JOURNAL OF BACTERIOLOGY, Oct. 2008, p. 6636–6645 Vol. 190, No. 20
0021-9193/08/$08.00+0 doi:10.1128/JB.00744-08

σ^{32}-Mediated Negative Regulation of *Salmonella* Pathogenicity Island 1 Expression▽

Mari Matsui, Akiko Takaya, and Tomoko Yamamoto*

Department of Microbiology and Molecular Genetics, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 263-8522, Japan

Received 26 May 2008/Accepted 9 August 2008

***Salmonella* pathogenicity island 1 (SPI1) enables infecting salmonellae to invade the intestinal epithelium and induce a proinflammatory response and macrophage cell death. SPI1 expression is controlled by a complex cascade with several transcriptional regulators within the island and global regulators outside it. Previously, we reported that DnaK-depleted salmonellae could neither invade epithelial cells nor secrete SPI1-encoded proteins, suggesting that DnaK is involved in the expression of SPI1. Here, we found that DnaK is involved in SPI1 expression through inhibition of σ^{32} protein, which directs the transcription of a group of genes in response to various global stresses. Overproduction of σ^{32} resulted in decreased levels of the SPI1-specific transcriptional regulators HilD and HilA. Further analysis demonstrated that the σ^{32}-mediated system negatively regulates HilD and HilA at the posttranslational and transcriptional levels, respectively. The executioner of this negative regulation was shown to be a σ^{32}-induced protein ATP-dependent Lon protease, which specifically degrades HilD. Since HilD can activate *hilA* transcription, is at the top of the hierarchical SPI1 regulatory loop, and has a dominant role, the posttranslational control of HilD by Lon is critically important for precise expression of SPI1. Consequently, we suggest that SPI1 expression is controlled by the feedback regulatory loop in which σ^{32} induces Lon to control turnover of HilD, and DnaK, which inhibits σ^{32} function, leading to the modulation of *lon* expression. This regulation in response to a specific combination of environmental signals would ensure that SPI1 expression is restricted to a few specific locations in the host.**

Salmonella serovars are facultative intracellular pathogens that cause a range of diseases in a wide variety of hosts. *Salmonella enterica* serovar Typhimurium causes gastroenteritis in humans and systemic diseases similar to typhoid fever in mice. *Salmonella* is typically ingested in contaminated food or water. The bacteria colonize the small intestine and invade normally nonphagocytic epithelial cells to gain access to the underlying lymph tissue. Invasion is mediated by a type III secretion system (TTSS) encoded on a 40-kb island at centisome 63 of the *Salmonella* genome, termed *Salmonella* pathogenicity island 1 (SPI1) (16, 21). The SPI1 TTSS forms a multiprotein secretion apparatus, called a needle complex, through which effector proteins are injected into the host cell cytosol, where they activate signal transduction pathways, rearrange the actin cytoskeleton, and cause membrane ruffling, ultimately inducing uptake of the bacteria. In addition to invasiveness, various functions have been attributed to the SPI1 TTSS, including *Salmonella*-induced macrophage death (8, 25, 26), enteropathogenesis (59), and secretion of a pathogen-elicited epithelial chemoattractant that directs polymorphonuclear neutrophil movement across epithelial monolayers (30, 38).

SPI1 expression is controlled by a complex cascade with several transcriptional regulators present within the island. These regulators, HilD, HilC, HilA, and InvF, act in an ordered fashion to activate coordinated expression of the SPI1 genes (2, 10, 12, 13). The regulatory circuit converges on the expression of *hilA*, which activates the expression of all of the SPI1 operons encoding the TTSS apparatus, chaperones, and some effectors either directly or indirectly by activating the expression of another regulator, *invF* (3, 29, 33). HilD and HilC, which are members of the AraC/XylS family of transcriptional regulators, can each individually bind to the DNA immediately upstream of *hilA*, and it is believed that this binding leads to *hilA* expression (44, 45). In addition, RtsA, which also belongs to the AraC/XylS family, has been shown to activate the expression of SPI1 genes by binding upstream of *hilA* (14). HilD leads to transcriptional activation of *hilC* and *rtsA*, which activate themselves (13, 34, 41). Therefore, it is suggested that HilD is at the top of the hierarchy of regulation of SPI1 expression.

Control of SPI1 also extends to global regulators encoded outside the island. Several global regulators respond to a specific combination of environmental signals that presumably act as cues that the bacteria are in the appropriate anatomical location (4). The present report suggests that all of the global regulators control SPI1 expression in a HilD-dependent manner, that is, through posttranscriptional or posttranslational control of HilD, which in turn activates *hilC* and *rtsA* (15). One such regulator is SirA in the BarA/SirA two-component regulatory system. It has been demonstrated that overproduction of SirA can activate transcription from the *hilA* promoter only when HilD is present (13). SirA acts by inducing the expression of two small RNA molecules, CsrB and CsrC. These small RNAs are antagonistic to the posttranscriptional regulatory protein CsrA, which binds to the message of its targets and alters mRNA stability (43). Thus, SirA induction of *csrBC* prevents CsrA action, indirectly activating *hilD* expression posttranscriptionally. Recently, we have shown that the flagel-

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan. Phone: (81) 43-290-2928. Fax: (81) 43-290-2929. E-mail: tomoko-y@p.chiba-u.ac.jp.

▽ Published ahead of print on 22 August 2008.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties[b]	Reference or source
S. enterica serovar Typhimurium strains[a]		
χ3306	Virulent strain; *gyrA*	20
CS2021	*dnaK*::Cm in χ3306	54
CS2022	*lon*::Cm in χ3306	53
CS2501	CS2021 harboring pTKY608	54
CS2937	χ3306 harboring pKD46	This study
CS3072	χ3306 harboring pDMI1 and pUHE21-2Δfd12	This study
CS3249	*hilA*::Km in CS2021	This study
CS3271	CS3249 harboring pZA4*lacI*^{q} and pUHE21-2Δfd12	This study
CS3273	CS3249 harboring pZA4*lacI*^{q} and pTKY748	This study
CS3586	χ3306 harboring pDMI1 and pUHE212-1(*rpoH*)	This study
CS3593	CS2022 harboring pDMI1 and pUHE21-2Δfd12	This study
CS3596	CS2022 harboring pDMI1 and pUHE212-1(*rpoH*)	This study
CS3613	χ3306 harboring pTKY821	This study
CS3614	CS2021 harboring pTKY821	This study
CS3641	CS2021 harboring pBB535	This study
CS3647	CS3641 harboring pTKY797	This study
CS3658	Δ*hilD* in CS2021	This study
CS3659	CS3658 harboring pTKY797 and pZA4*lacI*^{q}	This study
CS3660	CS3658 harboring pTKY797 and pBB535	This study
CS3756	χ3306 harboring pDM1 and pTKY720	This study
CS3800	*lon*::Sp in CS2021	This study
Plasmids		
pTKY608	pMW119 carrying the *dnaK-dnaJ* operon of strain χ3306; Ap	54
pTKY651	pUHE21-2Δfd12 with 1,050-bp *hilD* fragment; Ap	55
pTKY720	pUHE21-2Δfd12 with 2,352-bp *lon* gene	This study
pTKY748	pUHE21-2Δfd12 with 1,836-bp *hilA* gene	This study
pTKY797	pMPM-A4 carrying *hilD* gene; Ap	This study
pTKY821	pCB182 with 347-bp fragment containing *lon* promoter; Ap	This study
pBB535	p15A derivative with $P_{A1/lacO-1}$-*dnaKJ* operon; Sp	57
pUHE212-1-*rpoH*	pUHE212-1 carrying *rpoH* gene; Ap	17
pCB182	Promoter cloning vector; Ap	47
pKD4	Km	11
pKD46	λ Red (γ, β, *exo*)	11
pMPM-A4	Cloning vector with arabinose-inducible promoter; Ap	37
pUHE212-1	N-terminal His tag vector; Ap	17
pUHE21-2Δfd12	$P_{A1/lacO-1}$ system vector; Ap	17
pZA4*lacI*^{q}	*lacI*^{q}; Sp	Our collection

[a] All *Salmonella* derivatives are originally from strain χ3306.
[b] Ap, ampicillin resistance; Km, kanamycin resistance, Sp, spectinomycin resistance.

lum-related gene product FliZ positively regulates *hilD* expression at the posttranscriptional level (27). The systems that negatively regulate SPI1 expression are the two-component PhoP/PhoQ and PhoR/PhoB regulatory systems and FimZY for type 1 fimbrial expression (6, 35, 42). These seem to function primarily through HilE, which binds directly to HilD, presumably preventing its action (5, 15). We have previously demonstrated that ATP-dependent Lon protease is a powerful negative regulator of SPI1 expression; depletion of Lon increased *hilA* expression 40-fold and caused a 10-fold increase in the invasion of cultured epithelial cells (53). Lon regulates HilD posttranslationally by specifically degrading it (55). We have further demonstrated that the DnaK chaperone machinery is essential for invasion of epithelial cells by *Salmonella* depending on the SPI1 TTSS; DnaK-depleted *Salmonella* cells could neither invade cultured epithelial cells nor secrete any of the invasion proteins encoded in SPI1 (54).

In the present study, we demonstrated that SPI1 expression is tightly controlled in the network of the global response mediated by the σ^{32} factor for RNA polymerase. σ^{32} is the first alternative σ factor discovered in *Escherichia coli* and can direct the transcription of a group of genes upon heat shock stress and other general stresses (40, 62). These gene products, collectively termed heat shock proteins, include molecular chaperones such as DnaK and ATP-dependent proteases such as Lon, which constitute the cellular network for the de novo folding and quality control of proteins under normal and stress conditions (22, 57). On the basis of the present results, we gain insight into the complex network for regulating SPI1 expression modulated by the σ^{32}-initiated regulatory loop.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All of the strains and plasmids used in this study are shown in Table 1. Bacterial cells were grown in L broth (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% sodium chloride, pH 7.4) and L agar, which were supplemented when necessary with either chloramphenicol (20 μg ml^{-1}), ampicillin (25 μg ml^{-1}), or kanamycin (25 μg ml^{-1}).

Construction of plasmids. Plasmid pTKY720, in which the expression of *lon* is controlled by the $P_{A1/lacO-1}$ promoter, was constructed by amplifying a BglII-HindIII fragment carrying *lon* by PCR with the *lon*-F1 (5′-GAAAAGCGGAT CCGTAATCGTGT-3′) and *lon*-R1 (5′-CCGAAAAAGCTTGCCAGCCC-3′)

primers and subsequent cloning of the fragment into pUHE21-2Δfd12 cleaved with BamHI and HindIII. Plasmid pTKY748, in which the expression of *hilA* is controlled by the $P_{A1/lacO-1}$ promoter, was constructed by amplifying a BglII-PvuII fragment carrying *hilA* by PCR with the *hilA*-F (5′-GAGAGTACACTA GATCTATGCCACAT-3′) and *hilA*-R (5′-TCATCGCCGATTCCAGCTGGG CGATA-3′) primers and subsequent cloning of the fragment into pUHE21-2Δfd12 cleaved with BamHI and PvuII. Plasmid pTKY797, in which the expression of *hilD* is controlled by the P_{araBAD} promoter, was constructed by cloning the EcoRI-PstI fragment carrying *hilD* from pTKY651 into plasmid pMPM-A4 cleaved with EcoRI and PstI. To construct plasmid pTKY821 containing the *lon* promoter, the *lon* promoter locus was amplified from the chromosome of strain χ3306 with primers *lon*-F2 (5′-AAACAGGATCCGCAGGC TTCT-3′) and *lon*-R2 (5′-CGTAGAAGCTTCCAGACAACG-3′). The 347-bp fragment generated was cleaved with BamHI at the 5′ end and HindIII at the 3′ end and then cloned into the vector pCB182 cleaved with BamHI and HindIII.

Construction of a *hilA*::Km mutant and a *dnaK*::Cm Δ*hilD* double mutant. Insertion of a kanamycin resistance (Km) cassette flanked by a FLP recombination sequence into *hilA* on the chromosome of χ3306 was accomplished by λ Red-mediated recombination essentially as described by Datsenko and Wanner (11). PCR products used to construct gene replacements were generated with template plasmid pKD4 and the *hilA*P1-F (5′-TATTATAACTTTTCACCCTG TAAGAGAATACACTATTATCGTGTAGGCTGGAGCTGCTTC-3′) and *hilA*P2-R (5′-ACGATGATAAAAAAATAATGCATATCTCCTCTCTCAGAT TCATATGAATATCCTCCTTA-3′) primers. The 1,476-bp fragment generated was purified and then introduced into strain CS2937 carrying plasmid pKD46, encoding the λ Red recombinase, by transformation. The Km cassette insertion in *hilA* was verified by PCR amplification of the chromosomal DNA with the pKD4-P1 (5′-GTGGTAGGCTGGAGCTGCTTC-3′) and pKD4-P2 (5′-CATA TGAATATCCTCCTTAG-3′) primers and by Southern blotting.

To construct the *dnaK*::Cm Δ*hilD* double mutant CS3658, bacteriophage P22 was propagated on a *Salmonella* mutant in which *hilD* was replaced with the construct in which *hilD* is disrupted by a single-crossover event (55), and the resultant lysates were used to infect CS2021 (*dnaK*::Cm). The transductants were selected for ampicillin resistance. Subsequently, a double-crossover event in the Δ*hilD* mutant was assessed by its resistance to sucrose and sensitivity to ampicillin. Disruption of the *hilD* gene was checked by PCR with *hilD*-F (5′-CCGG GGATCCATGGAAAATGTAACCTTTG-3′) and *hilD*-R (5′-CAATCTGCAG GAATAGCCTCCCATCCTG-3′).

Immunoblotting analysis. To prepare whole-cell proteins, bacterial cells were harvested by centrifuging the culture and then suspended in sample buffer (28). Gel electrophoresis was carried out according to the method of Laemmli (28), with a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and Coomassie brilliant blue staining. The separated proteins were transferred onto Immun-Blot polyvinylidene difluoride membranes (Bio-Rad) and reacted with rabbit anti-HilD serum (1:12,500), anti-HilA serum(1:25,000), and anti-SipC serum (1:25,000) and mouse anti-DnaK monoclonal antibody (1:25,000; Stressgen), followed by alkaline phosphatase-conjugated anti-rabbit or anti-mouse immunoglobulin G. The enzymatic reactions were performed in the presence of 0.3 mg ml^{-1} nitroblue tetrazolium (Wako) and 0.15 mg ml^{-1} 5-bromo-4-chloro-3-indolylphosphate (Sigma). Anti-HilD, -HilA, and -SipC sera were previously established in our laboratory (50, 55).

Assay for invasion of epithelial cells. Intestine-407 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Cells (2×10^5) were seeded into 24-well tissue culture plates to obtain about 90% confluent monolayers on the following day. Bacterial cultures were grown to an optical density at 600 nm (OD_{600}) of 0.5, and *hilA* expression was induced by adding 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubating the mixture for 2 h. The bacterial cells were washed with Hanks' balanced salt solution (HBSS) and used to inoculate monolayers previously washed with HBSS at a multiplicity of infection of 10. The monolayers were centrifuged at 500 × *g* for 5 min, incubated for 2 h at 30°C, washed thoroughly with HBSS, and further incubated for 3 h in Dulbecco modified Eagle medium containing 100 μg gentamicin ml^{-1} to eliminate extracellular bacteria before lysis with Triton X-100. Bacterial numbers were determined by plating the lysates on L agar plates after appropriate dilution.

RNA extraction and quantitative real-time RT-PCR. Total RNAs were extracted from 1 ml of bacterial culture with the RNeasy Mini kit and the RNAprotect bacterial reagent according to the manufacturer's instructions. Reverse transcription (RT) was performed on 1 μg isolated total RNA with a QuantiTect RT kit by following the manufacturer's instructions. PCRs were performed with an Mx3000P QPCR system. For each PCR, the reaction mixture was prepared with Brilliant Sybr green QPCR Master Mix with 1 μl cDNA, forward and reverse primers (for *hilA* and *hilD*, 0.4 μM; for 16S rRNA, 1 μM), and the ROX reference dye supplied in a total of 25 μl. Thermal cycling conditions were an initial denaturation step for 10 min at 95°C, following 45 cycles of denaturing for 30 s at 95°C, annealing for 1 min at 55°C, and elongation for 30 s at 72°C. The following primer sequences were used: for *hilA*, sense primer CCGAGAGTCT GCATTACTCTATCGT and antisense primer TATCCTTAACACTGCGGCA GTTC; for *hilD*, sense primer ACTCGAGATACCGACGCAAC and antisense primer CTTCTGGCAGGAAAGTCAGG; for the 16S rRNA gene, sense primer GAATGCCACGGTGAATACGTT and antisense primer ACCCACTCCCAT GGTGTGA.

Stability of HilD protein in vivo. Cultures were grown to an OD_{600} of 0.5 in L broth containing 500 μM IPTG to induce *dnaK* expression, followed by the induction of *hilD* expression by adding 0.005% arabinose and incubation for 30 min. Tetracycline (100 μg ml^{-1}) and glucose (2%) were then added to the culture to block the translation and expression of *hilD*. Aliquots of the cells were taken at appropriate intervals and mixed with trichloroacetic acid (final concentration, 10%), chilled on ice for 15 min, and centrifuged at 10,000 × *g* for 15 min. The pellets were washed with acetone and resuspended in sample buffer (28). A portion of each sample was separated on a 10% polyacrylamide gel and immunostained with anti-HilD serum (1:12,500). The levels of protein were quantified with Quantity One.

RESULTS

The inability of the *dnaKJ* mutant to invade is due to the marked decrease in the *hilA* transcript level. In our previous study, we revealed that a *dnaK*::Cm mutant derived from *S. enterica* serovar Typhimurium lost the ability to invade cultured Intestine-407 cells (54). Several SPI1 proteins involved in invasion, such as SipA, SipC, and SipD, can be detected in the culture medium as proteins secreted by *Salmonella* (53). SipC and SipD are integral components of the translocation apparatus, which has been shown to be inserted into the host cell plasma membrane upon infection (46). Furthermore, SipC has been shown to initiate actin polymerization and anchor bundled filaments beneath invading bacteria (23). SipA is an effector that can bind actin directly to reduce the critical actin concentration required for polymerization (65). We have found that neither SipA nor SipC nor SipD was secreted in the culture medium of *dnaK*::Cm mutant cells (51, 54), suggesting that DnaK may be involved in the expression, stabilization, and/or secretion of SPI1-encoded proteins in *Salmonella*. The mutation was created by inserting a chloramphenicol resistance cassette including a stop codon and therefore resulted in a polar effect of *dnaJ* in the *dnaK-dnaJ* operon (54). Since DnaK functions as a molecular chaperone with the cochaperone DnaJ, we decided to use this mutant strain, CS2021, for further analysis.

To examine whether the disappearance of SPI1 proteins secreted from the cells by *dnaKJ* disruption is due to loss of expression of the proteins, we initially compared the cellular levels of SipC in Δ*dnaKJ* cells and isogenic $dnaKJ^+$ cells by immunoblotting. No SipC was detected in the Δ*dnaKJ* cells, while it was clearly detected in the $dnaKJ^+$ cells (Fig. 1). To confirm that the absence of SipC from the whole-cell lysate of this strain is due to the disruption of *dnaKJ*, the mutation was complemented by a functional *dnaKJ* operon from χ3306 in *trans* and tested for SipC. As shown in Fig. 1, SipC was restored in the complemented strain, suggesting that the DnaKJ chaperone machinery is involved in producing SipC. We then examined the cellular levels of HilA, which is the central regulator in the overall scheme of SPI1 gene expression. No HilA was detected in the Δ*dnaKJ* cells, but this defect was fully compensated for by a functional copy of *dnaKJ* in *trans*. The

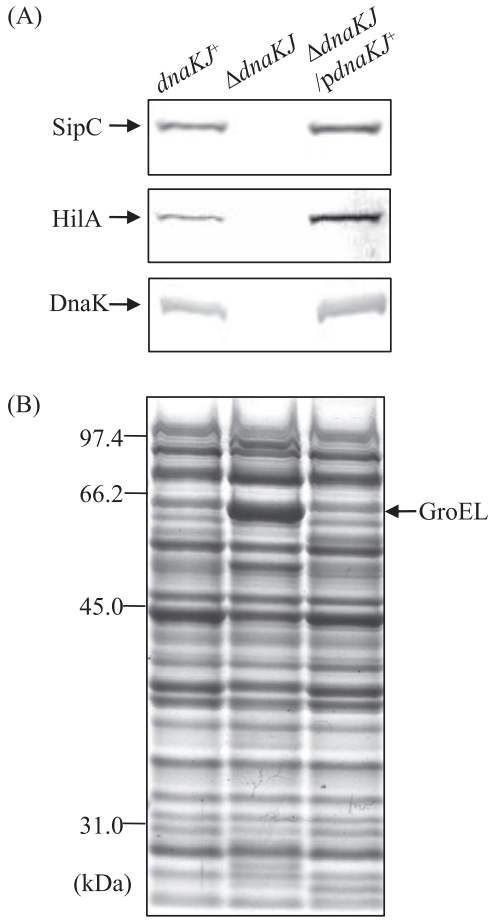

FIG. 1. Cellular levels of SPI1 proteins in wild-type cells and cells in which *dnaKJ* is disrupted. Bacterial cells of strains χ3306 (*dnaKJ*$^+$), CS2021 (Δ*dnaKJ*), and CS2501 (Δ*dnaKJ*/p*dnaKJ*$^+$) were used. (A) Immunoblotting of cellular lysates with anti-SipC, anti-HilA, and anti-DnaK sera. (B) Coomassie brilliant blue-stained SDS–10% polyacrylamide gel electrophoresis patterns of the samples used for immunoblotting. p*dnaKJ*$^+$, pTKY608.

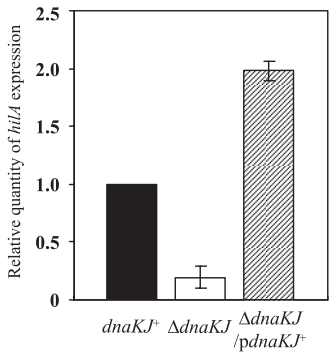

FIG. 2. Expression of *hilA* in wild-type cells and cells in which *dnaKJ* is disrupted. Total RNA was prepared from strains χ3306 (*dnaKJ*$^+$), CS2021 (Δ*dnaKJ*), and CS2501 (Δ*dnaKJ*/p*dnaKJ*$^+$) grown in L broth to an OD_{600} of 0.5 at 30°C. The levels of *hilA* transcripts were measured by quantitative real-time RT-PCR and then normalized to 16S rRNA gene expression. The values represent the means and standard deviations of *n*-fold changes in comparison with the transcription level in χ3306. p*dnaKJ*$^+$, pTKY608.

effect of *dnaKJ* disruption on the expression of *hilA* was also examined by measuring the *hilA* transcript by quantitative real-time RT-PCR (Fig. 2). The *dnaKJ* disruption markedly decreased the amount of *hilA* transcript, and this decrease was fully restored by a functional copy of *dnaKJ*, suggesting that the DnaKJ chaperone machinery is probably involved in *hilA* expression. The *hilA* transcript level in the Δ*dnaKJ* cells complemented in *trans* exceeds that in the wild-type control. This is probably due to DnaKJ in excess over the normal level, as shown by immunoblotting (Fig. 1A).

To determine whether the loss of the ability to invade epithelial cells by *dnaKJ* disruption is due to the great decrease in *hilA* transcript levels, we examined the efficiency of invasion of Δ*dnaKJ* cells expressing *hilA* under the control of the $P_{A1/lacO-1}$ promoter system on plasmid pTKY748, in a Δ*hilA* background on the chromosome. Bacterial cells exposed to 500 μM IPTG for 2 h produced sufficient HilA to be detected even in the *dnaKJ*-deficient background (Fig. 3A). Furthermore, the increased HilA levels found subsequent to the activation of *hilA* by IPTG resulted in a sufficient amount of the SPI1 product, SipC. The invasion assay with the cultured Intestine-407 cells demonstrated that the induced HilA level enhanced the efficiency of invasion 240-fold even in the *dnaKJ*-deficient background (Fig. 3B). Taking these findings together, it is suggested that the DnaKJ chaperone machinery is essential for *hilA* expression to ensure the production of SPI1 proteins required for the capacity of *Salmonella* to invade epithelial cells.

HilD, a transcriptional regulator of SPI1 expression, is destabilized in cells in which *dnaKJ* is disrupted. To reveal the involvement of the DnaKJ chaperone machinery in regulating *hilA* expression, we examined the cellular level of HilD, which has been shown to bind to the upstream sequence of *hilA* to activate its transcription directly (7, 13, 34). Immunoblotting of

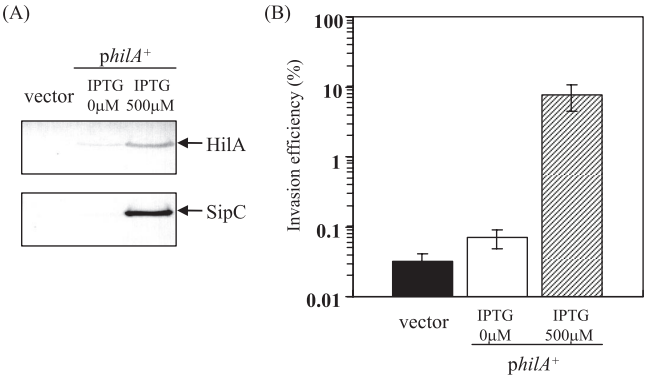

FIG. 3. Effect of overproduction of HilA on *Salmonella* invasiveness in a *dnaKJ*-deficient background. Bacterial cells of strains CS3271 (Δ*dnaKJ*Δ*hilA*/vector) and CS3273 (Δ*dnaKJ* Δ*hilA*/p*hilA*) were grown to an OD_{600} of 0.5 at 30°C in L broth, followed by the induction of *hilA* expression by adding 0 or 500 μM IPTG for 2 h. (A) Immunoblotting of cellular lysates with anti-HilA and anti-SipC sera. (B) Efficiency of invasion of cultured Intestine-407 cells. Bacterial cells in which *hilA* expression was induced by adding 500 μM IPTG for 2 h were used to inoculate monolayers. Invasion efficiency was examined as described in Materials and Methods. The data are the means and standard deviations for each strain tested in triplicate. Vector, pUHE21-2Δfd12; p*hilA*$^+$, pTKY748.

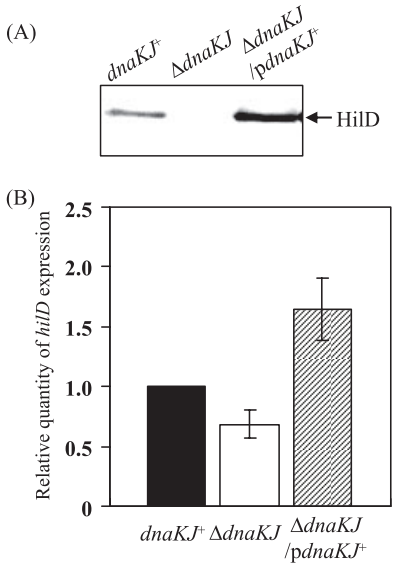

FIG. 4. Cellular levels of HilD and relative levels of *hilD* expression. (A) Whole-cell lysates were prepared from strains χ3306 (*dnaKJ*$^+$), CS2021 (Δ*dnaKJ*), and CS2501 (Δ*dnaKJ*/p*dnaKJ*$^+$) grown in L broth to an OD_{600} of 1.0 at 30°C and then separated on an SDS–10% polyacrylamide gel. The separated proteins were immunostained with anti-HilD serum. (B) Total RNAs were prepared from the strains used in panel A grown in L broth to an OD_{600} of 0.5 at 30°C. The levels of *hilD* transcripts were measured by quantitative real-time RT-PCR and then normalized to 16S rRNA gene expression. The values represent the means and standard deviations of *n*-fold changes in comparison with the transcription level in χ3306. p*dnaKJ*$^+$, pTKY608.

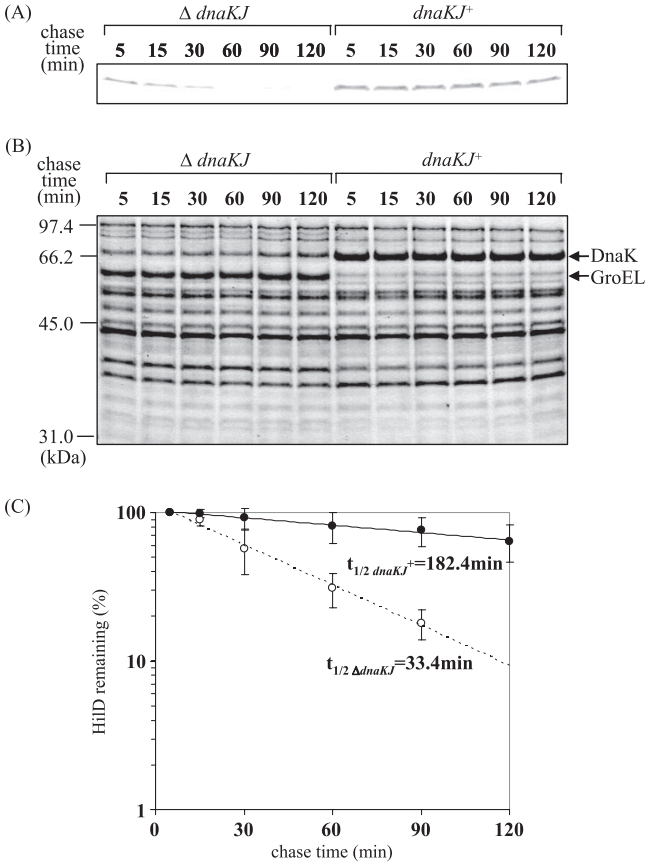

FIG. 5. In vivo stabilities of HilD protein in wild-type cells and cells in which *dnaKJ* is disrupted. (A) The bacterial strains used were CS3659 (Δ*dnaKJ*) and CS3660 (*dnaKJ*$^+$). Cells were grown to an OD_{600} of 0.5 at 30°C in L broth containing 500 μM IPTG to induce *dnaKJ* expression, followed by the induction of *hilD* expression by adding 0.005% arabinose for 30 min. Tetracycline (100 μg ml^{-1}) and glucose (2%) were added, and samples were added to prechilled trichloroacetic acid (final concentration, 10%) at the indicated times. The proteins were separated on an SDS–10% polyacrylamide gel and then immunostained with anti-HilD antibody. (B) Coomassie brilliant blue-stained gel patterns of the same samples used for immunoblotting. (C) Quantification of the precipitated proteins relative to the value at 5 min. Mean values of at least three independent experiments are given. $t_{1/2}$, half-life.

cell lysates prepared from the Δ*dnaKJ* mutant and isogenic *dnaKJ*$^+$ cells detected no significant amount of HilD in the Δ*dnaKJ* cells (Fig. 4A). The dramatic decrease in HilD caused by *dnaKJ* disruption was fully compensated for by providing a functional *dnaKJ* operon in *trans*. Therefore, it is possible that the DnaKJ chaperone machinery is involved in regulating *hilA* transcription by modulating the cellular level of HilD. The effect of *dnaKJ* disruption on *hilD* expression was then examined in cells with the genetic backgrounds used for immunoblotting (Fig. 4A) by quantitative real-time RT-PCR. The results (Fig. 4B) demonstrate that *dnaKJ* disruption moderately decreased the expression of *hilD*, notwithstanding the marked decrease in the amount of HilD protein caused by this mutation. It has been demonstrated that HilD is at the top of the hierarchy of the SPI1 regulatory loop and also activates its own promoter, leading to amplification of the regulatory loop (13). Therefore, the slightly lower level of *hilD* transcript in the Δ*dnaKJ* mutant cells compared to that in the isogenic *dnaKJ*$^+$ cells could be due to the absence of amplification of the regulatory loop by HilD in the Δ*dnaKJ* cells. The levels of both HilD protein and *hilD* transcript in the Δ*dnaKJ* cells complemented in *trans* exceed those in wild-type control cells. These are probably due to excess DnaKJ over the normal level, as shown in Fig. 1A. Taken together, these findings suggest that the DnaKJ chaperone machinery is possibly involved in the posttranscriptional and/or posttranslational regulation of *hilD*.

To test whether the DnaKJ chaperone machinery is involved in the posttranslational control of HilD, we decided to determine the in vivo half-life of HilD in the presence or absence of DnaKJ. In DnaKJ-producing cells, the *dnaKJ* genes were expressed under the control of the $P_{A1/lacO-1}$ promoter system. The expression of *hilD* was initiated from the P_{araBAD} promoter on plasmid pTKY797 in a Δ*hilD* background on the chromosome by adding arabinose. The results (Fig. 5A) show the cellular levels of HilD at the indicated times after addition of tetracycline to prevent de novo synthesis of proteins. Whereas HilD disappeared at 60 min after the arrest of de novo synthesis in cells in which *dnaKJ* is disrupted, it was clearly detectable up to 120 min in cells producing DnaKJ. The half-lives of HilD in the cells in which *dnaKJ* is disrupted and the *dnaKJ*-expressing cells were 33.4 and 182.4 min, respectively (Fig. 5C). These results indicate that the DnaK chaperone machinery is involved in the control of HilD turnover.

σ^{32} mediates the control of SPI1 gene expression. How is the DnaKJ chaperone machinery involved in turnover of

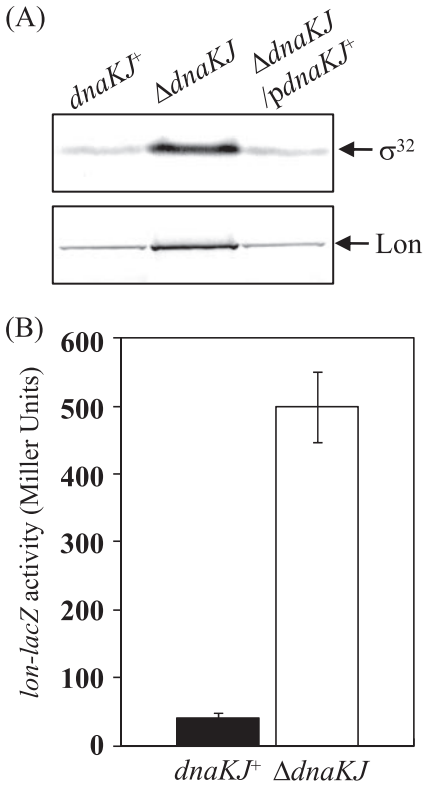

FIG. 6. Effect of *dnaKJ* disruption on expression of σ^{32} and *lon* in *Salmonella* cells. (A) Whole-cell extracts were prepared from strains χ3306 (*dnaKJ*$^+$), CS2021 (Δ*dnaKJ*), and CS2501 (Δ*dnaKJ*/p*dnaKJ*$^+$) grown in L broth to an OD_{600} of 1.0 at 30°C and separated on an SDS–10% polyacrylamide gel. The separated proteins were immunostained with anti-σ^{32} serum and anti-Lon serum. p*dnaKJ*$^+$, pTKY608. (B) The expression levels of *lacZ* fusion to the *lon* promoter in cells harboring pTKY821 were assayed for β-galactosidase activity. The values represent the means and standard deviations of samples tested at least in triplicate. The strains used were CS3613 (*dnaKJ*$^+$) and CS3614 (Δ*dnaKJ*).

HilD? It is unlikely that it directly degrades the HilD protein. We have previously reported that HilD is degraded by ATP-dependent Lon protease, leading to downregulation of SPI1 gene expression (55). The genes for the Lon protease and the DnaKJ chaperone are led by two consensus promoter sequences, each recognized by the σ^{70} factor involved in the transcription of most genes in cells and by σ^{32}, an alternative σ factor the level of which is increased in response to a temperature upshift and other stresses. σ^{32} preferentially binds to core RNA polymerase to form an Eσ^{32} holoenzyme complex, so the increased σ^{32} results in the accelerated transcription of σ^{32}-dependent genes. In *E. coli*, it has been demonstrated that the DnaKJ chaperone machinery negatively controls both the amount and the activity of σ^{32}, creating a feedback loop of the σ^{32} regulon (31, 49, 56, 64). In *S. enterica* serovar Typhimurium, we observed that the amount of σ^{32} was greatly increased by *dnaKJ* disruption (Fig. 6A), suggesting negative modulation of σ^{32} by the DnaKJ machinery. We also observed that the *dnaKJ* disruption stimulated the transcription from the *lon* promoter 10-fold (Fig. 6B) and significantly increased the cellular level of Lon in *S. enterica* serovar Typhimurium (Fig. 6A). The finding that the *dnaKJ* disruption resulted in an increase in the amount of σ^{32} and therefore stimulated the transcription of *lon* in *S. enterica* serovar Typhimurium raises the possibility that the cellular level of HilD may be controlled by the σ^{32}-initiated regulatory loop and this regulates SPI1 expression.

To examine this possibility, the cellular level of HilD was determined in σ^{32}-overproducing cells in which *rpoH*, encoding σ^{32}, is induced under the control of the $P_{A1/lacO-1}$ promoter system. Immunoblotting with lysates prepared from wild-type cells with or without $P_{A1/lacO-1}$-*rpoH* on the plasmid showed that overproduction of σ^{32} resulted in a marked decrease in the cellular level of HilD (Fig. 7A). Owing to the dramatic decrease in HilD, HilA disappeared from the σ^{32}-overproducing cells. On the other hand, the effect of overproducing σ^{32} on the transcription of *hilD* and *hilA* was comparatively determined by quantitative real-time RT-PCR. The results (Fig. 7B) demonstrate that overproduction of σ^{32} did not significantly affect *hilD* transcription but dramatically reduced *hilA* transcription. The decrease in *hilA* transcription could be due to the marked decrease in the cellular level of HilD. To check whether σ^{32} modulates the HilD level by controlling the induction of Lon, we examined the effect of σ^{32} overproduction on the levels of HilD in a *lon*-deficient background. The results (Fig. 7C) show that the increased level of σ^{32} does not result in decreased HilD if Lon protease was absent from the cells, suggesting that σ^{32} controls the cellular level of HilD through the induction of Lon protease, which specifically recognizes and degrades it. Similarly, overproduction of σ^{32} did not affect the cellular level of HilA in the *lon*-deficient cells.

Taking these findings together, it is suggested that SPI1 gene expression is regulated by the feedback regulatory loop in which σ^{32} induces Lon protease to control the turnover of HilD and the DnaKJ chaperone machinery, which can inhibit σ^{32} function, leading to the modulation of *lon* expression. Therefore, the extreme accumulation of Lon due to collapse of the negative control of σ^{32} regulon by *dnaKJ* disruption could explain the disappearance of SPI1 expression from the mutant cells. We confirmed that SPI1 gene expression is controlled in response to global stresses, leading to the induction of σ^{32}, by measuring the *hilA* transcription in cells exposed to a variety of stresses, including heat shock (42°C), acidic shock (pH 4.0), 5% ethanol, and 1 mM H_2O_2. The results (Fig. 8) demonstrate that *hilA* transcription significantly decreased after the exposure of cells to these stresses, suggesting that SPI1 gene expression is under the control of a σ^{32}-mediated stress response.

An alternative explanation for the role of the DnaKJ chaperone in the control of HilD turnover is that it is directly involved in the folding of HilD and its absence accelerates the degradation of HilD by Lon or other proteases, since unfolded proteins similar to irreversibly damaged proteins are nonspecifically degraded by proteases (24). To address this possibility, we examined whether overproduction of Lon in wild-type cells also decreases the amount of HilD similar to *dnaKJ* deletion. The results (Fig. 9A) demonstrate that the overproduction of Lon in wild-type cells markedly decreased the amount of HilD, as observed in Δ*dnaKJ* cells (Fig. 4A), suggesting that Lon can specifically degrade folded HilD. Furthermore, we determined the cellular level of HilD in a Δ*dnaKJ* Δ*lon* double mutant. As shown in Fig. 9B, the decreased effect of *dnaKJ* disruption on

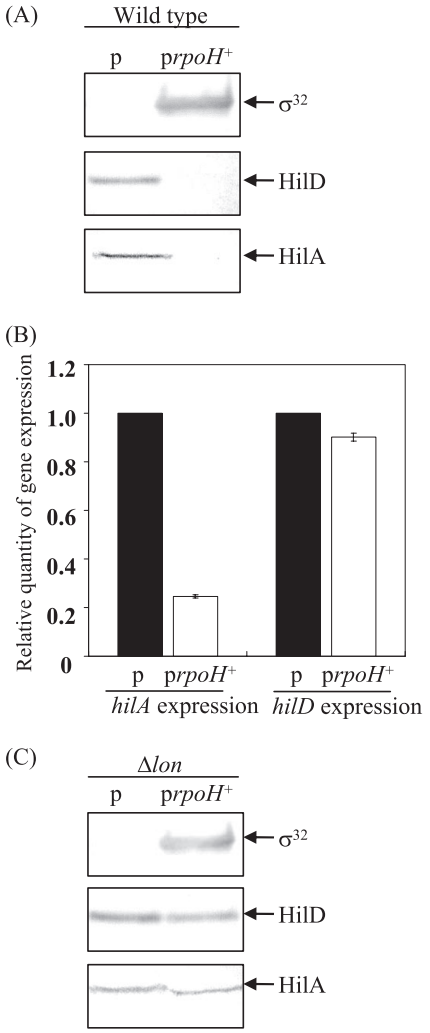

FIG. 7. Effects of σ^{32} overexpression on cellular levels of HilA and HilD proteins and *hilA* and *hilD* transcripts. (A) Cultures of strains CS3072 (p) and CS3586 (p*rpoH*$^+$) were grown in L broth to an OD_{600} of 0.5 at 37°C, followed by the induction of σ^{32} with 200 μM IPTG for 1 h. Whole-cell lysates were separated on an SDS–10% polyacrylamide gel and then subjected to immunoblotting with anti-σ^{32}, anti-HilD, and anti-HilA sera. (B) Cultures of strains used in panel A were grown in L broth to an OD_{600} of 0.5 at 37°C, followed by the induction of σ^{32} with 200 μM IPTG for 30 min. The levels of *hilD* and *hilA* transcripts were measured by quantitative real-time RT-PCR and then normalized to 16S rRNA gene expression. The values represent the means and standard deviations of *n*-fold changes in comparison with the transcription levels of the corresponding genes in CS3072. (C) Cultures of strains CS3593 (Δ*lon*/p) and CS3596 (Δ*lon*/p*rpoH*$^+$) were grown in L broth to an OD_{600} of 0.5 at 37°C, followed by incubation with 200 μM IPTG for 1 h to induce σ^{32}. Whole-cell lysates were separated on an SDS–10% polyacrylamide gel and then subjected to immunoblotting analysis. p, pUE212-1; p*rpoH*$^+$, pUE212-1-*rpoH*.

the cellular level of HilD was abolished by introducing a *lon* mutation. The results simultaneously indicate that the *lon* disruption resulted in the marked accumulation of HilD even in the absence of the DnaKJ chaperone. Therefore, it is unlikely that DnaKJ is directly involved in the folding of HilD and its absence accelerates the degradation of HilD by proteases.

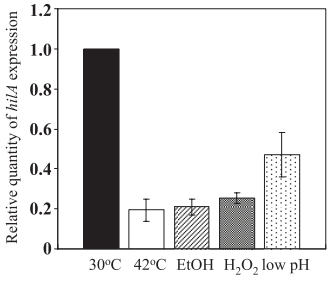

FIG. 8. Expression of *hilA* in *Salmonella* cells exposed to heat stress and other stresses. Cells of strain χ3306 grown in L broth to an OD_{600} of 0.5 at 30°C were exposed to heat shock (42°C), 5% ethanol (EtOH), 1 mM H_2O_2, and acidic shock (pH 4.0) for 10 min. Total RNAs were extracted, and *hilA* transcripts were measured by quantitative real-time RT-PCR and then normalized to 16S rRNA gene expression. The values represent the means and standard deviations of *n*-fold changes in comparison with the transcription levels of *hilA* in cells incubated at 30°C.

DISCUSSION

The expression of SPI1 genes is regulated by a variety of positive and negative regulator genes in response to environmental conditions. In the present study, we have demonstrated that SPI1 expression is tightly regulated in the σ^{32}-mediated regulatory loop. This is executed by the specific degradation of HilD by ATP-dependent Lon protease, a heat shock protein

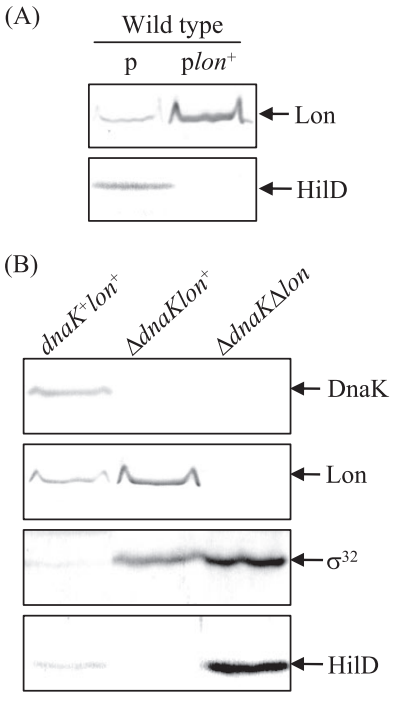

FIG. 9. Effects of *lon* overexpression in a *dnakJ*$^+$ background (A) and a Δ*dnakJ* Δ*lon* double mutation (B) on cellular levels of HilD protein. (A) Cultures of strains CS3072 (p) and CS3756 (p*lon*$^+$) were grown in L broth to an OD_{600} of 0.5 at 37°C, followed by the induction of *lon* with 200 μM IPTG for 1 h. p, pUE21-2Δfd12; p*lon*$^+$, pTKY720. (B) Cultures of strains χ3306 (*dnakJ*$^+$ *lon*$^+$), CS2021 (Δ*dnakJ lon*$^+$), and CS3800 (Δ*dnakJ* Δ *lon*) were grown in L broth to an OD_{600} of 0.5 at 30°C. Whole-cell lysates were separated on an SDS–10% polyacrylamide gel and then subjected to immunoblotting analysis.

induced by σ^{32}. Since HilD is at the top of the hierarchy of the SPI1 regulatory loop and has a predominant role in regulation, its degradation is essential for precise regulation of SPI1 expression. The levels of heat shock proteins are controlled primarily by σ^{32}, which senses the cellular protein folding environment through negative feedback control mediated by molecular chaperones that are also σ^{32} regulon members. It has been demonstrated that each of the DnaKJ and GroELS chaperones in *E. coli* constitutes a negative feedback loop that couples σ^{32} activity to the cellular protein folding state: overproduction of either chaperone machinery decreases both the amount and the activity of σ^{32}; conversely, chaperone depletion or overexpression of the chaperone substrates, misfolded and unfolded proteins, increases both the amount and the activity of σ^{32} (19, 48, 58). The homeostatic regulation model proposes that induction of the heat shock response relies on sequestering DnaKJ and GroEL by binding to the damaged proteins that accumulate during stress (19, 58). The present study has shown that the loss of the DnaKJ chaperone machinery results in the continuous expression of Lon because the negative feedback control is impaired (Fig. 6), therefore leading to severely diminished expression of SPI1 genes through excess degradation of HilD. Consequently, it is suggested that the expression of SPI1 genes is negatively regulated by a σ^{32}-mediated stress response. On the other hand, the question of whether any of the SPI1 genes are under the positive control of σ^{32} remains unanswered.

The heat shock proteins, which are alternatively called stress proteins, are believed to be induced during various stages of bacterial infection because pathogens are exposed to a variety of environmental stress conditions such as sudden elevated temperature and stomach acidity before reaching the low-oxygen, hyperosmotic environment of the small intestine and the bactericidal mechanisms associated with the host immune system. Actually, heat shock proteins such as DnaK, GroEL, and GroES have been identified among the proteins induced during the growth of bacteria, including *Salmonella* (9), *Yersinia* (61), *Legionella* (1), and *Brucella* (32), within macrophages. To cause systemic infection, *Salmonella* must grow inside macrophages and must overcome exposure to oxidative stress, acid pH, cationic peptides, and nutrient deprivation, suggesting that the σ^{32}-controlled regulon of *Salmonella* indeed responds to the hostile environment in the macrophage phagosome. We have previously reported direct evidence that the σ^{32} heat shock regulon is involved in the pathogenesis of *S. enterica* serovar Typhimurium; that is, the heat shock proteins DnaK, Lon, ClpX, and ClpP are essential for intracellular growth within macrophages and the systemic infection of mice (52, 54, 60).

In addition to its ability to grow within infected macrophages, *Salmonella* has been shown to induce macrophage cell death by mechanisms depending on caspase 1 but not by the classical mechanism depending on caspase 3, which is a key executioner caspase in the proteolytic cascade leading to cell death (36, 39). Rapid cell death is independent of intracellular bacterial multiplication but dependent on the SPI1-encoded SipB effector protein (25). Caspase 1-dependent programmed cell death is distinct from other forms of classical apoptosis that depend on caspase 3. One characteristic is that caspase 1 is a proinflammatory enzyme that cleaves the inactive precursors of interleukin-1β and -18 into their active cytokines (63). In contrast, we have found that the *lon* disruption-containing mutant of *S. enterica* serovar Typhimurium induces rapid, large-scale cell death by a mechanism involving both caspases 1 and 3 (50). Furthermore, we have demonstrated that *lon* disruption leads to the continuous expression of SPI1 genes within macrophages, where they are normally repressed, and that derepression of the SPI1 genes causes massive macrophage apoptosis. A recent report demonstrates that liver phagocytes can undergo apoptotic caspase 3-mediated cell death in vivo, with apoptosis being a rare event, more prevalent in heavily *Salmonella*-infected cells (18). Once *Salmonella* has established a systemic infection, excess macrophage apoptosis would be detrimental to the pathogen because it utilizes macrophages as vectors for systemic dissemination throughout the host. Thus, SPI1 gene expression must be suppressed to allow sufficient time for the bacteria to replicate, escape, and invade new macrophages. Induction of the σ^{32}-mediated heat shock regulon by *S. enterica* serovar Typhimurium that has met the hostile environment within macrophages would be necessary to restrict SPI1 expression. That is quite important for suppressing apoptosis sufficiently to allow time for *Salmonella* to replicate within macrophages. Since the increased DnaKJ and GroES chaperone machineries directly regulate σ^{32} by using a chaperone network (19), the heat shock response would ensure an appropriate level of Lon as a negative regulator of SPI1 expression. In addition, the induced levels of chaperone machineries would be required to cope with the accumulation of partially unfolded or denatured proteins in cells exposed to the intracellular stresses associated with phagocytosis. Bacterial pathogenesis generally depends on the environmental conditions inside host cells. Interaction of specific virulence factors, e.g., the SPI1 TTSS in *Salmonella*, with global regulators such as σ^{32} responding to environmental signals would contribute to the spatiotemporal regulation of multistage pathogenesis.

ACKNOWLEDGMENTS

We thank A. Tokumitsu 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.

REFERENCES

1. **Abu Kwaik, Y., B. I. Eisenstein, and N. C. Engleberg.** 1993. Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages. Infect. Immun. **61:**1320–1329.
2. **Akbar, S., L. M. Schechter, C. P. Lostroh, and C. A. Lee.** 2003. AraC/XylS family members, HilD and HilC, directly activate virulence gene expression independently of HilA in *Salmonella typhimurium*. Mol. Microbiol. **47:**715–728.
3. **Bajaj, V., C. Hwang, and C. A. Lee.** 1995. *hilA* is a novel *ompR*/*toxR* family member that activates the expression of *Salmonella typhimurium* invasion genes. Mol. Microbiol. **18:**715–727.
4. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. Mol. Microbiol. **22:**703–714.
5. **Baxter, M. A., and B. D. Jones.** 2005. The *fimYZ* genes regulate *Salmonella enterica* serovar Typhimurium invasion in addition to type 1 fimbrial expression and bacterial motility. Infect. Immun. **73:**1377–1385.
6. **Behlau, I., and S. I. Miller.** 1993. A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. J. Bacteriol. **175:**4475–4484.
7. **Boddicker, J. D., B. M. Knosp, and B. D. Jones.** 2003. Transcription of the *Salmonella* invasion gene activator, *hilA*, requires HilD activation in the absence of negative regulators. J. Bacteriol. **185:**525–533.
8. **Brennan, M. A., and B. T. Cookson.** 2000. *Salmonella* induces macrophage death by caspase-1-dependent necrosis. Mol. Microbiol. **38:**31–40.

9. **Buchmeier, N. A., and F. Heffron.** 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science **248:**730–732.
10. **Darwin, K. H., and V. L. Miller.** 2001. Type III secretion chaperone-dependent regulation: activation of virulence genes by SicA and InvF in *Salmonella typhimurium*. EMBO J. **20:**1850–1862.
11. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
12. **Eichelberg, K., and J. E. Galán.** 1999. Differential regulation of *Salmonella typhimurium* type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and HilA. Infect. Immun. **67:**4099–4105.
13. **Ellermeier, C. D., J. R. Ellermeier, and J. M. Slauch.** 2005. HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator *hilA* in *Salmonella enterica* serovar Typhimurium. Mol. Microbiol. **57:**691–705.
14. **Ellermeier, J. R., and J. M. Slauch.** 2003. RtsA and RtsB coordinately regulate expression of the invasion and flagellar genes in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **185:**5096–5108.
15. **Ellermeier, J. R., and J. M. Slauch.** 2007. Adaptation to the host environment: regulation of the SPI1 type III secretion system in *Salmonella enterica* serovar Typhimurium. Curr. Opin. Microbiol. **10:**24–29.
16. **Galán, J. E.** 1999. Interaction of *Salmonella* with host cells through the centisome 63 type III secretion system. Curr. Opin. Microbiol. **2:**46–50.
17. **Gamer, J., H. Bujard, and B. Bukau.** 1992. Physical interaction between heat shock proteins DnaK, DnaJ, and GrpE and the bacterial heat shock transcription factor σ^{32}. Cell **69:**833–842.
18. **Grant, A. J., M. Sheppard, R. Deardon, S. P. Brown, G. Foster, C. E. Bryant, D. J. Maskell, and P. Mastroeni.** 20 February 2008. Caspase-3-dependent phagocyte death during systemic *Salmonella enterica* serovar Typhimurium infection of mice. Immunology **125:**28–37.
19. **Guisbert, E., C. Herman, C. Z. Lu, and C. A. Gross.** 2004. A chaperone network controls the heat shock response in *E. coli*. Genes Dev. **18:**2812–2821.
20. **Gulig, P., and R. Curtiss III.** 1987. Plasmid-associated virulence of *Salmonella typhimurium*. Infect. Immun. **55:**2891–2901.
21. **Hansen-Wester, I., and M. Hensel.** 2001. *Salmonella* pathogenicity islands encoding type III secretion systems. Microbes Infect. **3:**549–559.
22. **Hartl, F. U.** 1996. Molecular chaperones in cellular protein folding. Nature **381:**571–580.
23. **Hayward, R. D., and V. Koronakis.** 1999. Direct nucleation and bundling of actin by the SipC protein of invasive *Salmonella*. EMBO J. **18:**4926–4934.
24. **Herman, C., and R. D'Arit.** 1998. Proteolysis and chaperones: the destruction/reconstruction dilemma. Curr. Opin. Microbiol. **1:**204–209.
25. **Hersh, D., D. M. Monack, M. R. Smith, N. Ghori, S. Falkow, and A. Zychlinsky.** 1999. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. Proc. Natl. Acad. Sci. USA **96:**2396–2401.
26. **Jesenberger, V., K. J. Procyk, J. Yuan, S. Reipert, and M. Baccarini.** 2000. *Salmonella*-induced caspase-2 activation in macrophages: a novel mechanism in pathogen-mediated apoptosis. J. Exp. Med. **192:**1035–1046.
27. **Kage, H., A. Takaya, M. Ohya, and T. Yamamoto.** 2008. Coordinated regulation of expression of *Salmonella* pathogenicity island 1 and flagellar type III secretion systems by ATP-dependent ClpXP protease. J. Bacteriol. **190:**2470–2478.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
29. **Lee, C. A., B. D. Jones, and S. Falkow.** 1992. Identification of *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. Proc. Natl. Acad. Sci. USA **89:**1847–1851.
30. **Lee, C. A., M. Silva, A. M. Siber, A. J. Kelly, E. Galyov, and B. A. McCormick.** 2000. A secreted *Salmonella* protein induces a proinflammatory response in epithelial cells, which promotes neutrophil migration. Proc. Natl. Acad. Sci. USA **97:**12283–12288.
31. **Liberek, K., and C. Georgopoulos.** 1993. Autoregulation of the *Escherichia coli* heat shock response by the DnaK and DnaJ heat shock proteins. Proc. Natl. Acad. Sci. USA **90:**11019–11023.
32. **Lin, J., and T. A. Ficht.** 1995. Protein synthesis in *Brucella abortus* induced during macrophage infection. Infect. Immun. **63:**1409–1414.
33. **Lostroh, C. P., and C. A. Lee.** 2001. The *Salmonella* pathogenicity island-1 type III secretion system. Microbes Infect. **3:**1281–1291.
34. **Lucas, R. L., and C. A. Lee.** 2001. Roles of *hilC* and *hilD* in regulation of *hilA* expression in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **183:**2733–2745.
35. **Lucas, R. L., C. P. Lostroh, C. C. Dirusso, M. P. Spector, B. L. Wanner, and C. A. Lee.** 2000. Multiple factors independently regulate *hilA* and invasion gene expression in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **182:**1872–1882.
36. **Lundberg, U., U. Vinatzer, D. Berdnic, A. von Gabain, and M. Baccarini.** 1999. Growth phase-regulated induction of *Salmonella*-induced macrophage apoptosis correlates with transient expression of SPI-1 genes. J. Bacteriol. **181:**3433–3437.
37. **Mayer, M.** 1995. A new set of useful cloning and expression vectors derived from pBlueScript. Gene **163:**41–46.
38. **McCormick, B. A., C. A. Parkos, S. P. Colgan, D. K. Carnes, and J. L. Madara.** 1998. Apical secretion of a pathogen-elicited epithelial chemoattractant activity in response to surface colonization of intestinal epithelia by *Salmonella typhimurium*. J. Immunol. **160:**455–466.
39. **Monack, D. M., B. Raupach, A. E. Hromockyj, and S. Falkow.** 1996. *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. Proc. Natl. Acad. Sci. USA **93:**9833–9838.
40. **Morimoto, R. I.** 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev. **12:**3788–3796.
41. **Olekhnovich, I. N., and R. J. Kadner.** 2002. DNA-binding activities of the HilC and HilD virulence regulatory proteins of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **184:**4148–4160.
42. **Pegues, D. A., M. J. Hantman, I. Behlan, and S. I. Miller.** 1995. PhoP/Q transcriptional repression of *Salmonella typhimurium* invasion genes: evidence for a role in protein secretion. Mol. Microbiol. **17:**169–181.
43. **Romeo, T., M. Gong, M. Y. Liu, and A. M. Brun-Zinkernagel.** 1993. Identification and molecular characterization of *csrA*, a pleiotropic gene from *Escherichia coli* that affects glycogen biosynthesis, gluconeogenesis, cell size, and surface properties. J. Bacteriol. **175:**4744–4755.
44. **Schechter, L., and C. A. Lee.** 2001. AlaC/XylS family members, HilC and HilD, directly bind and derepress the *Salmonella typhimurium hilA* promoter. Mol. Microbiol. **40:**1289–1299.
45. **Schechter, L., S. Damrauer, and C. A. Lee.** 1999. Two AlaC/XylS family members can independently counteract the effect of repressing sequences upstream of the *hilA* promoter. Mol. Microbiol. **32:**629–642.
46. **Scherer, C. A., E. Cooper, and S. I. Miller.** 2000. The *Salmonella* type III secretion translocon protein SspC is inserted into the epithelial cell plasma membrane upon infection. Mol. Microbiol. **37:**1133–1145.
47. **Schneider, K., and C. Beck.** 1986. Promoter-probe vectors for the analysis of divergently arranged promoters. Gene **42:**37–48.
48. **Straus, D., W. Walter, and C. A. Gross.** 1989. The activity of σ^{32} is reduced under conditions of excess heat shock protein production in *Escherichia coli*. Genes Dev. **3:**2003–2010.
49. **Straus, D., W. Walter, and C. A. Gross.** 1990. DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis and stability of σ^{32}. Genes Dev. **4:**2202–2209.
50. **Takaya, A., A. Suzuki., Y. Kikuchi, M. Eguchi, E. Isogai, T. Tomoyasu, and T. Yamamoto.** 2005. Derepression of *Salmonella* pathogenicity island 1 genes within macrophages leads to rapid apoptosis via caspase-1- and caspase-3-dependent pathways. Cell. Microbiol. **7:**79–90.
51. **Takaya, A., M. Matsui, T. Tomoyasu, M. Kaya, and T. Yamamoto.** 2006. The DnaK chaperone machinery converts the native $FlhD_2C_2$ hetero-tetramer into a functional transcriptional regulator of flagellar regulon expression in *Salmonella*. Mol. Microbiol. **59:**1327–1340.
52. **Takaya, A., M. Suzuki, H. Matsui, T. Tomoyasu, H. Sashinami, A. Nakane, and T. Yamamoto.** 2003. Lon, a stress-induced ATP-dependent protease, is critically important for systemic *Salmonella enterica* serovar Typhimurium infection of mice. Infect. Immun. **71:**690–696.
53. **Takaya, A., T. Tomoyasu, A. Tokumitsu, M. Morioka, and T. Yamamoto.** 2002. The ATP-dependent *lon* protease of *Salmonella enterica* serovar Typhimurium regulates invasion and expression of genes carried on *Salmonella* pathogenicity island 1. J. Bacteriol. **184:**224–232.
54. **Takaya, A., T. Tomoyasu, H. Matsui, and T. Yamamoto.** 2004. The DnaK/DnaJ chaperone machinery of *Salmonella enterica* serovar Typhimurium is essential for invasion of epithelial cells and survival within macrophages, leading to systemic infection. Infect. Immun. **72:**1364–1373.
55. **Takaya, A., Y. Kubota, E. Isogai, and T. Yamamoto.** 2005. Degradation of the HilC and HilD regulator proteins by ATP-dependent Lon protease leads to downregulation of *Salmonella* pathogenicity island 1 gene expression. Mol. Microbiol. **55:**839–852.
56. **Tilly, K., J. Speuce, and C. Georgopoulos.** 1989. Modulation of stability of the *Escherichia coli* heat shock regulatory factor σ^{32}. J. Bacteriol. **171:**1585–1589.
57. **Tomoyasu, T., A. Mogk, H. Langen, P. Goloubinoff, and B. Bukau.** 2001. Genetic dissection of the roles of changes and proteases in protein folding and degradation in the *Escherichia coli* cytosol. Mol. Microbiol. **40:**397–413.
58. **Tomoyasu, T., T. Ogura, T. Tatsuya, and B. Bukau.** 1998. Levels of DnaK and DnaJ provide tight control of heat shock gene expression and protein repair in *Escherichia coli*. Mol. Microbiol. **30:**567–581.
59. **Wallis, T. S., and E. E. Galyov.** 2000. Molecular basis of *Salmonella*-induced enteritis. Mol. Microbiol. **36:**997–1005.
60. **Yamamoto, T., H. Sashinami, A. Takaya, T. Tomoyasu, H. Matsui, Y. Kikuchi, T. Hanawa, S. Kamiya, and A. Nakane.** 2001. Disruption of the genes for ClpXP protease in *Salmonella enterica* serovar Typhimurium results in persistent infection in mice, and development of persistence requires endogenous gamma interferon and tumor necrosis factor alpha. Infect. Immun. **69:**3164–3174.

61. **Yamamoto, T., T. Hanawa, and S. Ogata.** 1994. Induction of *Yersinia enterocolitica* stress proteins by phagocytosis with macrophage. Microbiol. Immunol. **38:**295–300.
62. **Yura, T., H. Nagai, and H. Mori.** 1993. Regulation of the heat shock response in bacteria. Annu. Rev. Microbiol. **47:**321–350.
63. **Zeuner, A., A. Eramo, C. Peschle, and R. De Maria.** 1999. Caspase activation without death. Cell Death Differ. **6:**1075–1080.
64. **Zhao, K., M. Liu, and R. R. Burgess.** 2005. The global transcriptional response of *Escherichia coli* to induced σ^{32} protein involves σ^{32} regulon activation followed by inactivation and degradation of σ^{32} *in vivo*. J. Biol. Chem. **280:**17758–17768.
65. **Zhou, D., M. S. Mooseker, and J. E. Galán.** 1999. Role of the *S. typhimurium* actin-binding protein SipA in bacterial internalization. Science **283:**2092–2095.