

RESEARCH PAPER



Deletion of the *lon* gene augments expression of *Salmonella* Pathogenicity Island (SPI)-1 and metal ion uptake genes leading to the accumulation of bactericidal hydroxyl radicals and host pro-inflammatory cytokine-mediated rapid intracellular clearance

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ABSTRACT

In the present study, we characterized the involvement of Lon protease in bacterial virulence and intracellular survival in *Salmonella* under abiotic stress conditions resembling the conditions of a natural infection. Wild type (JOL401) and the *lon* mutant (JOL909) *Salmonella* Typhimurium were exposed to low temperature, pH, osmotic, and oxidative stress conditions and changes in gene expression profiles related to virulence and metal ion uptake were investigated. Expression of candidate genes *invF* and *hilC* of *Salmonella* Pathogenicity Island (SPI)-1 and *sifA* and *sseJ* of SPI-2 revealed that Lon protease controls SPI-1 genes and not SPI-2 genes under all stress conditions tested. The *lon* mutant exhibited increased accumulation of hydroxyl (OH[•]) ions that lead to cell damage due to oxidative stress. This oxidative damage can also be linked to an unregulated influx of iron due to the upregulation of ion channel genes such as *fepA* in the *lon* mutant. The deletion of *lon* from the *Salmonella* genome causes oxidative damage and increased expression of virulence genes. It also prompts the secretion of host pro-inflammatory cytokines leading to early clearance of the bacteria from host cells. We conclude that poor bacterial recovery from mice infected with the *lon* mutant is a result of disrupted bacterial intracellular equilibrium and rapid activation of cytokine expression leading to bacterial lysis.

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Introduction

Salmonella enterica causes a spectrum of diseases in animals and humans, ranging between mild infection to severe infection that can be fatal¹. Different strains of *Salmonella enterica* Typhimurium cause enteric infections in humans and other warm-blooded animals, whereas the serovar Typhi is restricted to humans causing typhoid fever. All *Salmonella* species possess *Salmonella* Pathogenicity Island I (SPI-1) despite variations in virulence factors among different serotypes. Coordinated expression of genes belonging to SPI-1 and SPI-2 are important for initial entry and the subsequent establishment of infection in a target host. The SPI-1 encodes proteins including a *Salmonella* type III secretion system (TTSS) that is responsible for injecting effector molecules that alter cytoskeletal arrangement in the host and help bacterial internalization. Once the bacterium is in the host

cell, genes in SPI-2 are expressed that encodes another type of TTSS that secretes effectors diverting endocytic trafficking to prevent lysosome recruitment and fusion of *Salmonella*-containing vacuoles. This process helps host-adapted *Salmonella* reach extraintestinal organs and establish a systemic infection. *Salmonella* invasion is a complex and intricate process that essentially controls virulence. Concerning invasion and virulence of *Salmonella*, studies have revealed that Lon protease plays a significant role during early infection and colonization of *Salmonella*.² As a member of the AAA+ superfamily of proteases that also includes ClpA, ClpB, and FtsH, Lon has been extensively studied in *Escherichia coli*.^{3–5} The Lon protease plays a vital role in protein quality control by eliminating faulty proteins during stress responses and activating pathways to prevent any further damage to the bacterial cell.⁶

Moreover, recent reports indicate the involvement of Lon protease in antimicrobial resistance, further broadening its role in bacterial survival.⁷

The involvement of Lon in *Salmonella* SPI-1 gene expression has been previously reported, which proposed a link between the SPI-1 regulator HilA and Lon protease. HilA is an OmpR/ToxR family protein⁸ that is activated by the transcriptional regulator InvF, which in turn regulates the transcriptional activation of effector genes. In *Salmonella*, *hilA* expression is modulated by the HilC and HilD transcriptional regulators.⁹ Therefore, the interaction of Lon with the SPI-1 axis of virulence modulation is worth further elaboration under various environmental and physiological stress conditions to investigate the survival and virulence behavior of *Salmonella*.² During the natural infection cycle, *Salmonella* encounters various stress conditions including a transition to increased body temperature, acidic pH in the stomach, high salt and osmolarity in bile salts, and oxidative stress in the lumen as a result of the host inflammatory response.¹⁰ During systemic infection, the host attempts to starve *Salmonella* by limiting nutrient and metal ion uptake; in response, *Salmonella* manipulates ion and nutrient uptake gene expression to counter the host restrictions.^{11,12} Further, *Salmonella* attempts to circumvent the recruitment of antigen-presenting cells to the site of infection by interacting with the host immune pathways¹³ and exploits the metabolic pathways of the host for its survival.¹⁴

Apart from manipulating these host mechanisms, the bacteria modulate their biosynthesis machinery that determines their fitness and virulence.^{14,15} As a facultative anaerobe, *Salmonella* undergoes metabolic reprogramming depending on the presence or absence of oxygen. One of the principal determinants of this process is redox homeostasis.¹⁶ On the other hand, reactive oxygen species (ROS) produced during microbial metabolism and the stress response can alter the redox state of a cell.¹⁷ Gene deletions or mutations hindering intracellular iron regulation is known to cause the accumulation of excess free cytosolic iron, leading to iron-catalyzed production of ROS.^{18,19} This endogenous oxidative stress is regarded as an unwanted and damaging by-product of cellular metabolism.

Here, we describe the quantification of gene expression involved in pathogenesis including genes that

encode SPI effector proteins, genes regulating iron uptake, and levels of hydroxyl (OH·) radical accumulation in *lon*-mutant *Salmonella*; JOL 909 after exposure to various conditions including acidic, cold, osmotic, and oxidative stress treatments. The changes in gene expression were compared with that of parental wild type strain *Salmonella* Typhimurium; JOL 401 and *lon* complemented *Salmonella* strain JOL 909::*lon*. We analyzed gene expression after 5 h of exposure and found that JOL 909 exhibited the upregulation of SPI-1 virulence-associated and iron uptake genes, followed by increased accumulation of OH· in the *lon* mutant suggesting the participation of Lon in modulating bacterial internal stability. Further, a distinct pro-inflammatory cytokine response in the host shows that Lon alters bacterial interaction with the host TLR; a hypothesis that needs further validation.²⁰

Although a previous study reports the inability of a *lon* mutant to survive phagocytic lysis, the *lon*-specific molecular mechanism was not elucidated¹. Here, our observations show that *lon* regulates intracellular homeostasis when *Salmonella* maneuvers through the host cell to establish an infection. In the absence of this regulator, an unchecked influx of iron cations occurs that reacts with H₂O₂ produced within the bacteria, as well as inflow from the host cells, leading to the formation of OH· radicals. Hydroxyl ion-mediated oxidative stress leads to DNA damage and subsequent bacterial lysis. The present work adds a new level of information about Lon protease and its role in tightly regulated bacterial stress-response pathways. Here, we show that Lon in *Salmonella* is essential for mediating regulated virulence behavior that may ensure the successful establishment of systemic infection.

Results

Characterization of a *lon* mutant in *Salmonella*

The Lon protease is a global regulator that regulates the expression of early virulence genes located in SPI-1. Mechanistic studies have revealed Lon as a negative regulator of SPI-1 that regulates and ensures the successful establishment of *Salmonella* in a target host cell. Herein, we investigated the involvement of the *lon* gene in virulence modulation

of *Salmonella* upon exposure to various abiotic stress conditions. To examine this phenomenon, we used wild type strain ST JOL 401 and a *lon* deleted strain ST JOL 909. To further confirm that the ability to adhere and invade epithelial cells and metal ion uptake associated genes in JOL 909 are modulated as a result of *lon* knockout, a functional *lon* gene was cloned in a low copy-number vector, pWSK 29, and the resultant plasmid was used to transform JOL 909, resulting in strain JOL 909::*lon*. To assess whether the *lon* deletion causes growth defects in *Salmonella*, a growth curve analysis of all three strains was carried out. Results demonstrated that the *lon* deletion did not significantly alter the growth characteristics of *Salmonella* (Supplementary Figure 1). The JOL 909 strain exhibited enhanced adhesion and invasion compared to JOL 401. Such enhanced adhesion and invasion were inhibited by *lon* complementation in JOL 909, showing that Lon plays a crucial role in regulating both adhesion and invasion. The adhesive and invasive phenotype of the *lon* mutant JOL 909 was demonstrated to be similar in all three cell lines HeLa, HepG2, and Caco-2 (Figure 1a). However, when the JOL 401 and JOL 909 were exposed to anaerobic conditions their adhesion and invasion ability increased drastically. The increased invasion by JOL 909 was further evident in fluorescent microscopy conducted using CellTracker-labeled *Salmonella*; however, their population was significantly reduced in both non-phagocytic (data not shown) and phagocytic cells (Figure 1b,c) earlier than the wild type and complemented strains.

Actin rearrangement by *Salmonella*

Salmonella induced actin rearrangement in a host cell is essential for *Salmonella* entry and survival. Such manipulation helps *Salmonella* in better vacuolization and prevents lysosomal engagement allowing them to thrive in the host. Such phenotypic changes in the host cell induced by JOL 401 and JOL 909 were recorded during the entry phase, characterized by the trigger or zipper mechanisms. In HeLa cells (Figure 1a), both JOL 401 and JOL 909 induced lamellipodia formation characterized by membrane ruffling and formation of appendages engulfing the bacteria. However, in HepG2 cells (Figure 2b), the strains did not induce the formation of membrane extensions most likely due to the

engagement of the zipper mechanism. Although no lamellipodia formation was evident in Caco-2 cells, a mild yet distinct membrane ruffling was observed when infected with JOL 401 and JOL 909 (Figure 2c). These results show that the regulation of host actin polymerization is independent of a Lon-mediated regulatory pathway. We hypothesize that this rearrangement is also partly dependent on the host cell type. To further ensure independent behavior on actin rearrangement, microscopic observations were conducted at varying time intervals of 15, 30, 45, 60, 90, and 150 min post-inoculation.

Effect of *lon* on bacterial hydroxyl ion generation

The generation of reactive oxygen species (ROS) is a hallmark of the bacterial stress response. In bacteria, ROS are readily detoxified by enzymatic conversion of ROS ($\text{OH}\cdot$) into hydrogen peroxide and subsequently to water and oxygen. However, in the presence of iron ions, the Fenton reaction can generate $\text{OH}\cdot$ that are lethal to bacterial cells.²¹ To elucidate the kinetics of $\text{OH}\cdot$ generation in JOL 401, JOL909, and JOL909::*lon*, cells were exposed to various naturally-occurring stress conditions for 5 h. JOL 401 was able to effectively mitigate $\text{OH}\cdot$ generation under cold and oxidative stress; however, the production of $\text{OH}\cdot$ was significantly higher under osmotic and acidic pH conditions. Under all stress conditions, JOL 909 appeared to have produced higher levels of $\text{OH}\cdot$. The maximum level of $\text{OH}\cdot$ production in JOL 909 was observed under oxidative stress, suggesting the role of Lon protease in mitigating oxidative stress. The involvement of Lon seems to be comparatively less with osmotic and acidic stress conditions. The results were corroborated with JOL 909::*lon*, which effectively diminished $\text{OH}\cdot$ production. These results imply the critical involvement of Lon protease activity in mitigating oxidative stress in the phagosomes of host cells²² (Figure 3). It is worthwhile to note that JOL 909::*lon* exhibited lower levels of $\text{OH}\cdot$ compared to JOL 401. As the overproduction of Lon can prove fatal for bacterial cells, these low levels may be due to slightly higher levels of Lon protein in JOL 909 complemented in *trans* by a low-copy-number plasmid.

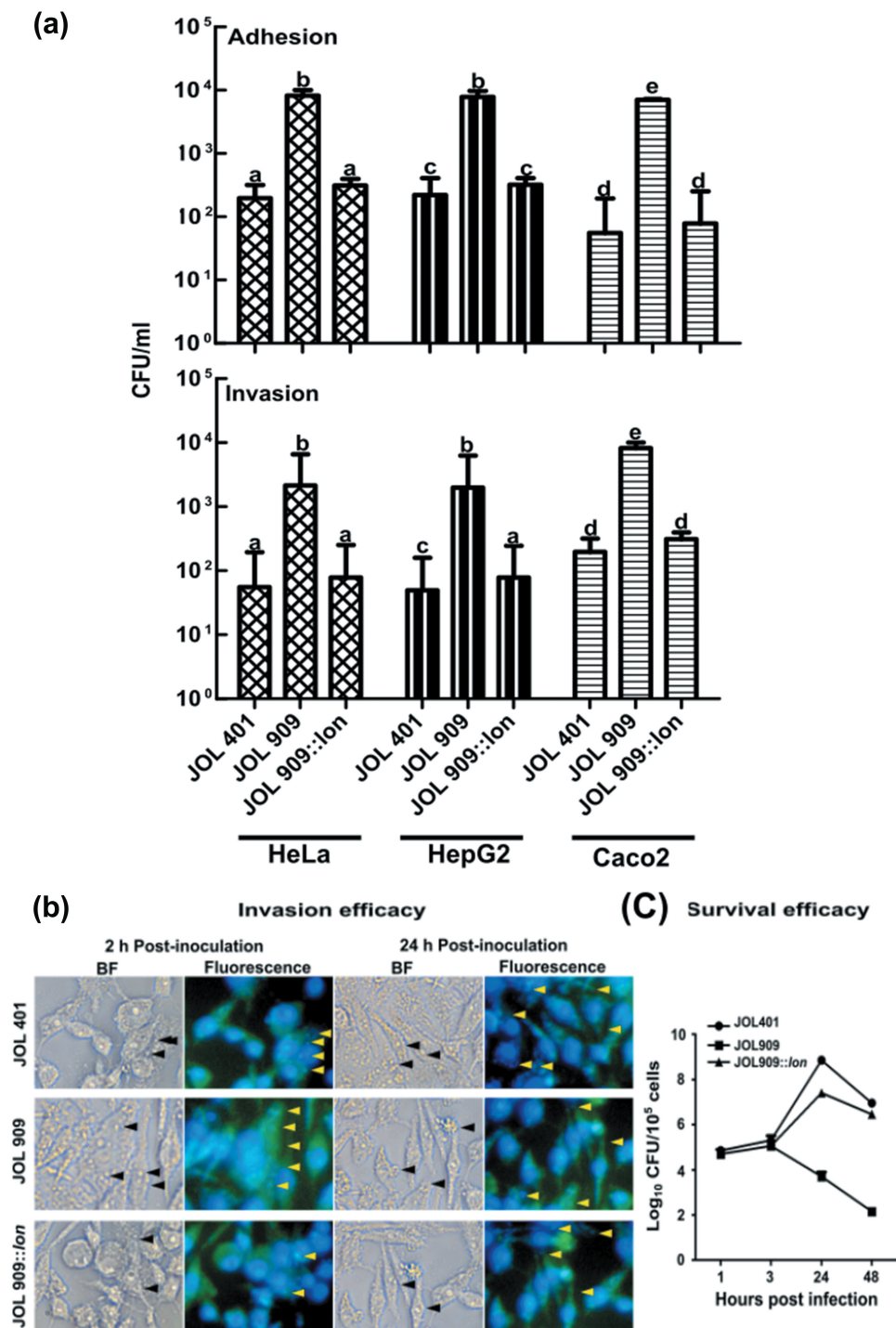


Figure 1. Role of *lon* in Adhesion and Invasion of HeLa and HepG2 and macrophage survival. (A) Adhesion and Invasion of HeLa, HepG2, and Caco-2 monolayer were observed post-infection with JOL 401, JOL 909, and JOL 909::lon (MOI = 20). The data represent the adhesion and invasion (Log CFU/mL) \pm the SD from at least three independent experiments performed in duplicates. *P* values of <0.05 were considered significantly different, and bars with the same lowercase letter are not significantly different from one another. (B) Fluorescent microscopy images of RAW cells infected with *Salmonella* (JOL 401, JOL 909, and JOL 909::lon) containing pHLJ65::gfp plasmid to visualize the live (green fluorescent) bacteria within the cells. (C) Intracellular survival of *Salmonella* wild type, *lon* mutant, and complemented strain in RAW cells.

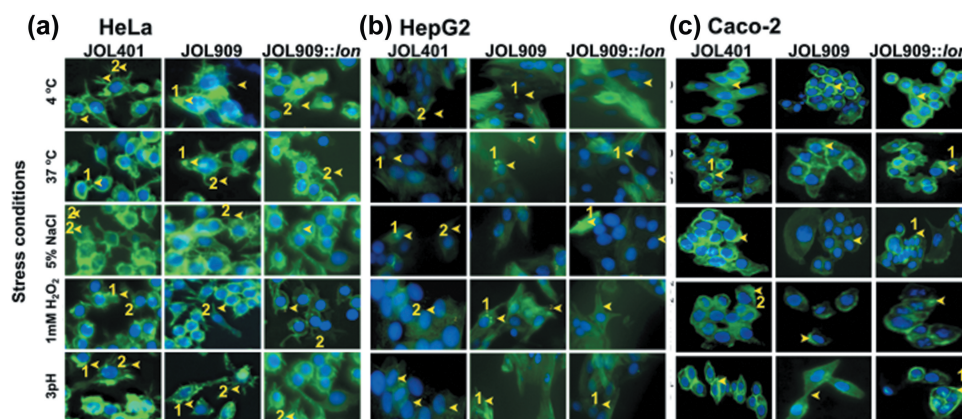


Figure 2. Actin polymerization is a distinctive attribute of individual host cells. Microscopic images show membrane ruffling and lamellipodia formation. (A) HeLa cells (B) HepG2 cells (C) Caco-2 cells were infected with stress exposed *Salmonella* (JOL 401, JOL 909, and JOL 909::*lon*; MOI = 20) for 30 min and stained with phalloidin (for F-actin) and DAPI (nuclear stain); 1-actin condensation, 2-lamellipodia. These observations demonstrate that *Salmonella* enters the HeLa cells vigorously rearranging the host actin filaments via a trigger mechanism. The interaction with HepG2 cells results in a zipper-type of actin rearrangement. The interaction with Caco-2 cells results in a minimum ruffling of the membrane.

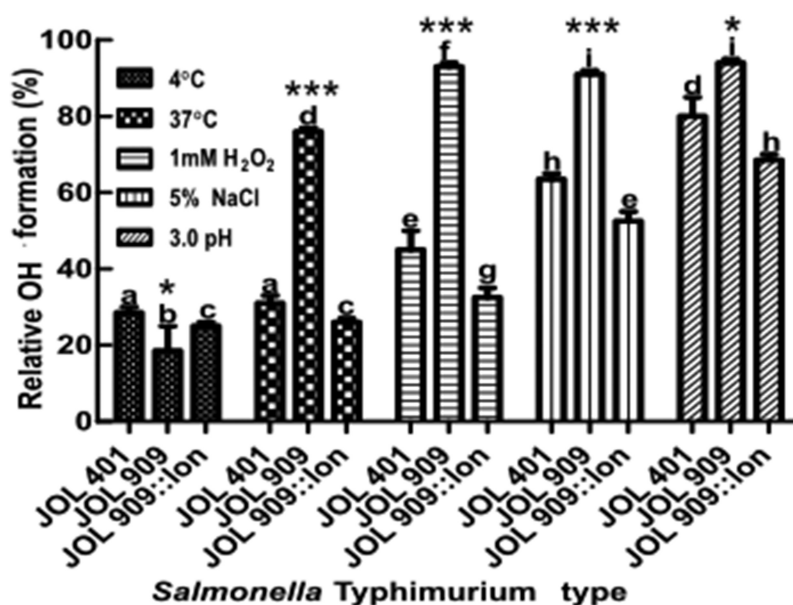


Figure 3. Endogenous Hydroxyl radical (OH•) formation during stress treatments in the *lon* mutant. JOL 401, JOL 909 and JOL 909::*lon* were exposed to different abiotic stress (cold, oxidative, osmotic, and acidic) for 5 h and their endogenous hydroxyl radical levels were measured and the values were compared with the bacterial cells grown at 37°C without any stress inducers. The data was represented as relative OH• formation \pm the SD from at least three independent experiments performed in duplicates. Means were compared by Tukey's multiple comparison test. Similar lowercase letters indicate the non-significant difference of means ($p > .05$). Significant difference (* $p < .05$, *** $p < .0001$) compared to the wild type control.

Effect of *lon* deletion on metal ion uptake and virulence gene expression

To investigate the role of *lon* in *Salmonella* virulence under the aforementioned stress conditions, the expression profile of four genes encoding outer membrane proteins, namely *ompD*, *ompF*, *fepA*, and *csgB* was studied by qRT-PCR. Among these

genes, *csgB* gene expression was significantly upregulated under cold stress, which lead us to believe that Lon protease, a member of the heat shock protein (Hsp) family, also regulates the expression of genes that are primarily activated below 37°C. The other three outer membrane genes were downregulated in response to cold stress. Under osmotic

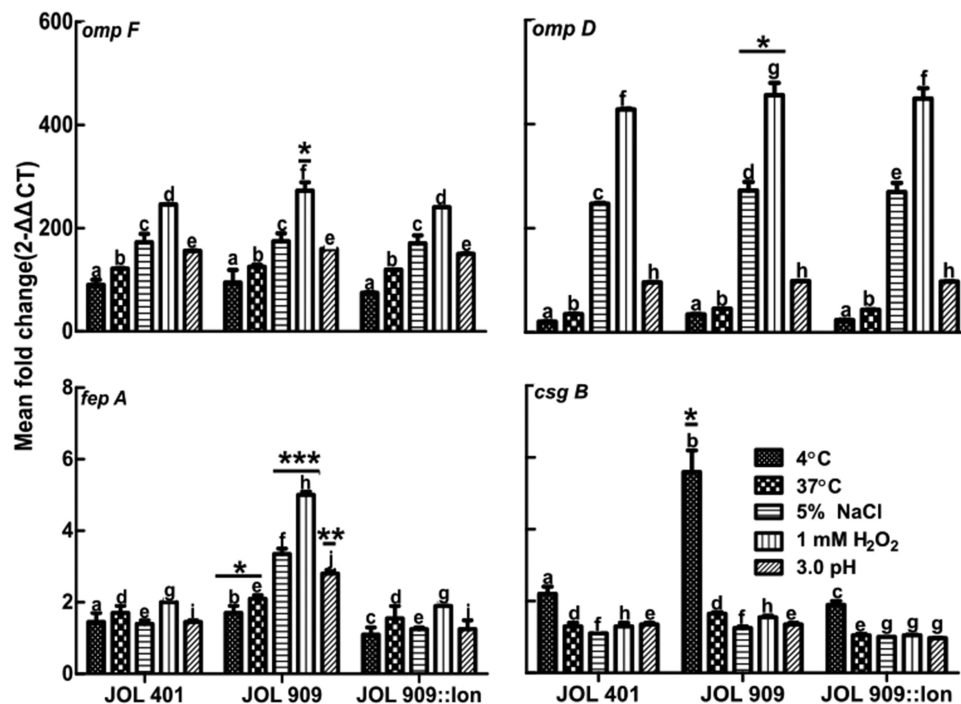


Figure 4. Expression of bacterial cell surface protein genes during stress treatments. JOL 401, JOL 909 and JOL 909::lon were subjected to abiotic (oxidative-1 mM H₂O₂; acidic-3.0 pH; osmotic- 5% NaCl and cold-4°C) stress treatment for 5 h. The regulation of the *ompF*, *ompD*, *fepA*, and *csgB* were evaluated post-stress treatments. The results are expressed as mean fold change in the transcription levels of *ompF*, *ompD*, *fepA*, and *csgB* genes compared to unstimulated bacterial cells. The data was represented as relative mean fold change \pm the SD from at least three independent experiments performed in duplicates. Means were compared by Tukey's multiple comparison test. Similar lowercase letters indicate the non-significant difference of means ($p > .05$). Significant difference (* $p < .05$, ** $p < .01$, *** $p < .0001$) compared to the wild type control. *ompD*- Outer membrane protein D; *ompF*- Outer membrane protein F; *fepA*- Ferric Enterobactin Transporter; *csgB*- Minor Curlin subunit.

and acidic stress conditions, only *fepA* expression appeared to be regulated by Lon. When exposed to oxidative stress, *ompF*, and *fepA* expression were upregulated (Figure 4).

The upregulation of iron uptake regulator genes (*fur* regulon and *sitA*) and iron sequestration gene (*dps*) under various stress conditions was distinct. In particular, the *fur* regulon was highly upregulated in JOL 909 under osmotic and oxidative stress conditions. The increase in the mRNA level was similar to the increased expression levels of *fepA*, which is an iron ion channel on the bacterial cell surface (Figure 5). Also, JOL 909 exhibited a surge in the expression of *corA*, *mgtA*, and *mgtB* compared to JOL 401 (Figure 6), all three of which are transporters of divalent magnesium ions while *corA* is also associated with the uptake of ferrous ions.

Apart from modulating gene expression levels of outer membrane proteins under various stress conditions, Lon regulates the expression of SPI-1 transcriptional factors. The *invF* and *hilC* (Figure 7a)

genes were significantly upregulated under all stress conditions in JOL 909 compared to JOL 401, attributing it to be Lon-dependent. On the other hand, the SPI-2 genes *sifA* and *sseJ* were upregulated uniformly in both JOL 401 and JOL 909 (Figure 7b). The levels of *sifA* were observed to have increased up to 4-fold during oxidative and osmotic stress treatments. A similar trend was observed for *sseJ*, whose levels were upregulated 2-fold during oxidative, osmotic, and acidic stress. These observations suggest that the regulation of a cluster of genes in SPI-2 is partly independent of pathways involving Lon protease. SPI-1 is essential for invasion whereas SPI-2 is crucial for intracellular survival and systemic persistence.^{23,24}

fepA knockout and lon expression

Based on the aforementioned observations, we theorized that *fepA* is transcriptionally regulated by *lon* under stress conditions especially during oxidative

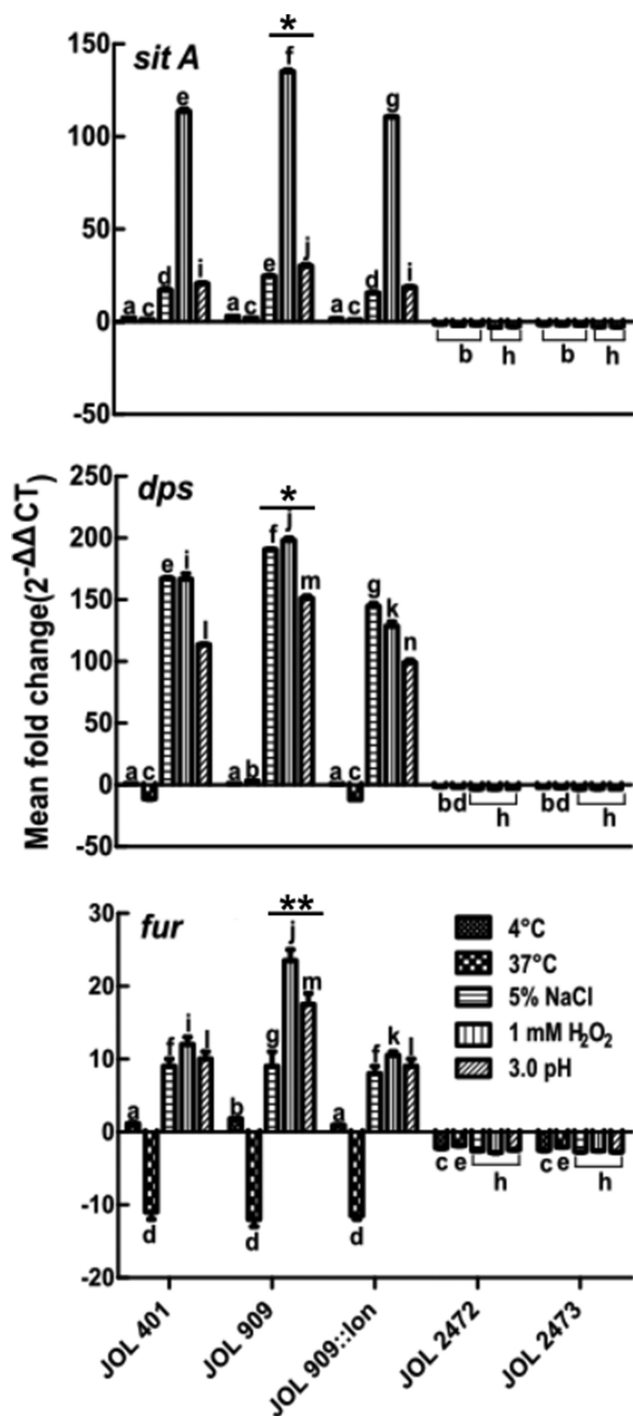


Figure 5. Expression of regulatory genes that participate in iron uptake and sequestration during stress exposure. Post-exposure to the abiotic stress factors the transcription levels of *sitA*, *dps*, and *fur* were evaluated in JOL 401, JOL 909, JOL 909::lon, JOL 2472, and JOL 2473. The results are expressed as mean fold change in the transcription levels of *sitA*, *dps*, and *fur* genes compared to unstimulated bacterial cells. The data was represented as relative mean fold change \pm the SD from at least three independent experiments performed 2 compared by Tukey's multiple comparison test. Similar lowercase letters indicate the non-significant difference of means ($p > .05$). Significant difference (* $p < .05$, ** $p < .01$) compared to the wild type control.

stress. To further understand the *fepA-lon* interaction, we constructed JOL 401 Δ *fepA* and JOL 909 Δ *fepA* and refer to them as JOL 2472 and JOL 2473, respectively (Supplementary Figure 2). To study the role of *fepA* in the regulation of *lon* expression, JOL 401 and JOL 2472 were exposed to the above-mentioned stress conditions for 5 h and their RNA was isolated to evaluate *lon* expression (Supplementary Figure 3). The expression of *lon* was upregulated in JOL 401 by \sim 10-fold under all the stress treatments, peaking at 15-fold changes on average during oxidative stress. On the other hand, *lon* expression increased up to 2-fold on average in JOL 2472 after treatment (Supplementary Figure 3). This was further followed by the downregulation of iron uptake and sequestration genes in JOL 2472 and JOL 2473 (Figure 5). Additionally, under the anaerobic condition, the *lon* expression decreased by 2-fold in both JOL 401 and JOL 2473.

In vitro bacterial survival and recovery from mice

The presence of colony-forming units (CFU) on BGA agar showed the ability of JOL 401 and JOL 909 to survive *in vitro* after exposure to abiotic stress conditions. Oxidative stress caused characteristic damage to JOL 909 wherein <4 log CFU/mL bacteria were observed compared to a log CFU close to 6 per mL for JOL 401 (Figure 8a,b). Although all the stress conditions hampered JOL 909 survival, the oxidative stress-induced damage was distinct. To study if the *in vitro* observations were consistent with the *in vivo* bacterial clearance, the spleens of infected mice were aseptically collected and bacterial load was evaluated. The results corroborated with the *in vitro* survival pattern wherein JOL 909 seemed to be eliminated by host phagocytosis and exerting oxidative stress on the invading bacteria. By day 12 post-infection, splenomegaly was evident, and <3 log CFU/mL JOL 909 were recovered from the spleen (Figure 8c).

Host cytokine responses

The role of Lon in eliciting the host immune response is crucial as it determines the fate of invading bacteria. The *in vitro* studies showed that JOL 909 upregulated the expression of pro-

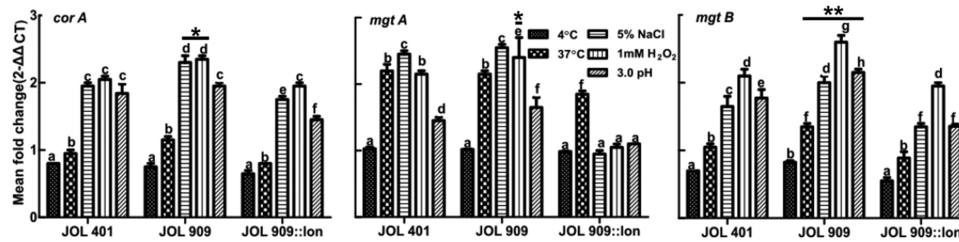


Figure 6. Regulation of Magnesium uptake associated genes. The mRNA expression of Magnesium uptake genes was studied after the bacterial cells were subjected to stress treatments for 5 h. The mean fold change in the levels of *corA*, *mgtA*, and *mgtB* in JOL 401, JOL 909, and JOL 909::lon were recorded. The data was represented as relative mean fold change \pm the SD from at least three independent experiments performed in duplicates. Means were compared by Tukey's multiple comparison test. Similar lowercase letters indicate the non-significant difference of means ($p > .05$). Significant difference (* $p < .05$, ** $p < .01$) compared to the wild type control.

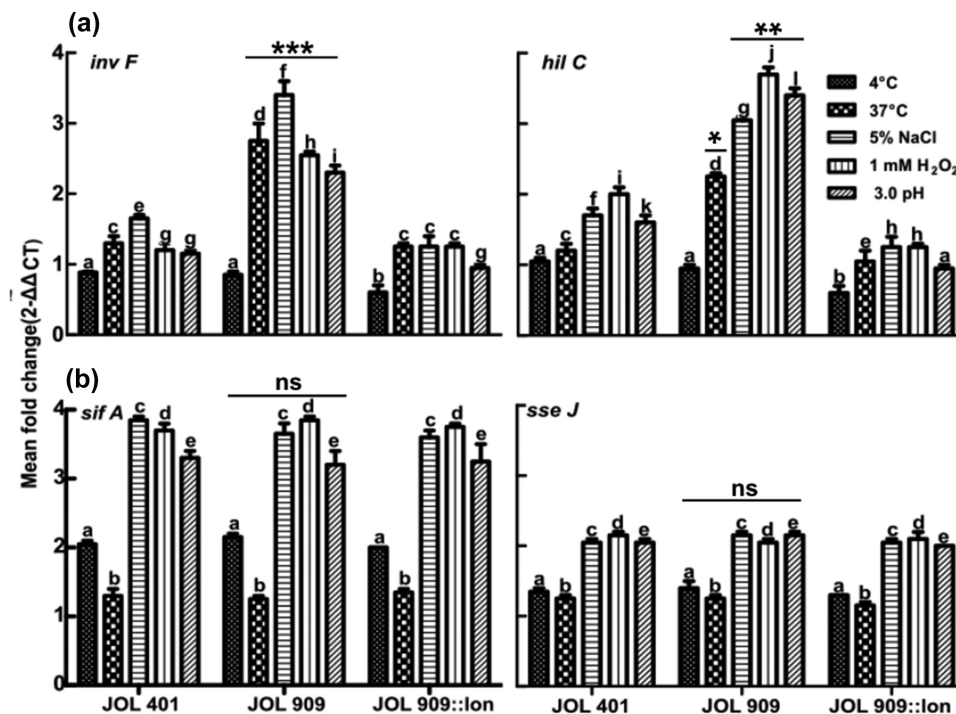


Figure 7. Expression of a virulence-associated gene from SPI 1 and 2 gene clusters. mRNA levels of (A) SPI1 genes-*invF* and *hilC* and (B) SPI2 genes-*sifA* and *sseJ* were recorded as relative mean fold changes using *rrsG* as the endogenous control. The data was represented as relative mean fold change \pm the SD from at least three independent experiments performed in duplicates. Means were compared by Tukey's multiple comparison test. Similar lowercase letters demonstrate the non-significant difference of means ($p > .05$). Significant difference (* $p < .05$, ** $p < .01$, *** $p < .001$) or no significant difference (ⁿ $p > .05$) compared to the wild type control.

inflammatory cytokines up to 48 hours post-infection (hpi). The levels of IL-2 and IFN- γ increased by about 80- and 200-fold on average in both non-phagocytic (HeLa and HepG2) and phagocytic (RAW) cells infected with JOL 909. Levels of the potent anti-inflammatory cytokine IL-10 were within the same range (up to 12-fold change) in cells infected with JOL 401 and JOL 909 (Figure 9). The levels of IL-4 increased in cells infected with JOL 909, which activates the adaptive immune response proving advantage to the host.

Discussion

The present study investigates the involvement of the Lon protease in *Salmonella* virulence and intracellular survival with respect to the ability to establish systemic infection. To dissect the details of Lon-mediated *Salmonella* virulence, we exposed a *lon* mutant JOL 909, wild type JOL 401, and *lon* complemented *Salmonella* JOL 909::lon to a range of abiotic stress conditions including temperature, osmolarity, oxidative and pH-induced stress and

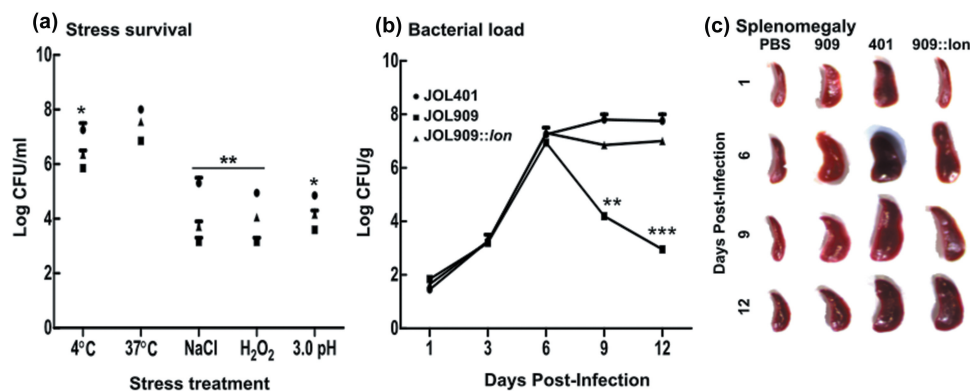


Figure 8. Bacterial recovery and survival to study the involvement of *lon* in bacterial longevity. (A) *In vitro* bacterial survival post-stress exposure was evaluated by serial dilution and plating of stress endured JOL 401, JOL 909, and JOL 909::*lon* on BGA plate. (B) The bacterial recovery was calculated by plating the serially diluted homogenized spleen (1 g) on BGA plates. (C) The spleens were collected aseptically from mice ($n = 3$; infected with JOL 401, JOL 909, and JOL 909::*lon*) on days 1, 6, 9, and 12 post-infection for evaluation of gross pathological lesions including splenomegaly. The data was represented as log CFU/mL \pm the SD from at least three independent experiments performed in duplicates. Means were compared by Tukey's multiple comparison test. Significant difference (* $p < .05$, ** $p < .01$, *** $p < .001$) compared to the wild-type control.

quantitatively assessed the expression of virulence and nutrient uptake genes, their interaction with host cells, and fate during an *in vivo* infection.¹ Survival during stress requires proficient manipulation of a myriad of genes; thus, the effect of Lon may be better elucidated under stress-induced virulence studies. Growth analysis experiments revealed that deletion of the *lon* gene was not detrimental to host cells and did not cause significant growth rate alteration as compared to the wild type strain. Therefore, the mutant could be utilized in subsequent studies comparable to the wild type strain. During the infection process, *Salmonella* will make contact with the host cell by attaching to the host cell through their receptors, and the level of adhesion and invasion are the very first virulence determinants for successful infection.^{25,26} The adhesion and invasion studies conducted on non-phagocytic HeLa, HepG2 and Caco-2 cells revealed the *lon* deletion significantly increased (approximately 2-fold increase) both adhesion and invasion into the host cell. This was confirmed by CFU counting and by fluorescence microscopy. When we examined persistence of the various *Salmonella* strains within host cells, it was evident that the *lon* mutant was significantly defective in intracellular survival compared to the wild type strain. Such a dramatic increase in virulence yet defective intracellular fitness in *lon* mutant hints at a link between Lon protease and SPI-1 genes that are important for the early stage of infection,^{27,28}

but not with SPI-2 genes that are primarily responsible for *Salmonella* survival in the host cell.^{29,30} The SPI-1 gene region encodes elements of Type III secretory system (TTSS) machinery that bacteria use to inject effector proteins at early stages of infection that cause actin rearrangement in the host cytoskeleton facilitating *Salmonella* entry into the host cell.^{31,32} This phenomenon is demarcated by membrane ruffling and the formation of protrusions called lamellipodia (Trigger mechanism). In certain cells like the HepG2, actin condensation is less profound, and the process is termed the Zipper mechanism. A similar observation was evident in Caco-2 cells wherein distinct membrane ruffling was seen without the formation of lamellipodia. These studies conducted on HeLa, HepG2, and Caco-2 cells for entry mechanism did not show significant alteration due to *lon* deletion. This suggests, even though Lon causes enhancement of *Salmonella* virulence, Lon may not influence the entry mechanism into the host cell.

Under normal physiological conditions, cells produce ROS that is harmful to cellular components.^{33–35} In healthy cells, the levels of ROS are maintained at harmless levels by enzymes such as superoxide dismutase, peroxidase, and catalase that convert reactive oxygen into H₂O₂ or water as the terminal product. However, in the presence of iron ions, H₂O₂ can revert to form OH· free radicals that are again harmful to cellular components. On the other hand, mammalian phagocytic cells use H₂O₂ as a defense

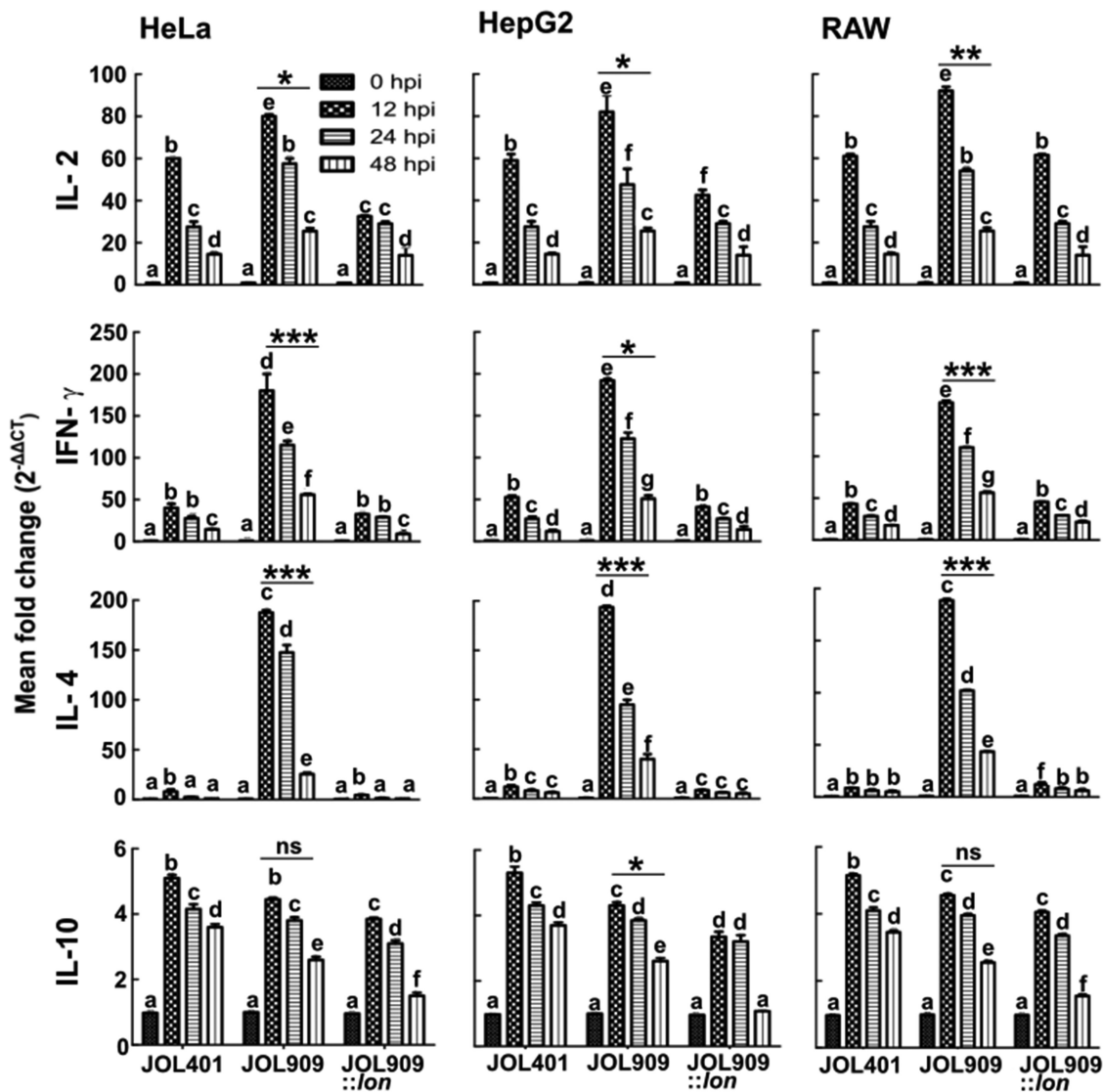


Figure 9. The kinetics of cytokine gene expression in the phagocytic and non-phagocytic epithelial cells. The *IL-2*, *IL-4*, *IFN- γ* , and *IL-10* mRNA expression in JOL 401, JOL 909, and JOL 909::lon infected HeLa, HepG2, and RAW cells were evaluated at various time points post-infection. The data was represented as relative mean fold change \pm the SD from at least three independent experiments performed in duplicates. The experiment was performed using GAPDH as the endogenous control. IL- Interleukin; IFN- Interferon. Means were compared by Tukey's multiple comparison test. Similar lowercase letters indicate the non-significant difference of means ($p > .05$). Significant difference (* $p < .05$, ** $p < .01$, *** $p < .001$) compared to the wild-type control.

tool against invading pathogens.^{35,36} The results of the present study suggest that the *lon* mutant extensively produced OH \cdot free radicals relative to the wild type counterpart, demonstrating that the mutant lost the ability to mitigate oxidative stress. It also strengthens the notion that the *lon* mutant may enhance internal and host-derived oxidative stress due to an enhanced virulence phenotype. One strategy bacteria utilize to avoid the accumulation of H₂O₂ is active secretion *via* outer membrane proteins.³⁷ The expression of genes encoding outer membrane proteins was elevated in the *lon* mutant as

we measured four representative outer membrane protein-encoding genes *ompF*, *ompD*, *fepA*, and *csgB*.³⁸⁻⁴⁰ The encoded proteins are important for nutrient and ion uptake. Interestingly, the *lon* mutant resulted in a sharp increase in *csgB* at cold shock (4°C) that encodes for a minor curling subunit in *Salmonella* that confirms Lon protease function as a cold-shock protein similarly to what has been demonstrated in *E. coli* in previous studies.^{41,42}

Herein, a link between the Lon protease and iron uptake regulation can be proposed due to the hyperexpression of the *fepA* gene in the *lon* mutant.

To investigate the connection between *lon* and iron uptake regulation, we evaluated the level of expression of iron homeostasis related genes *fur* (ferric uptake regulator), *sitA* (Iron/manganese ABS transporter substrate-binding protein), and *dps* (DNA protection during starvation protein) genes. Expression kinetics demonstrated significant upregulation of these genes in the *lon* mutant ST, suggesting that the mutation may result in an influx of iron ions. These observations corroborate the previously described phenotype of increased OH \cdot generation by the *lon* mutant (Fenton reaction). This suggests *lon* is involved in iron-dependent oxidative stress response pathways in *Salmonella*. Interestingly, a *fepA* deletion in the *lon* mutant ST (JOL2472) and the ST wild type strain (JOL2473) completely abolished the expression of *fur*, *sitA*, and *dps* in both strains, possibly due to “*lon*-mediated” and “*fepA*-mediated” iron uptake is controlled by two independent pathways or these genes may activate upon a certain threshold of iron in the cytoplasm that is never reached in the absence of *fepA*. In addition to iron, the magnesium ion transporters^{43,44} *mgtA*, *mgtB*, and *corA* also demonstrated an increasing trend in the *lon* mutant. Herein, *corA* expression is particularly important due to its involvement in iron transport along with magnesium. Therefore, it can be concluded that the *lon* mutant naturally experiences an increased level of oxidative stress that is generated by its cellular machinery.

When examining the virulence gene expression profile of the *lon* mutant and the wild type strain, it is possible to propose that Lon protease activity is linked to SPI-1 rather than SPI-2,⁴⁵ as indicated by a sharp increase in the SPI-1 candidate *invF* and *hilC* genes but not the SPI-2 *sifA* and *sseJ* candidate genes. This allows us to hypothesize that Lon activity is essential during the early phases of infection to maintain the virulence phenotype of *Salmonella* until the cells have entered and established infection in the host. This was also evident in the *lon* mutant whereby it lost virulence regulation, which is a key failure for a successful intracellular pathogen. In turn, such an enhanced virulence phenotype may elicit enhanced cytokine response from the host perspective, which was confirmed by assaying cytokine profiles in phagocytic (RAW 264.7) and non-phagocytic (HeLa and

HepG2) cells.^{46,47} Results confirmed that the *lon* mutant is hyper immunogenic relative to the wild type strain, possibly due to the aforementioned overexpressed virulence genes. However, according to our hypothesis, the *lon* mutant should be unable to mitigate stress-induced damage and must be rapidly eliminated from the host cell compared to the wild type strain due to weakened virulence homeostasis. To further investigate the idea of early clearance, we exposed *Salmonella* mutant and wild type strains to all the tested stress conditions for a predetermined time (5 h) and verified their survival by plate counting. Besides, *in vivo* inoculation studies were also conducted to evaluate bacterial stress survival using a mouse model. As expected, the mutant resulted in rapidly dwindling numbers in both *in vitro* and *in vivo* conditions, suggesting that the activity of Lon protease is indispensable for *Salmonella* systemic infection and phagocytic survival during dissemination stages in the host.^{48–50}

In the present study, the expression of *lon* was studied under normal laboratory conditions where the *Salmonella* was allowed to grow aerobically. Oxygen deprivation is yet another virulence determinant of wild type *Salmonella*. Being a facultative anaerobe, *Salmonella* can effectively survive under partial or complete anaerobic conditions in the gut. Low oxygen tension in the anaerobic environment upregulates the SPI-1 genes which in turn enhances the invasiveness of the bacteria.⁵¹ On the other hand, such a reducing environment causes downregulation of *lon* expression. Absence of oxygen hampers the synthesis of ATP thereby decreasing the activity of Lon protease (AAA+ ATPase). Once the *Salmonella* maneuvers through the gut and reaches an oxidizing environment, the Lon protease activity increases. Our observation of a reduction in *lon* expression under anaerobic condition corroborates with the earlier studies in *Escherichia coli*. Lon activity is controlled by a redox switch which adjusts the size of the enzyme's exit pore depending on the presence or absence of oxygen⁵² and thereby regulates the *lon*-dependent pathways in *Salmonella*. Additionally, the exposure to the anaerobic environment helps *Salmonella* to survive in the extra-intestinal oxygen-rich conditions.⁵³ Further, *Salmonella* virulence is influenced by multiple genes and pathways other than *lon*.^{54,55}

Summarizing the results presented here, we conclude that Lon protease is essential for *Salmonella* oxidative stress regulation followed by acid stress, both of which are important aspects of *Salmonella* survival in macrophages and the hostile gastric tract. The role of Lon protease goes beyond its cellular homeostasis and quality control role; rather it is a key element in *Salmonella* pathogenesis and is indispensable for systemic infection. We propose that Lon protease is a mediator of virulence genes hosted in the SPI-1 genomic region. This study adds mechanistic insight to the fate of *Salmonella* in the absence of Lon protease and highlights its essentiality as a virulence mediator on top of its already known function as a housekeeping protease.

Materials and methods

Bacterial strains, plasmids, and primers

The bacterial strains, plasmids, and primers used in this study are listed in Tables 1 and 2. *Salmonella* strains were routinely grown in Luria broth (LB) at 37°C with constant agitation or on LB agar. For all stress-induced experiments, *Salmonella* Typhimurium (ST) grown to log phase was utilized unless otherwise indicated.

Construction of a *fepA* *Salmonella* mutant and a *lon* complemented strain

The mutant strain was constructed as previously described.⁵⁷ Briefly, prior to the target gene deletion from the host strain, JOL 401 and JOL 909 were electroporated with the helper plasmid pKD46, which provides the inducible red lambda components required for recombination. The target gene *fepA* was replaced with the *cat*^R gene contained on a linear PCR product amplified from the pKD3 plasmid. Recombinant clones were selected by plating on LB agar containing 25 µg/mL chloramphenicol. Successful *fepA* gene deletion was confirmed using flanking (*fepA*-F) and inner (*fepA*-I) PCR primers (Table 2). The *lon* gene was amplified for complementation experiments using the *Lon*-C primers (Table 2). The general PCR steps used for amplification consisted of a 3-min denaturation step at 95°C, 30 cycles of denaturation for 45 s at 90°C, annealing for 45 s at 55°C, an extension for the 60 s at 73°C, followed by a final elongation of 10 min at 73°C. The PCR product was cloned into the pWSK29 low copy number plasmid using EcoRI and HindIII as restriction enzymes.

Cell culture

The human epithelial cancer cell lines HeLa, HepG2, and Caco-2 were procured from the

Table 1. List of bacterial strains and plasmids used in this study.

Bacteria/Plasmid	Genotypic characteristics	Reference
<i>S. Typhimurium</i>		
JOL 401	<i>Salmonella</i> Typhimurium wild type, SPI-1 <i>invAE</i> ⁺ <i>hliA</i> ⁺ <i>avr</i> ⁺ ; SPI-2, amino acid permease; SPI-3, <i>mgtC</i> ⁺ ; SPI4, ABC transporter; SPI5, <i>pipB</i> ⁺	Lab stock
JOL 909	JOL 401 Δlon	Lab stock
JOL 909:: <i>lon</i>	JOL 909 carrying pWSK29+ <i>lon</i> gene	This study
JOL 2472	JOL 401 $\Delta fepA$:: <i>cat</i>	This study
JOL 2473	JOL 909 $\Delta fepA$:: <i>cat</i>	This study
<i>E. coli</i>		
DH5 α	<i>E. coli</i> F ⁻ Φ 80dlacZ Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _k ⁻ , m _k ⁺) <i>phoA supE44 thi-1 gyr A96 relA1λ⁻</i>	Invitrogen
Plasmid		
pWSK29	Low copy cloning vector, Ap ^R	42
pKD46	oriR101-repA101ts; encodes lambda red genes (<i>exo</i> , <i>bet</i> , <i>gam</i>); native terminator (tL3); arabinose-inducible promoter for expression (ParaB); <i>bla</i> oriR6Kgamma, <i>bla</i> (<i>ampR</i>), <i>rgnB</i> (Ter), <i>catR</i> , FRT	57
pKD3	<i>asd</i> ⁺ , pBR ori, lactamase signal sequence-based	57
pHLJ 65:: GFP	periplasmic secretion plasmid, 6 His tag containing GFP	Lab stock

Table 2. List of primers used in this study.

Gene	Sequence (5'-3')	Reference
<i>cat^R</i>	F:ACTTCCCGTTGCCAGCGCCTCTTTCAT TATAACCTGTGTTTATTATGAAGGTAGG CTGGAGCTGCTTC R:AGCATCGTTTTTGCCAATTCCTCCC CGAATGAGGGAGGGAAGTTGCCAATG GGAATTAGCCATGGTCC	This study
<i>FepA-F¹</i>	F:ACTTCCCGTTGCCAGCGCCTCTTTC R:AGCATCGTTTTTGCCAATTCCTCCC	This study
<i>FepA-I²</i>	F:GACGGTAAACCCGTCACCAG R:ACTCCCGGTACGTTCCAGACT	This study
<i>invF</i>	F:GACGAAATATTACGCTTTC R:AGTCTTCTCCAGCATTCTC	This study
<i>hilC</i>	F:GCAATAATCACCTTCAACAGCC R:GCCCTTCTATTTCCGCTCATA	This study
<i>sifA</i>	F:ATTACCACCACCGCATACC R:GCTTCGACTATCTCAGCTAAC	This study
<i>sseJ</i>	F:TCAATAAATCACATCCCAAGCC R:CCCTTCAGCAAATTAAGCATC	This study
<i>ompD</i>	F:GCAACCGTACTGAAAGCCAGGG R:GCCAAGAAGTCAGTGTACGGT	56
<i>ompF</i>	F:CGTGCTGGCGGTTTGTGAC R:TTGCTGTACGCTGCGGTGAC	56
<i>fepA</i>	F:TTTTGCTCCGCTCAGTC R:GTTACGCCATTATCCAGCC	This study
<i>csgB</i>	F:AGGAAATAATCGGGCGAAAG R:GCGAATAGCCATATGCGAC	This study
<i>sitA</i>	F:AAAGCACCATTATCCCGCC R:AATCGACATACAGCACGCC	This study
<i>dps</i>	F:ACCACGCAAGTTATCAACAG R:CGAACATCATTAGCGACAACAG	This study
<i>Fur</i>	F:CAGGAACCAGATAACCATCAC R:CCGCAATCAAGGAGATAAG	This study
<i>regulon</i>	F:GCATATCCACTCTTCTTCTTC R:TTTCATCCGCCAAGTGTTC	This study
<i>corA</i>	F:ACTGCTTTTTGTGCGCGCC R:TACCGTGTGTCGGTGTTC	This study
<i>mgtB</i>	F:AACAAATCAAAAGCCGACAG R:CAGTAATCCACCACAAACC	This study
<i>lon</i>	F:CGCTGAAACTGGCGGATAAA R:TAGTACTCGCGCTGGGATT	This study
<i>Lon-C³</i>	F:attGAATTCCTGACCATTACGAACTTGC R:tacAAGCTTGTTCGGAATAAAAGCGTT	This study
<i>rrsG</i>	F:GTTACCCGCAAGAAGACAC R:CACATCCGACTTGACAGACC	56
<i>IL-2</i>	F:TTCAAGCCCCACTCAAGCC R:TGAGTCAAATCCAGAACATGCC	This study
<i>IL-4</i>	F:ACGGATGCGACAAAATCAC R:ACCTTGAAGCCCTACAGAC	This study
<i>IFN-γ</i>	F:AGACAATGAACGCTACACAC R:TCTTTCTTCCACATCTATGCC	This study
<i>IL-10</i>	F:GACAACATACTGCTAACCGAC R:ATCACTTTCACCTGCTCC	This study
<i>GAPDH</i>	F:AGAGGAGAAAGTGGGAAAAG R:AAATCCGTTACACCCGACC	This study

¹-primers for the flanking region of *fepA*; ²- primers for the internal gene region of *fepA*; ³-primers used to amplify *lon* for complementation experiment

American Type Culture Collection (Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, MA, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and L-glutamine (0.3 mg/mL). When the cells reached 60–80% confluence, cells were harvested and seeded in 96-well tissue culture plates at

a rate of 2.5×10^5 /mL in 10% DMEM. Plates were incubated at 37°C in a 5% CO₂ atmosphere.

Growth curve analysis

Growth of the ST wild type strain (JOL 401), ST *lon* mutant (JOL 909), and ST *lon* complemented strain (JOL 909::*lon*) was analyzed by sub-culturing overnight grown cultures at a 1:100 ratio in LB broth. Cultures were incubated at 37°C on a shaking platform (225 rpm). Aliquots of 1 mL were collected every hour for a period of 12 h and OD₆₀₀ was determined.

Bacterial growth in stress conditions

Log phase bacterial cultures were subjected to various stress conditions with slight modifications.⁵⁸ Briefly, bacterial cells were grown to mid-log phase (0.4 OD₆₀₀) and cells were collected by centrifugation at 3,500 x g for 15 min. Bacterial cells were re-suspended in pre-warmed LB medium at pH 3.0 (adjusted with hydrochloric acid) for acid stress, into pre-chilled LB medium at 4°C for cold stress, into pre-warmed LB with 5 mM H₂O₂ for oxidative stress, and LB with 5% NaCl (wt/v) for osmotic stress. Bacteria were exposed to respective stress conditions for 5 h.

Adhesion and invasion assays

Adhesion and invasion assays were conducted using HeLa, HepG2, and Caco-2 cell lines according to a previously described procedure.⁵⁹ Confluent cell monolayers were infected with *Salmonella* strains JOL401, JOL909, and the JOL909::*lon* complemented strain at 20 multiplicity of infection (MOI) in 24-well tissue culture plates. After 45 min of incubation, cells were washed three times with PBS, and adhered bacterial cells were enumerated by lysis of monolayers with 0.1% Triton X-100 for 10 min. For invasion assays, bacterial interaction with cell monolayers was conducted for 2 h and subsequently treated with gentamycin (100 µg/mL) for 1.5 h to eliminate any extracellular bacteria. Then, the cells were lysed with Triton X-100 for 10 min. Invaded cells were enumerated by plating on LB agar using decimal dilutions. The data from three

independent experiments are presented as CFU/mL with standard deviation.

Intracellular survival in phagocytic cells

To assess the intracellular survival of the *lon* mutant in phagocytic cells, the *Salmonella* strains were labeled using a CFSE Cell Division Tracking Kit (BioLegend, CA, USA). Then, the labeled *Salmonella* strains were injected into the mouse macrophage cell line RAW and incubated at 10^5 cells/mL for 24 h in 24-well plates. Cells were observed under fluorescent microscopy (Leica, Wetzlar, Germany) at 400 x magnification. Further, actual CFU counts were taken at 1, 3, 24 and 48 h post-inoculation to confirm the reduction in cell numbers in phagocytic cells.⁴⁵

Phalloidin staining of actin

HeLa, HepG2 and Caco-2 cells growing on a coverslip were infected with stress exposed JOL 401, JOL 909, or JOL 909::*lon* cells at 20 MOI. Uninfected cells at $t = 0$ were considered as the control. Cells were incubated for 20 min and washed three times with PBS. Slides containing infected cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed three times with 1X PBS. Cells were permeabilized with 0.01% Triton X-100 in PBS for 10 min at room temperature. After washing, cells were blocked with 5% bovine serum albumin. To stain actin filaments, 100 μ L of 1X phalloidin (Abnova, Taiwan) was prepared in 1% BSA and incubated with cells for 1 h in the dark. Nuclear staining was carried out using DAPI (Sigma, CA, USA) and observed under fluorescence microscopy (Leica). The extent of actin polymerization by each ST strain was compared in three independent experiments.

Hydroxyl radical (OH \cdot) measurement

Production of OH \cdot was measured following the manufacturer's protocol (Abcam, Cambridge, UK). After exposure to stress treatments, *Salmonella* strains were plated in 96-well plates at a concentration of 1×10^4 cells/90 μ L per well. Plates were centrifuged at 800 rpm for

2 min. The supernatant was removed, and wells were supplemented with 100 μ L/well OH580 Stain Working Solution and incubated at 37°C for 1 h. Then, cells were washed three times with DPBS followed by the addition of 100 μ L assay buffer to each well. Fluorescence was recorded at Ex/Em = 540/590 nm. As a positive control, HeLa and HepG2 cells treated with 10 μ M CuCl₂ and 100 μ M H₂O₂ (Fenton reaction) at 37°C for 1 h. Cells without any treatment were considered as a negative control.

In vitro bacterial survival

To evaluate the ability of bacterial cells to withstand stress treatment, cells were collected by vacuum filtration using a 47-mm filter membrane with 0.45- μ m pores (Thermo Fisher Scientific) after 5 h. Filtered cells were resuspended in pre-warmed LB broth by vortexing. The viable bacterial counts were performed through serial dilutions and plating on BGA agar plates.

In vitro bacterial virulence gene expression

The expression of virulence genes in *Salmonella* strains after stress treatment was investigated at the mRNA level at 5 hpi under each stress condition. The total RNA was isolated, and cDNA was synthesized (Toyobo, Osaka, Japan). Virulence gene expression was quantitatively analyzed using qRT-PCR (Table 2). The PCR conditions were 95°C for 5 min, 40 cycles at 95°C for 30 s, 56°C for 30 s, 72°C for 1 min, and final annealing and extension of 55°C for 5 s and 95°C for 30 s. The expression results were normalized against the housekeeping gene *rrsG* of *Salmonella* and data are presented in the $2^{-\Delta\Delta CT}$ method.⁶⁰

In vitro cytokine gene expression

The cytokine gene expression in HeLa, HepG2, and RAW cells in response to bacterial infection (oxidative stress treated) were carried out for IL-2, IFN- γ , IL-4, and IL-10 (Table 2). Herein, infected HeLa and HepG2 cells were cultured for 12, 24, and 48 h. The cells were harvested at each time point and the total RNA was isolated and cDNA was synthesized as described above. The expression of

cytokine genes was normalized against GAPDH and non-infected control. The changes in the relative expression of cytokine genes were determined using the $2^{-\Delta\Delta CT}$ method.⁶⁰

Survival and growth in epithelial cells

HeLa and HepG2 cells were grown in DMEM complete medium. A total of 4×10^5 cells were seeded per well in 24-well plates. When the cells reached 80% confluence, monolayers were infected with JOL401, JOL909, and JOL909::*lon* at 10 MOI for 2 h. After incubation, cells were washed three times with PBS and the extracellular bacteria were removed by gentamicin treatment (100 $\mu\text{g}/\text{mL}$) for 2 h. Then, cells were washed three times with PBS and replenished with complete medium. The bacteria-induced cytotoxicity was assessed using IncuCyte (Thermo Fisher Scientific) live-cell imaging system using Cytotox Green reagent at 2 ng/mL.

Assessment of virulence in mice

All animal experimental procedures were approved (CBNU2015-00085) by the Jeonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care and Korean Animal Protection Law, 2007; Article 13 (experiments with animals). Specific pathogen-free, female BALB/c inbred mice (4–5 weeks average age) were purchased from Koatech (Pyeongtaek, Gyeonggi-do, Korea). The animals were provided with water and antibiotic-free food *ad libitum*. Animals were monitored twice daily for behavioral and physiological signs.

To assess *in vivo* virulence of *Salmonella* strains, mice ($n = 10$) were orally inoculated at 1×10^9 CFU/mice/100 μl of PBS with JOL401, JOL909, or JOL909::*lon*. Mice were monitored for *Salmonella* induced clinical signs including ruffled fur, weight loss, reduced feed intake, and mortality. Mice were sacrificed on days 1, 6, 9, and 12 post-infection, and enlargement of the spleen was recorded. Further, the level of bacterial colonization in the spleen was assessed by plating tissue homogenate (1 g) on BGA agar after serial dilutions.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software, CA, USA) and IBM SPSS software. Student's t-test and analysis of variance (ANOVA) followed by Turkey's multiple comparison test was used to compare means among the treatment groups and to compute the *p*-value. The difference was considered significant if *p* was ≤ 0.05 .

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Data availability statement

The fluorescent microscopy imaging conducted for *Salmonella* mediated actin cytoskeleton rearrangement compiled and has been deposited in the Mendeley Data repository and can be accessed using the following link. Lee, John Hwa (2020), "Salmonella mediated actin condensation upon entry into HeLa and HepG2 cells", Mendeley Data, V1, doi: 10.17632/czng5n4fdk.1.

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