻¹), and/or nalidixic acid (25 μ g ml⁻¹) when necessary. Bacterial cells were grown aerobically in L broth.

DNA techniques. DNA purification, ligation, restriction analysis, PCR amplification, DNA sequencing, and agarose gel electrophoresis were carried out as previously described (66).

Macrophage infection. Cultured RAW264.7 macrophages were seeded onto a 24-well plate at a density of 2×10^5 cells per well and incubated overnight at 37°C. Prior to infection, the macrophages were washed with Hanks balanced salt solution (HBSS) and challenged with *Salmonella* strains at a multiplicity of infection of 10. The plates were centrifuged for 5 min at $500 \times g$ to enhance and synchronize infection, followed by incubation for 30 min at 37°C to permit phagocytosis. The free bacteria were removed by three washes with HBSS. Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum and 100 μ g of gentamicin per ml was added, and the cells were incubated for 1.5 h at 37°C. The cells were washed with HBSS three times, followed by

TABLE 1. Bacterial strains used in this study

Strain	Relevant properties ^a	Reference
χ 3306	Virulent wild type	25
CS2007	<i>clpP::Cm</i> in χ 3306	66
CS2110	<i>hilA::Tn5 lacZY</i> in χ 3306	60
CS2120	<i>hilA::Tn5 lacZY</i> in CS2007	This study
CS2462	<i>fliZ::Km</i> in χ 3306	This study
CS2464	<i>fliZ::Km</i> in CS2007	This study
CS2609	<i>flhD::Tn10</i> in χ 3306	61
CS2610	<i>flhD::Tn10</i> in CS2007	61
CS2724	Δ <i>hilC</i> in χ 3306	60
CS2725	Δ <i>hilD</i> in χ 3306	60
CS2732	<i>hilA::Tn5 lacZY</i> in CS2724	60
CS2733	<i>hilA::Tn5 lacZY</i> in CS2725	60
CS2802	Δ <i>hilC</i> Δ <i>hilD</i> in χ 3306	60
CS2815	<i>hilA::Tn5 lacZY</i> in CS2802	60
CS3222	<i>hilA::Km</i> in χ 3306	This study
CS3319	<i>clpP::Cm</i> in CS2732	This study
CS3320	<i>clpP::Cm</i> in CS2733	This study
CS3321	<i>clpP::Cm</i> in CS2815	This study
CS3322	<i>clpP::Cm</i> in CS2724	This study
CS3325	<i>clpP::Cm</i> in CS2725	This study
CS3328	<i>clpP::Cm</i> in CS2802	This study
CS3329	<i>fliZ::Km</i> in CS2802	This study

^a Cm, chloramphenicol resistance; Km, kanamycin resistance.

incubation with DMEM containing 10% fetal bovine serum and 10 μ g of gentamicin per ml at 37°C.

Detection of histone-associated cytoplasmic DNA fragments after *Salmonella*-induced macrophage cell death. At 6 h after infection, the macrophages were washed with HBSS and treated with 0.5 ml of lysis buffer for 30 min at room temperature. The lysates were centrifuged at $200 \times g$ for 10 min at 4°C, and a 0.3-ml portion of the supernatant was taken for further assay. The samples were diluted 1:100, and the cytoplasmically located histones bound to fragmented DNA were quantified colorimetrically using Cell Death Detection ELISA^{PLUS} (Roche Diagnostics).

Assay for invasion of epithelial cells. Intestine-407 cells were invaded by *Salmonella* in 24-well tissue culture plates as described previously (58, 59) with some modifications. Bacterial cells were washed with HBSS and used to inoculate monolayers previously washed with HBSS, at a multiplicity of infection of 10. The monolayers were incubated for 2 h at 37°C and 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 cultured cells. Alternatively, to assess the number of intracellular bacteria, the infected tissue culture cells were further incubated for 3 h in DMEM containing 100 μ g of gentamicin per 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 appropriate dilution.

Collection of whole-cell proteins and secreted proteins. Bacterial cells were grown aerobically in 10 ml of L broth to an optical density at 600 nm of 1.0 at 37°C. To prepare whole-cell proteins, bacterial cells were harvested by centrifuging 1 ml of the culture and then suspended in 200 μ l of sample buffer (36). Cell lysates (20 μ l) were applied to each lane of a sodium dodecyl sulfate (SDS)-polyacrylamide gel. To prepare the proteins secreted into the medium, 7 ml of the same culture was centrifuged to remove cells. The filtered supernatant was mixed with prechilled trichloroacetic acid (final concentration, 10%), chilled on ice for 15 min, and centrifuged at $10,000 \times g$ for 20 min. The pellet was washed once with acetone and then solubilized in 70 μ l sample buffer (36). The sample (15 μ l) was loaded on each lane and subjected to immunoblotting analysis.

Immunoblot analysis. Equivalent numbers of bacterial cells were suspended in sample buffer (36), boiled for 5 min, and subjected to SDS-10% polyacrylamide gel electrophoresis. Separated proteins on the gels were transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). Proteins were reacted with rabbit anti-SipB serum (1:12,500), anti-SipC serum (1:12,500), anti-HilA serum (1:12,500), anti-HilC serum (1:12,500), and anti-HilD serum (1:12,500), followed by alkaline phosphatase-conjugated anti-rabbit immunoglobulin G as the secondary antibody. The enzymatic reactions were performed in the presence of 300 μ g ml⁻¹ of nitroblue tetrazolium (Dojin, Kumamoto,

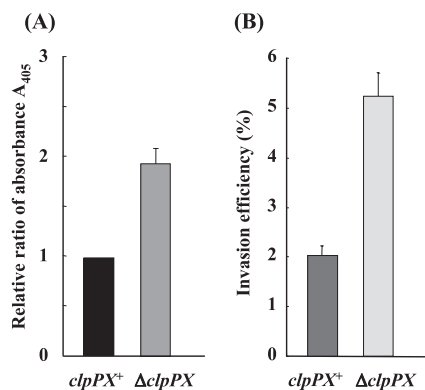


FIG. 1. (A) Levels of cytoplasmic nucleosomal fragments in macrophages infected with *S. enterica* serovar Typhimurium strains χ 3306 (*clpPX*⁺) and CS2007 (Δ *clpPX*). RAW264.7 cells were infected with bacteria at a multiplicity of infection of 10. At 6 h after infection, the levels of histone-associated cytoplasmic DNA fragments were measured by quantitative enzyme-linked immunosorbent assay using an antihistone antibody as described in Materials and Methods. The data are the means and standard derivations for each strain tested in triplicate. (B) Invasion efficiency of strains χ 3306 (*clpPX*⁺) and CS2007 (Δ *clpPX*). Cultured Intestine-407 cells were used to assess invasiveness as described in Materials and Methods. The data are the means and standard derivations for each strain tested in triplicate. Invasion efficiency was expressed as the ratio of the number of intracellular bacteria to the number of bacteria adhering to the cultured cells.

Japan) and 150 μ g ml⁻¹ of bromochloroindolylphosphate (Amresco, Solon, OH). Anti-HilA, -HilC, -HilD, and -SipC antisera were previously established in our laboratory (60).

β -galactosidase assay. The activity of β -galactosidase was determined by the method of Platt et al. (49). The enzyme units presented here are the averages from at least three independent assays.

RESULTS

Depletion of the ClpXP protease in *S. enterica* serovar Typhimurium stimulates the induction of apoptosis in macrophages.

In a previous study, we characterized the genomic organization of the *clpP* region of *S. enterica* serovar Typhimurium pathogenic strain χ 3306, *clpP-clpX-lon*, and constructed an insertional mutation in the *clpP* gene (66). The insertion of a chloramphenicol resistance cassette into *clpP* resulted in a polar mutation in *clpX*, which together with *clpP* constitutes an operon. However, the insertion evidently did not block the expression of *lon* preceded by its own promoter, which is downstream of *clpX*, because anti-Lon antiserum detected the corresponding protein in Δ *clpPX* mutant cells (66). Disruption of the *clpP* gene did not affect the cell growth rate (62, 66). During characterization of the virulence phenotype of the Δ *clpPX* mutant, we found cytoplasmic histone-associated DNA fragments, which constitute a marker for apoptotic cells (Fig. 1A), indicating that the mutant induces rapid and excessive macrophage apoptosis (6 h after infection). Rapid macrophage apoptosis by *Salmonella* infection is known to be mediated by an SPI1-encoded protein, SipB (26). Although *Salmonella* also induces SPI2-dependent, SPI1-independent apoptosis of macrophages, the execution of SPI2-dependent apoptosis is considerably delayed (18 to 24 h after infection) and is masked when the SPI1 genes are expressed (44, 64). Another aspect of SPI1-related virulence, the invasion of epithelial cells, was as-

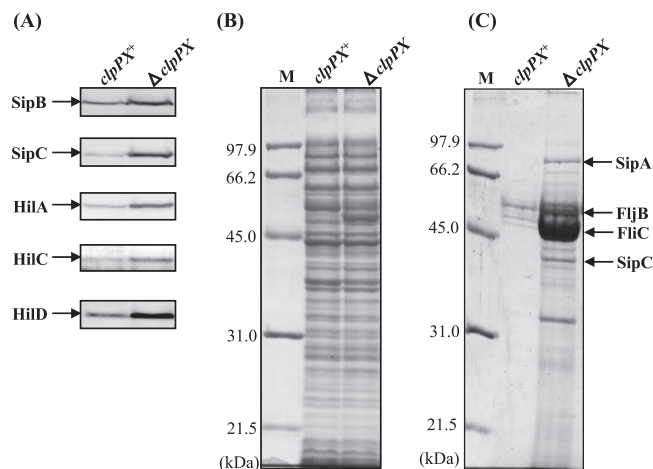


FIG. 2. Effect of depletion of ClpXP protease on cellular levels of various SPI1 proteins. Bacterial cells of strains χ 3306 (*clpPX*⁺) and CS2007 (Δ *clpPX*) were used. (A) Immunoblotting analysis of the cellular lysates using anti-SipB, anti-SipC, anti-HilA, anti-HilC, and anti-HilD sera. (B) Coomassie brilliant blue-stained SDS-10% polyacrylamide gel electrophoretic patterns of the same samples used for immunoblotting. Lane M contains the molecular mass standard. (C) Coomassie brilliant blue-stained gel patterns of the proteins secreted into the medium. The proteins were prepared as described in Materials and Methods.

essed in both the Δ *clpPX* mutant and the parental cells using cultured Intestine-407 cells (Fig. 1B). The level of invasion by Δ *clpPX* was significantly higher than that of invasion by the parental *clpPX*⁺ strain, suggesting that ClpXP is involved in the control of *Salmonella* invasiveness.

ClpXP negatively regulates the expression of the SPI1 regulon.

The enhanced ability of Δ *clpPX* to induce macrophage apoptosis and to invade epithelial cells suggested that *clpPX* deletion results in increased expression of SPI1 proteins. To examine this possibility, we initially used immunoblotting to compare the cellular levels of SipB and SipC, which are known to translocate into epithelial cells, in Δ *clpPX* cells and the isogenic *clpPX*⁺ cells. SipB is necessary and sufficient for the induction of rapid macrophage apoptosis (26). SipC itself is known to cause bundling of actin filaments directly (19). The amounts of SipB and SipC in Δ *clpPX* cells were apparently higher than those in the *clpPX*⁺ cells (Fig. 2A). We have previously identified the SPI1 proteins SipA and SipC by mass spectrometry of secreted proteins separated by SDS-polyacrylamide gel electrophoresis (58). The Coomassie blue-stained gel patterns of the secreted proteins indicate that significantly higher levels of SipA and SipC are secreted in the Δ *clpPX* mutant (Fig. 2C). The 50-kDa band that accumulates in the Δ *clpPX* strain (Fig. 2B) is a flagellin protein, FliC, as previously identified (62). *S. enterica* serovar Typhimurium expresses two antigenically distinct flagellins encoded by *fliC* and *fliB*, and the alternative expression of these two genes results in an oscillation of phenotype known as phase variation, which occurs with frequencies ranging from 10⁻³ to 10⁻⁵ per bacterium per generation (55). Mass spectrometry demonstrated that cultures of strain χ 3306 (*clpPX*⁺) include both the FliC and FljB proteins (62). The profile of secreted proteins shows that FliC and FljB are greatly increased by the disruption of *clpPX* (Fig. 2C). We then examined the cellular levels of HilA, a transcriptional

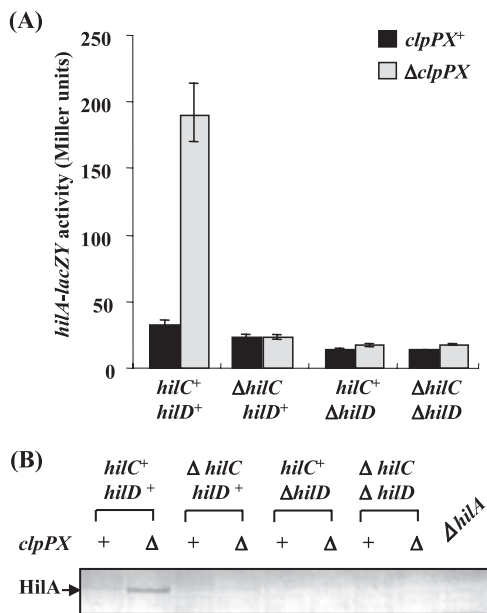


FIG. 3. Effect of depletion of ClpXP protease on the expression of the *hilA* gene in the absence of the *hilC* and/or *hilD* gene. (A) The expression levels of a *lacZ* fusion to the *hilA* promoter were assayed. The values represent the means and standard deviations for samples tested at least in triplicate. The strains used were CS2110 (*clpPX⁺ hilC⁺ hilD⁺*), CS2120 (Δ *clpPX hilC⁺ hilD⁺*), CS2732 (*clpPX⁺ Δ hilC hilD⁺*), CS3319 (Δ *clpPX Δ hilC hilD⁺*), CS2733 (*clpPX⁺ hilC⁺ Δ hilD*), CS3320 (Δ *clpPX hilC⁺ Δ hilD*), CS2815 (*clpPX⁺ Δ hilC Δ hilD*), and CS3321 (Δ *clpPX Δ hilC Δ hilD*). (B) Immunoblotting analysis of the cellular lysates using anti-HilA serum. The strains used were χ 3306 (*clpPX⁺ hilC⁺ hilD⁺*), CS2007 (Δ *clpPX hilC⁺ hilD⁺*), CS2724 (*clpPX⁺ Δ hilC hilD⁺*), CS3322 (Δ *clpPX Δ hilC hilD⁺*), CS2725 (*clpPX⁺ hilC⁺ Δ hilD*), CS3325 (Δ *clpPX hilC⁺ Δ hilD*), CS2802 (*clpPX⁺ Δ hilC Δ hilD*), CS3328 (Δ *clpPX Δ hilC Δ hilD*), and CS2110 (Δ *hilA*).

activator with a central role in the regulatory hierarchy of SPI1 gene expression. The results show that the level of HilA is greatly increased in Δ *clpPX* cells. Two SPI1 gene products, HilC and HilD, have been shown to bind to the upstream repressing sequence of *hilA* to induce its transcription (12, 52, 53). In addition, overproduction of HilC and HilD has been shown to increase *hilA* expression significantly (13, 40). It is suggested that HilD directly activates *hilA* transcription (6). Therefore, the amounts of HilC and HilD in Δ *clpPX* cells were compared with those in the *clpPX⁺* cells. The levels of both HilC and HilD were significantly increased by disruption of *clpPX*, suggesting that the ClpXP protease may down-regulate *hilA* transcription by a mechanism dependent on both HilC and HilD.

To examine this possibility, the effect of *clpPX* disruption on the expression of *hilA* was examined by use of a chromosomal *lacZY* fusion in strains with Δ *hilC* and/or Δ *hilD* backgrounds. As shown in Fig. 3A, disruption of *clpPX* increased *hilA* transcription approximately sixfold in *hilC⁺ hilD⁺* cells. In contrast, the stimulation of *hilA* expression in Δ *clpPX* cells was completely suppressed by introducing the Δ *hilC* and/or Δ *hilD* mutation. The results of immunoblotting of cell lysates prepared from various strains with the mutation backgrounds used for transcriptional analysis (Fig. 3A) are shown in Fig. 3B. The apparent levels of HilA are consistent with the transcriptional

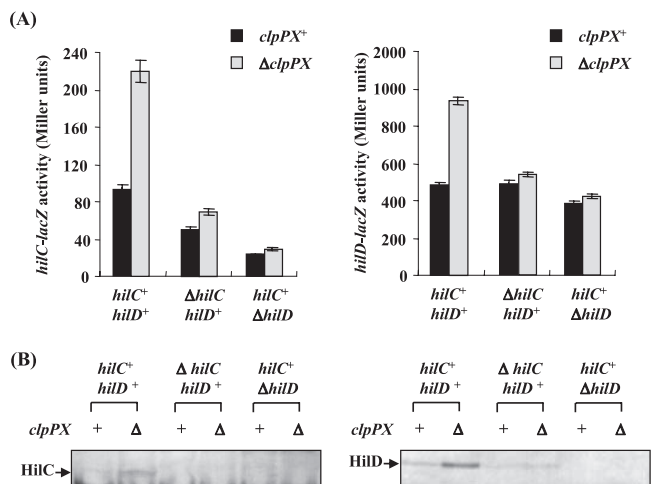


FIG. 4. Effect of depletion of ClpXP protease on the expression of the *hilC* and *hilD* genes in the absence of *hilC* or *hilD* gene. (A) The expression levels of *lacZ* fusions to *hilC* and *hilD* promoters were assayed. The values represent the means and standard deviations for samples tested at least in triplicate. Plasmid pTKY559 (*hilC* promoter-*lacZ* fusion) or pTKY562 (*hilD* promoter-*lacZ* fusion) was introduced into bacterial strains χ 3306 (*clpPX⁺ hilC⁺ hilD⁺*), CS2007 (Δ *clpPX hilC⁺ hilD⁺*), CS2724 (*clpPX⁺ Δ hilC hilD⁺*), CS3322 (Δ *clpPX Δ hilC hilD⁺*), CS2725 (*clpPX⁺ hilC⁺ Δ hilD*), and CS3325 (Δ *clpPX hilC⁺ Δ hilD*). The resultant strains were used for determination of β -galactosidase activity. (B) Immunoblotting of the cellular lysates using anti-HilC and anti-HilD sera. The strains used were χ 3306, CS2007, CS2724, CS3322, CS2725, and CS3325.

analysis results. That is, the amount of HilA in the Δ *clpPX* mutant cells was larger than that in *clpPX⁺* cells, and the accumulation of HilA in Δ *clpPX* cells was suppressed by introducing the *hilC* and/or *hilD* mutation. These results strongly suggest that ClpXP regulates *hilA* transcription through a pathway dependent on both HilC and HilD.

We then examined the effect of *clpPX* disruption on the transcription from *hilC* and *hilD* promoters using *lacZ* fusion on plasmids. As shown in Fig. 4A, disruption of *clpPX* increased *hilC* transcription 2.6-fold and *hilD* transcription 1.9-fold. HilC and HilD each activate the expression of the other as well as inducing their own promoters (39, 47), so we then measured transcription from the *hilC* and *hilD* promoters on plasmids in *hilC*- or *hilD*-disrupted backgrounds on the chromosome. The results show that the enhanced *clpPX* disruption effect on the transcription from *hilC* and *hilD* promoters was abolished by introducing either Δ *hilC* or Δ *hilD* mutations on the chromosome. The immunoblotting analyses show that the increased *clpPX* disruption effect on cellular levels of HilC and HilD disappeared when either Δ *hilD* or Δ *hilC* was introduced (Fig. 4B). Together, these findings suggest that the both HilC and HilD need to be accumulated to cause a significant stimulation of *hilA* expression by *clpPX* disruption.

ClpXP regulates SPI1 gene expression via control of flagellar gene expression. How does ClpXP control the levels of HilC and HilD? Our previous finding that it negatively regulates the expression of the flagellar regulon by controlling the turnover of the master regulator, FlhD/FlhC (62), allowed us to hypothesize that it may regulate HilC and HilD by controlling flagellar gene expression. In *S. enterica* serovar Typhi-

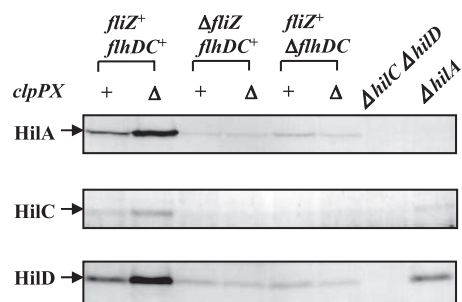


FIG. 5. Effects of disruption of *fliZ* or *flhDC* on enhancement of cellular levels of SPI1 regulator proteins by depletion of ClpXP. Whole-cell lysates were separated on SDS–10% polyacrylamide gels and then subjected to immunoblotting using anti-HilA, anti-HilC, or anti-HilD serum. The bacterial strains used were χ 3306 (*clpPX*⁺ *fliZ*⁺ *flhDC*⁺), CS2007 (Δ *clpPX* *fliZ*⁺ *flhDC*⁺), CS2462 (*clpPX*⁺ Δ *fliZ* *flhDC*⁺), CS2464 (Δ *clpPX* Δ *fliZ* *flhDC*⁺), CS2609 (*clpPX*⁺ *fliZ*⁺ Δ *flhDC*), CS2610 (Δ *clpPX* *fliZ*⁺ Δ *flhDC*), CS2802 (Δ *hilC* Δ *hilD*), and CS3222 (Δ *hilA*).

murium, the transcription of more than 50 genes related to flagellum biogenesis forms a highly ordered cascade divided into three classes, 1, 2, and 3 (35). At the apex of the transcription hierarchy lies the sole operon *flhDC*. Its products, FlhD and FlhC, act together in a FlhD/FlhC hetero-oligomer to activate the promoter of class 2 genes (28, 38). Our previous study demonstrated that ClpXP recognizes and degrades the FlhD/FlhC hetero-oligomer, resulting in down-regulation of the flagellar regulon (61). One of seven operons in class 2, *fliAZY*, encodes an alternative sigma factor, FliA (σ^{28}), that is required for the transcription of class 3 genes, producing FliZ and FliY. Previous studies have reported that the *fliA*::Tn5 mutation significantly decreases expression of the *hilA-lacZ* transcriptional fusion and that expression is restored by a trans-complementing plasmid expressing *fliZ*, suggesting that FliZ is required for *hilA* expression (29, 41). Therefore, ClpXP may possibly control the cellular levels of the transcriptional regulators HilC and HilD by a pathway strongly dependent on FliZ. To examine this possibility, we measured the levels of HilC and HilD in *clpPX*⁺ and Δ *clpPX* cells after introducing a Δ *fliZ* mutation. The results (Fig. 5) show that the enhancement of cellular HilC and HilD levels by *clpPX* disruption disappeared in the Δ *fliZ* mutant, suggesting that the control of HilC and HilD levels by ClpXP depends on the function of FliZ. Owing to the effect of the Δ *fliZ* mutation on HilC and HilD levels, the enhancement of HilA by *clpPX* disruption was also extinguished. As expected, disruption of the *flhDC* operon, encoding the master regulator for expression of the flagellar regulon, blocked the increase of HilC and HilD levels caused by *clpPX* disruption. Taking these results together, it is suggested that SPI1 expression is regulated by ClpXP through a mechanism by which FliZ controls the cellular levels of the transcriptional regulators HilC and HilD.

Transcriptional analysis of *hilC* and *hilD* in *fliZ*⁺ and Δ *fliZ* cells. To examine whether FliZ controls the transcription of *hilC* and *hilD*, we measured the efficiency of transcription from *hilC* and *hilD* promoters using *lacZ* fusions in cells with different genetic backgrounds. The results (Fig. 6) show that the Δ *fliZ* mutation moderately decreased the transcription of *hilD* in the *clpPX*⁺ background, notwithstanding the marked de-

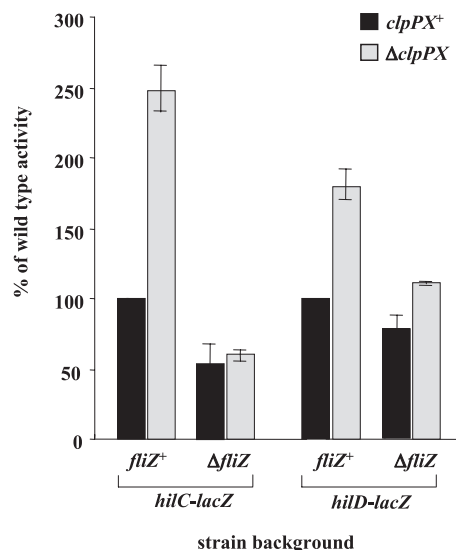


FIG. 6. Levels of *hilC* and *hilD* transcription in the absence of ClpXP and/or FliZ in cells. The levels of expression of a *lacZ* fusion to the *hilC* or *hilD* promoter in cells with different genetic backgrounds were assayed. Plasmid pTKY559 (*hilC* promoter-*lacZ* fusion) or pTKY562 (*hilD* promoter-*lacZ* fusion) was introduced into bacterial strains χ 3306 (*clpPX*⁺ *fliZ*⁺), CS2007 (Δ *clpPX* *fliZ*⁺), CS2462 (*clpPX*⁺ Δ *fliZ*), and CS2464 (Δ *clpPX* Δ *fliZ*). The resultant strains were used for determination of β -galactosidase activity. The values represent the means and standard deviations for samples tested at least in triplicate.

crease in the amount of HilD protein caused by this mutation (Fig. 5), suggesting that FliZ may regulate HilD production at a posttranscriptional and/or posttranslational level.

In contrast, the Δ *fliZ* mutation significantly decreased the transcription of *hilC* in cells with a *clpPX*⁺ background. It has been demonstrated that HilC and HilD each significantly activate the expression of the other when they are overproduced (13, 40). It has also recently been reported that loss of HilD decreases *hilC* transcription but that loss of HilC does not significantly alter *hilD* expression, so it is proposed that HilD is the apex of regulation of SPI1 expression (13, 15). Therefore, the decrease of *hilC* transcription caused by the Δ *fliZ* mutation in cells with a *clpPX*⁺ background could be explained by the decreased amount of HilD.

FliZ modulates HilD at the posttranscriptional level. To examine whether FliZ is involved in the posttranscriptional control of HilD, we decided to compare the levels of HilD protein in *fliZ*⁺ and Δ *fliZ* cells where *hilD* is expressed under the regulation of the $P_{A11acO-1}$ promoter system. For this purpose, strains CS2802 (*fliZ*⁺ Δ *hilC* Δ *hilD*) and CS3329 (Δ *fliZ* Δ *hilC* Δ *hilD*) were transformed with the plasmid pTKY651, in which the *hilD* promoter is replaced by the $P_{A11acO-1}$ promoter. The immunoblotting results in Fig. 7 show that HilD was detectable in the absence of IPTG (isopropyl- β -D-thiogalactopyranoside), a condition in which *hilD* was expressed by read-through from the $P_{A11acO-1}$ promoter. The results show that disruption of *fliZ* significantly decreased the cellular level of HilD even when the corresponding gene was expressed by the $P_{A11acO-1}$ promoter system, suggesting that HilD is controlled posttranscriptionally by FliZ. To test this inference, we analyzed the in vivo stability of HilD. There was no difference in

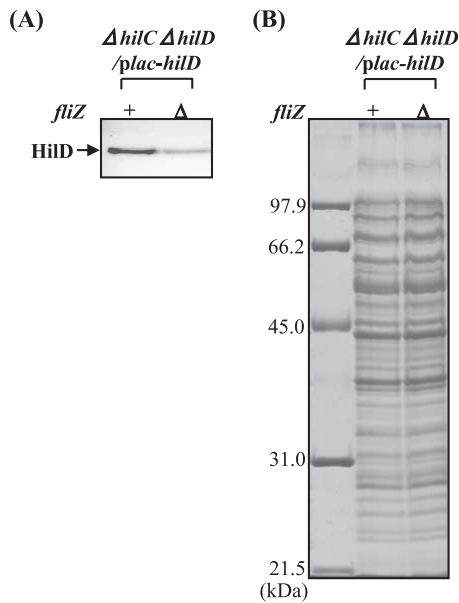


FIG. 7. Cellular levels of HilD protein expressed by a $P_{A11acO-1}$ promoter-*hilD* fusion in wild type and FliZ-depleted cells. Plasmid pTKY651 ($P_{A11acO-1}$ promoter-*hilD* fusion) was introduced into bacterial strains CS2802 (*fliZ*⁺ Δ *hilC* Δ *hilD*) and CS3329 (Δ *fliZ* Δ *hilC* Δ *hilD*). Cells of the resultant strains were grown to an optical density at 600 nm of 1.0, collected, lysed, and run on SDS-10% polyacrylamide gels. The separated proteins were transferred to a membrane and then immunostained with anti-HilD antiserum (A). Coomassie brilliant blue-stained gel electrophoretic patterns of the same samples used for immunoblotting are also shown (B). The leftmost lane in panel B contains molecular mass standards.

HilD half-life between the *fliZ*⁺ and Δ *fliZ* cells, suggesting that HilD is not controlled posttranslationally by FliZ (data not shown).

These findings, coupled with our previous results (61, 62), suggest that the overproduction of FliZ due to accumulation of the FlhD/FlhC master regulator of the flagellar regulon in *clpPX*-deleted cells results in the accumulation of HilD by posttranscriptional control and consequently leads to increased SPI1 expression.

DISCUSSION

Expression of SPI1 genes is tightly regulated at several stages in a complex manner by regulators within and outside the island. As summarized in Fig. 8, HilA is the central regulator in the overall scheme of SPI1 regulation and is known to activate expression of the *prg/org* and *inv/spa* operons. Read-through from *inv/spa* leads to the activation of *sic/spa*. InvF, in a complex with the chaperone protein SicA, also induces expression of the *sic/sip* operon, so HilA activates genes encoding all the components necessary for a functional SPI1 type III secretion system (2, 11, 12, 15, 37). The products of other genes within SPI1, *hilC* and *hilD*, have been shown to activate *hilA* expression (12, 50, 53). HilC and HilD are also capable of activating *hilC* and *hilD* expression independently of each other, and they act in a complex regulatory loop to control *hilA* expression (13, 50, 53).

In the present study, we have demonstrated that ClpXP negatively regulates SPI1 gene expression. We observed that depletion of ClpXP caused a significant increase in the amount

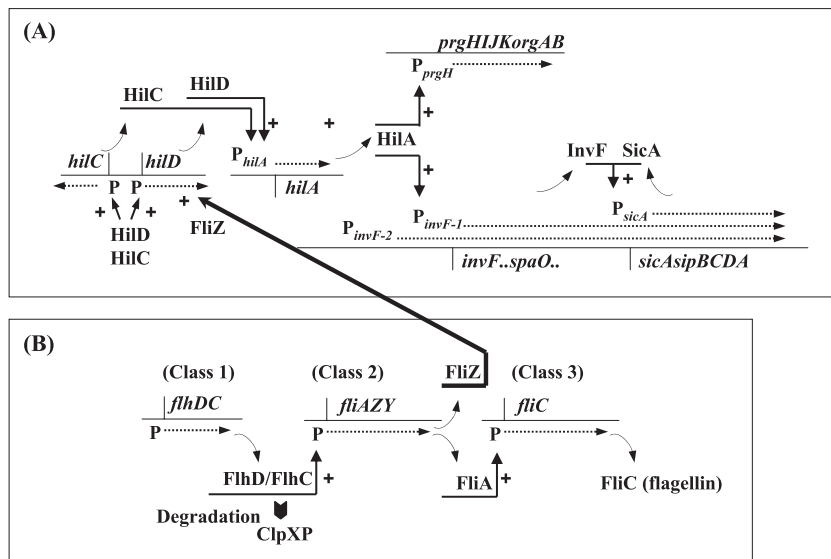


FIG. 8. A model of the coordinated regulation of SPI1 gene expression and flagellar gene expression by ClpXP protease. (A) Regulatory cascade of SPI1 gene expression. HilC and HilD bind directly upstream of the master regulator gene *hilA* to induce its expression. HilA directly activates the SPI1-encoded *prg/org* and *inv/spa* operons by binding just upstream of P_{prgH} and P_{invF-1} . The *inv/spa* transcript reads through the *sic/sip* operon. Activation of P_{invF-1} leads to production of InvF, in a complex with the chaperone protein SicA, and then induces expression of the *sic/sip* operon. (B) Regulatory cascade of flagellar gene expression. The products, FlhD and FlhC, act together in a FlhD/FlhC complex as the master regulator at the apex of a transcription hierarchy comprising three classes of genes. FliA, which is an alternative sigma factor, σ^{28} , is required to transcribe the class 3 genes. ClpXP degrades the FlhD/FlhC complex, leading to the down-regulation of flagellar regulon expression. FliZ, which is encoded by the *fliAZY* operon of the flagellar regulon, activates the expression of *hilD* at the posttranscriptional level, leading to the stimulation of SPI1 gene expression. ClpXP negatively regulates the expression of SPI1 genes through the repression of flagellar regulon expression. See the text for details and references.

of HilC and HilD proteins (Fig. 2), which is associated with increased expression of all SPI1 genes. Immunoblotting actually revealed that HilA and proteins encoded by genes in the *sic/sip* operon, SipB and SipC, are greatly increased in the $\Delta clpPX$ mutant. We hypothesized that ClpXP may regulate HilC and HilD through the control of the flagellar regulon, since ClpXP has been shown to regulate its expression negatively by degrading the FlhD/FlhC master regulator, which functions at the apex of the transcription hierarchy of the regulon (61, 62). Subsequent experiments showed that the enhancement of cellular levels of HilC and HilD by *clpPX* disruption seemed to be caused by an increase in the flagellar protein FliZ, encoded by the class 2 *fliAZY* operon. That is to say, disruption of *fliZ* abolished the increase of HilC and HilD caused by deleting *clpPX* (Fig. 5). Transcriptional analysis of *hilC* and *hilD* in the *fliZ*⁺ and $\Delta fliZ$ backgrounds suggested that FliZ controls HilC at the transcriptional level and may regulate HilD production at the posttranscriptional level (Fig. 6). Immunoblotting demonstrated that the $\Delta fliZ$ mutation decreased the cellular level of HilD even when *hilD* was transcribed from the P_{A11acO-1} promoter (Fig. 7), suggesting that *hilD* is controlled posttranscriptionally. Furthermore, it seems unlikely that FliZ controls HilD at the posttranslational level (our unpublished data). Therefore, it can be concluded that FliZ controls HilD at the posttranscriptional level. The function of FliZ is unknown, and its predicted product shows no significant homology to any known protein or structural motif.

When the levels of transcription from the *hilC* and *hilD* promoters are compared between *clpPX*⁺ cells and $\Delta clpPX$ cells in the *fliZ*⁺ background, it is evident that *clpPX* disruption increases the expression of both *hilC* and *hilD* (Fig. 4A and Fig. 6). It has been demonstrated that HilD is at the top of the hierarchy of the SPI1 regulatory loop and has a predominant role, though apparently it is not sufficient on its own to activate SPI1 (13, 15). Production of HilD leads to transcriptional activation of HilC, and each activates the expression of both (13, 40). Therefore, the increased transcription of *hilC* and *hilD* in the $\Delta clpPX$ cells could be explained by amplification of the regulatory loop in which FliZ triggered an increase in the amount of HilD via posttranscriptional control. On the other hand, the stimulation of *hilA* expression by *clpPX* disruption was abrogated by introducing the $\Delta hilC$ mutation (Fig. 3). This could be because HilD did not accumulate in the $\Delta hilC$ $\Delta clpPX$ double mutant cells (Fig. 4). As stated above, it is known that HilC and HilD can activate the expression of *hilC* and *hilD* independently of each other as well as inducing their own promoters (40, 47). Therefore, it is likely that the moderate effect on HilD production by FliZ needs to be amplified in the regulatory loop to cause significant stimulation of *hilA* expression in the $\Delta clpPX$ mutant cells.

In addition to HilC and HilD, RtsA, which belongs to the AraC/XylS family of transcriptional regulators, has been shown to activate expression of SPI1 genes by binding upstream of *hilA* to induce its expression (13, 14). HilC, HilD, and RtsA are all also capable of activating expression of *hilC*, *hilD*, and *rtsA* independently of each other, constituting a complex feed-forward regulatory loop that controls *hilA* expression (13, 14, 15). Therefore, it can be speculated that RtsA acts as an amplifier, leading to the overexpression of all SPI1 genes in the $\Delta clpPX$ mutant cells.

SPI1 is a complex regulatory system, and many different signals have been shown to feed into the network. The current report suggests that all the global regulators seem to control *hilA* expression in a HilD-dependent manner (15). One such regulator is the posttranscriptional regulatory protein CsrA, which binds to the messages of its targets and alters mRNA stability (51). In the $\Delta csrA$ mutant, the expression of SPI1 genes is greatly reduced (1). SirA in the BarA/SirA two-component regulatory system acts by inducing the expression of two small RNAs, the CsrB and CsrC RNAs, which are antagonistic to CsrA. Overproduction of SirA induces the expression of a *hilA-lacZ* transcriptional fusion only when HilD is present (13). Thus, SirA induction of *csrBC* prevents CsrA action, indirectly activating *hilD* expression posttranscriptionally (17). The RNA chaperone Hfq has recently been recognized as a major posttranscriptional regulator of bacterial gene expression that participates in numerous regulatory pathways (63). A recent report demonstrates that the Δhfq mutation drastically reduces *Salmonella* invasiveness (54). It has also demonstrated that Δhfq cells have sevenfold-reduced levels of *hilC*, *hilD*, and *rtsA* mRNAs, suggesting that Hfq affects signal transmission further upstream in the SPI1-activating cascade.

HilE is a major negative regulator of SPI1 expression. Deletion of *hilE* increases expression of *hilA* only when HilD is present. Although the mechanism of *hilE* action is not well understood, the results from bacterial two-hybrid studies suggest that HilE binds directly to HilD, preventing its action (3). *hilE* is regulated by several systems that feed into the SPI1 regulation circuit. The systems that negatively regulate SPI1 expression, e.g., the two-component PhoP/PhoQ and PhoR/PhoB regulatory systems and FimZY for type 1 fimbrial expression, seem to function primarily through HilE (3, 15). Lon protease is a powerful negative regulator of SPI1 expression (58, 60). Depletion of Lon increases *hilA* expression 40-fold and causes a 10-fold increase in invasiveness. Lon has been shown to regulate HilD posttranslationally, specifically by degradation. Taking these findings together, we suggest that HilD production is controlled largely at both the posttranscriptional and posttranslational levels and that this is the key to SPI1 regulation. To understand SPI1 regulation completely, the molecular mechanism by which *hilD* is regulated at the posttranscriptional level must be studied in detail.

ClpXP is also known to be a member of the family of stress proteins, which are induced in response to hostile environments (for a review, see reference 45). It has been demonstrated that stress proteins are selectively induced in *S. enterica* serovar Typhimurium growing within macrophages, where the bacteria are exposed to a variety of bactericidal mechanisms (8). We previously reported that *hilA* expression was repressed in macrophages after phagocytosis but that this gene was continuously expressed in a *S. enterica* serovar Typhimurium strain with a defect in Lon, another member of the stress protein family, growing within macrophages (57). Furthermore, we demonstrated that this derepression of SPI1 genes by a Lon-depleted mutant led to rapid and massive macrophage apoptosis by a mechanism involving caspases 1 and 3 (57). Once a systemic *Salmonella* infection has been established, excess apoptosis of macrophages, upon which the organism is reliant, would be detrimental to the bacteria. Thus, SPI1 gene expression must be controlled in bacteria growing within macro-

phages to suppress apoptosis sufficiently to allow time for the bacterium to replicate, escape, and invade new macrophages, leading to systemic infection. To ensure this, *Salmonella* can take advantage of a unique condition in the macrophage to repress SPI1. The ClpXP and Lon proteases may contribute to the down-regulation of SPI1 expression within macrophages. A recent report has demonstrated that expression of both *fliC*, encoding a flagellar filament, and *fliA*, encoding σ^{28} , is repressed during intracellular growth and that this repression depends on functional ClpXP (10). The increase in ClpXP in *S. enterica* serovar Typhimurium by its response to the hostile environment within macrophages after phagocytosis would ensure the repression of both SPI1 genes and the flagellar regulon.

ACKNOWLEDGMENTS

We thank T. Tomoyasu, A. Tokumitsu, and A. Suzuki for technical assistance.

This work was supported by grants-in-aid for scientific research (17390125) and research on priority areas (19041015) from the Ministry for Education, Culture, Sports, Sciences and Technology of the Japanese Government.

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