Coordinated interaction between Lon protease and catalase-peroxidase regulates virulence and oxidative stress management during Salmonellosis

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ABSTRACT
This study investigates the interplay between Lon protease and catalase-peroxidase (KatG) in relation to virulence modulation and the response to oxidative stress in Salmonella Typhimurium (ST). Proteomic comparison of ST wild-type and lon deletion mutant led to the recognition of a highly expressed KatG protein product among five other protein candidates that were significantly affected by lon deletion. By employing a bacterium two-hybrid assay (B2H), we demonstrated that the catalytic domain of Lon protease potentially interacts with the KatG protein that leads to proteolytic cleavage. Assessment of virulence gene expression in single and double lon and katG mutants revealed katG to be a potential positive modulator of both Salmonella pathogenicity Island-1 (SPI-1) and –2, while lon significantly affected SPI-1 genes. ST double deletion mutant, ΔlonΔkatG was more susceptible to survival defects within macrophage-like cells and exhibited meager colonization of the mouse spleen compared to the single deletion mutants. The findings reveal a previously unknown function of Lon and KatG interaction in Salmonella virulence. Taken together, our experiments demonstrate the importance of Lon and KatG to cope with oxidative stress, for intracellular survival and in vivo virulence of Salmonella.

Introduction
Salmonella enterica serovar Typhimurium (ST) is a facultative Gram-negative bacterium causing gastroenteritis and zoonotic infections. During an infection, the sequential expression of Salmonella virulence genes modulates bacterial entry and colonization by circumventing the host immune system. During the process of infection, Salmonella senses various environmental stress conditions and responds accordingly to survive in the host environment. Elimination of various misfolded and faulty proteins is a vital physiological function for Salmonella survival. Additionally, the ability of the bacteria to sense and respond to environmental stress is important for their survival and replication in host cells. Under unfavorable environmental conditions, bacteria express proteins for the elimination of stress-damaged proteins. In bacteria, most intracellular proteolysis is initiated by members of four families of ATP-dependent proteases – including the Clp family (ClpAP and ClpXP), HslVU, FtsH and Lon. Among these, Lon is responsible for more than half of all the energy-dependent proteolysis in Escherichia coli, hence, similar functions of Lon can be expected in ST signifying its importance in virulence modulation.

The Lon protease plays a key role in Salmonella virulence, as it regulates the expression of virulence genes located in Salmonella pathogenicity Island I (SPI-1) during the early stages of systemic infection. It does not seem to have a crucial impact on SPI-2 genes, which are regulated during later phases of infection. Lon protease is shown to potenti ate bacteria evolution and antimicrobial resistance. The dysregulation of Lon protease, a negative regulator of SPI-1 genes, leads to increased expression and orchestration of early virulence genes. Therefore, assessing the overexpressed proteins in the lon-deleted mutants may allow us to recognize key proteins that are regulated by lon and are crucial for bacterial survival and replication in the host.

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Previous studies have shown that the inactivation of lon leads to increased bacterial sensitivity to hydrogen peroxide (H₂O₂).⁹ Studies have shown that catalases; KatE, KatG, and KatN as well as alkyl hydroperoxide reductases; TsaA and AhpF play a vital role in combating the oxidative stress encountered by the bacteria in the host phagocytes.¹⁰ Among the catalases, KatE and KatG are heme catalases, whereas KatN is an Mn-catalase. The catalases scavenge endogenous and exogenous H₂O₂ and catalyze the decomposition of hydrogen peroxide to water and molecular oxygen.¹¹ In ST, KatE and KatN are stationary-phase catalases that are induced by RpoS regulon¹²,¹³ whereas KatG is modulated by OxyR regulator.¹⁰,¹⁴ Furthermore, KatG has been reported to impart protection against aminoglycoside antibiotics and is essential for the oxidation of kanamycin antibiotic.¹⁵ The alkyl hydroperoxide reductases scavenge the H₂O₂ by directly converting it into water. Unlike other enteric bacteria, ST contains genes encoding both the alkyl hydroperoxide reductases; tsA and ahpF. The catalytic efficiency of tsA/ahpC was reported to be better than the aforementioned catalases thus it is crucial in combating the oxidative stress induced by lower levels of H₂O₂.¹⁶–¹⁸ TsaA plays a vital role in protecting the gut microbes against toxicity and helps in the systemic dissemination of the infection.¹⁹,²⁰

Although previous reports from our lab and others have shown that the deletion of lon increases the ST susceptibility to H₂O₂,⁷,⁹ the role of Lon protease in regulating the levels of crucial enzymes associated with H₂O₂ scavenging is yet to be elucidated. To study the effect of Lon protease on the whole Salmonella proteome with special emphasis on the H₂O₂ degrading enzymes, we conducted a proteome comparison of wild-type (WT) and lon mutant Salmonella strains intending to recognize differentially expressed proteins using 2D gel electrophoresis. The level of mRNA expressions does not directly represent the proteomic profile. However, direct proteomic assessment can be advantageous to elucidate differentially affected proteome.²¹ Once the candidate proteins are recognized, they can precisely be identified by mass spectrometry analysis or by sequencing.²² Here, we compare the proteomes of wild-type ST, JOL 401, to its lon inactivated derivative (JOL 909) grown under standard culture conditions. We aim to better understand the role of KatG in pathogenesis as well as its intersecting link with Lon protease if any. To this end, a double deletion mutant, ΔkatG/Δlon was constructed, besides lack of catalase activity, this mutant will possess a global dysregulation of bacterial proteins. We hypothesized that the lack of both katG and lon would lead to a severely impaired phenotype of ST. We, therefore, characterized the phenotypes of single deletion mutants, Δlon and ΔkatG, and a double deletion mutant, Δlon/ΔkatG, in comparison to the parental ST strain.

Figure 1. Proteomic comparison of Salmonella by 2-D gel electrophoresis. The whole proteomes were isolated from Salmonella Typhimurium wild-type (WT) and lon deletion mutant. Resolved gels were stained with Coomassie blue. Molecular standards represent myosin- 220 kDa; phosphorylase A- 94 kDa; catalase- 60 kDa; actin- 43 kDa; carbonic anhydrase- 29 kDa and lysozyme- 14 kDa. The selected differentially overexpressed protein spots are demarcated by numbers 1–6 (Table 1).
Results

Deletion of lon influences the ST proteomic landscape

The effects of lon deletion on the ST proteomic landscape were analyzed using 2-D gel electrophoresis. The wild-type (WT) strain JOL 401 and its lon inactivated derivative JOL 909 were grown under identical conditions in LB broth until the late logarithmic phase of growth. Total proteins were extracted, resolved by 2-D gel electrophoresis, and stained with Coomassie blue (Figure 1). A comparison of the protein profile led to the identification of six protein spots that were more intense in the lon mutant than in the wild-type, suggesting that the genes encoding these proteins may be directly or indirectly regulated by lon. The six proteins were in abundance in lon-deleted mutants were designated using their molecular weights and are listed in Table 1. Then, the six prominent proteins were subjected to amino-terminal sequencing, and their sequence data were obtained. The amino acid sequences were compared against the NCBI database of Salmonella enterica serovar Typhimurium proteins (Table 1) for identification. Spots 1 and 2 were revealed to be catalase-peroxidase and manganese catalase, respectively. These are H2O2-dismutating enzymes that have a protective role against environmental H2O2 and are involved in stress adaptation. Spot 3 was alkyl peroxide reductase subunit C, a major protein in ST that protects bacteria from reactive nitrogen intermediates. Spot 4 was revealed to be OsmY, osmoprotectant import permease protein in ST. Spots 5 and 6 were found to be glucosamine-6-phosphate deaminase and D-ribose ABC transporter substrate-binding protein, respectively.

KatG protein levels controlled by Lon protease

We hypothesized that one or more of the cytoplasmic proteases were responsible for the degradation of KatG in Gram-negative bacteria, including Lon protease. If the hypothesis is true, the absence of Lon protease will stabilize KatG in the bacterial cytoplasm. To test the aforementioned hypothesis, KatG was produced from the pBAD vector in a katG-deleted strain carrying an additional lon, hslUV, ftsH, or clpP gene deletion. After inhibition of protein synthesis using chloramphenicol, KatG stability was quantified by immunoblot. It was evident that KatG was efficiently degraded in all Lon protease intact Salmonella strains but was significantly stable in the lon-deleted mutant (Figure 2). The inactivation of ftsH and clpP increased KatG stability only partially. On the other hand, the deletion of hslUV did not affect KatG stability. These observations suggest that KatG is most likely degraded by the Lon protease.

The proteolytic active site of Lon protease interacts with KatG

The Lon proteolytic domain (Lon PD) contains a conserved lysine (Lys722), located 43 residues beyond the catalytic serine (Ser679) to carry out catalytic activities. To understand how Lon protease is involved in the degradation of KatG, we investigated whether KatG physically interacts with the proteolytic active site of Lon protease. We used a bacterial two-hybrid assay to assess the interaction between KatG and the PD of Lon protease by expressing the katG gene with a C-terminal fusion of the cyaA-T18 fragment and Lon PD genes with N-terminal fusions of the cyaA-T25 fragment in an E. coli strain lacking CyaA adenylate cyclase. We

Table 1. Effect of the lon deletion on proteins expressed in Salmonella Typhimurium as observed by 2-D gel analysis of total proteins.

| Protein designation (kDa) | Expression in Δlon | N-terminal protein | Identical protein or homologue |
|--------------------------|-------------------|-------------------|-----------------------------|
| 79                       | Increased         | MSTDDTHTNLSTGKCPFHQ | Catalase/Peroxidase          |
| 31                       | Increased         | MFRHVQLOQYTRVSEPnP | Manganese catalase           |
| 20                       | Increased         | MSLINTKIPFKNOAFKNGE | alkyl peroxide reductase subunit C |
| 21                       | Increased         | MTMTLKLKSTLLAVMLTS | Molecular chaperon OsmY      |
| 29                       | Increased         | MRLPLSTAEVGKWARRHI | glucosamine-6-phosphate deaminase |
| 30                       | Increased         | MNNMKKLATLWSAVALSATV5 | D-ribose ABC transporter substrate-binding protein |
then spotted cells on a MacConkey agar plate containing maltose and measured β-galactosidase production from a cAMP-dependent promoter produced when T18 and T25 fragments of the cyaA gene are functionally complemented by a physical interaction between fused KatG and the PD of Lon protease. The strain expressing KatG-T18 and the T25-Lon proteolytic domain showed a strong red color on the MacConkey-maltose plate, indicating that KatG interacts with Lon PD (Figure 3a). By contrast, no interaction was observed in KatG-T18 co-expressing the empty T25 fragment. The interaction was confirmed by measuring the β-galactosidase activity. The KatG-Lon PD interaction was further analyzed by in vitro cross-linking of purified proteins. We observed an additional band corresponding to roughly 99 kDa (79 + 20) on polyacrylamide gel when KatG and the Lon proteolytic domain were incubated with a cross-linker (Figure 3a).

**The C-terminus domain beyond Arg207 of the KatG protein is essential for interaction with Lon PD**

Given that the mechanism of Lon is similar to that of *E. coli* Type 1 signal peptidase (SPase) (MEROPS, clan SK; PDB codes: 1kn9, 1b12), we predicted KatG cleavage sites for Lon PD using PROSPER (PROtease substrate SSpecificity servER) (Supplementary information). We then constructed a series of T18-fused katG derivatives deleted from their C-termini to determine the minimal requirement for Lon PD interaction. Interestingly, katG derivatives that harbored the coding region up to the amino acid 207 or more (up to 207, 208, 209, or 211) retained the ability to interact with Lon PD, but the derivatives with coding regions up to amino acid 206 or less (up to 206, 205, or 204) did not exhibit any interaction. These results indicate that the amino acid at position 207, which corresponds to arginine, in KatG is essential for interaction with Lon PD (Figure 3b,c).

**katG and lon deletion prevents in vitro ST survival**

The ability of ST to adhere, invade, and replicate inside macrophage cells is directly linked with virulence and systemic colonization of the host. We hypothesized that the inactivation of KatG might impact the ability of this mutant to invade and survive within macrophages. The deletion of only *lon* enhanced bacterial adhesion and invasion (Figure 4a,b). The adhesion and invasion assay revealed that the ΔkatG mutants were associated and internalized into RAW cells less than the wild-type bacteria. Upon further comparison, the adhesion and invasion capacity of the bacteria was reduced in ΔkatG mutants carrying an additional *lon* deletion.
Systemic dissemination is dependent on survival within phagocytic cells. To determine whether KatG contributes to intracellular replication, we assessed the intracellular replication of wild-type and mutants after 1, 2, and 3 h post-infection in RAW264.7 cells (Figure 4c). Compared to the wild-type, the ΔkatG mutant exhibited defective intracellular persistence. Additionally, we observed a significant decrease in the replication of Δlon and ΔkatGΔlon mutants compared to the wild-type in the macrophage cell line. These data support the hypothesis that KatG is needed for intracellular survival and growth in macrophages.

**Deletion of katG and lon enhances ST susceptibility to exogenous H₂O₂**

Despite the reduced or complete absence of catalase activity, ΔkatG, Δlon, and ΔkatGΔlon grew well in LB broth. To evaluate the adaptation of the bacterial strains to oxidative stress, we added H₂O₂, the substrate of the catalase, to the culture. The bacterial growth in the presence of 0, 1, 2, and 4 mM H₂O₂ was investigated. Compared to that of the wild-type, the growth of ΔkatG and Δlon was reduced at 2 mM and 4 mM H₂O₂, and the growth of ΔkatGΔlon was further inhibited (Figure 5a).
deletion of stress regulating genes led to decreased bacterial survival upon exposure to H$_2$O$_2$ wherein each of the strains exhibited slightly different growth. This pattern was observed at 3 and 6 h post H$_2$O$_2$ exposure (Figure 5a). Additionally, the sensitivity of bacteria to H$_2$O$_2$ was indicated by the zone of inhibition. As shown in Figure 5b, the inhibitory zone diameters for ΔkatG and Δlon were bigger than that of the wild-type at each concentration of H$_2$O$_2$, while no differences were observed between the wild-type and the complementary strain. Further, the diameters of inhibitory zones for ΔkatGΔlon were larger compared to those of the other strains (Figure 5b).

As the mutants were highly susceptible to H$_2$O$_2$ stress, we asked if the deletion has affected their catalase activity, the enzyme required to detoxify H$_2$O$_2$ to water and oxygen. Therefore, we monitored the catalase activity through the enzymatic decomposition of hydrogen peroxide (Figure 5c). We also evaluated the expression of katE, katG, katN, tsA, and ahpF in the mutants. The expression levels of genes, such as katE, katN, and tsA were downregulated in the lon mutant and an upregulated expression of katG and ahpF was observed. On the other hand, an increase in expression of tsA and ahpF was noted in katG and double deletion lon/katG mutants (Supplementary Figure 1A).

Absence of katG and lon causes the accumulation of intracellular ROS in ST

As the mutants exhibited high sensitivity to H$_2$O$_2$ with reduced or no detectable catalase activity, we studied the accumulation of intracellular ROS in wild-type and the mutants by fluorescent staining with DCFH-DA. The DCFH-DA is a non-fluorescent compound, however, upon oxidation by ROS forms a fluorescent product, 2’, 7’ – dichlorofluorescein (DCF) and the accumulation of DCF is widely applied to quantify the intracellular ROS. The ΔkatG and Δlon mutants exhibited a noticeably increased fluorescence compared to the wild-type bacteria (Figure 5d). The microscopic images of DCF fluorescence were higher in the double deletion mutant, ΔkatGΔlon, compared
Figure 5. H$_2$O$_2$ sensitivity, ROS production, and Catalase activity. (a) Susceptibility of Salmonella strains WT, Δlon, ΔkatG, ΔlonΔkatG, and ΔkatG::katG to oxidative stress was assessed following H$_2$O$_2$ exposure. An equal number of cells were exposed to 0, 1, 2, 4 mM H$_2$O$_2$ for 6 h, and the live cell numbers were determined by plating on agar. (b) Disk diffusion assay. A hundred microliters of each strain was spread on LB agar. Disks were saturated with 0, 2, and 4 mM and placed on bacterial lawns. Plates were incubated at 37°C for 12 h. Inhibition zones were compared. (c) Catalase activity of Salmonella strains was compared following exposure to 30% H$_2$O$_2$. An equal number of mid-log phase cultures were treated with 100 μl of 1% Triton X 100 and 30% H$_2$O$_2$. Foaming was compared after a 15-min reaction time. (d) Intracellular ROS assessment. The generation of ROS was assessed by DCF fluorescence assay. DCFH without cells was used as a negative control. DCF fluorescence intensities from WT, Δlon, ΔkatG, ΔlonΔkatG, ΔkatG::katG and ΔlonΔkatG::katG were measured by a spectrofluorophotometer. Significant differences compared to the wild-type control are indicated (*non-significant, *p < .05, **p < .01, ***p < .001, ****p < .0001).
KatG and lon oppositely regulate the expression of SPI-2 not SPI-1 genes

Given that SPI-1 genes are upregulated during bacterial uptake by macrophages and SPI-2 genes are required for ST intra-macrophage survival, we studied the impact of katG deletion on SPI-1 and SPI-2 gene expression. The deletion of lon led to the upregulation of SPI-1 genes, whereas the mRNA levels of SPI-2 genes were insignificant compared to those of wild-type (Figure 6a). Inactivation of katG led to the...
downregulation of SPI-1 genes. The mRNA levels of invA and sopE were decreased by 4- and 6-folds in the adherent ΔkatG mutant, respectively (Figure 6a). The expression of SPI-2 genes was studied in bacteria collected after 2 hours of infection. Our results showed that the transcriptional levels of sseJ and sifA were also downregulated in the absence of katG. The ΔkatG mutant exhibited a 3-fold reduction in the expression levels of sseJ and a 4-fold decrease in sifA after 2 hours of infection (Figure 6a). The double deletion mutant ΔkatGΔlon failed to upregulate both SPI-1 and -2 effector genes. Additionally, evaluation of the gene expression in bacteria grown in LB medium revealed a similar trend (Supplementary Figure 1B and 1C).

Inactivation of katG and lon influences the host inflammatory response

Given that bacterial antioxidants harness H₂O₂ to restrict the host immune function, we hypothesized that the deletion of katG and lon could increase the secretion of host pro-inflammatory cytokines. To test this hypothesis, we infected RAW264.7 and Caco-2 cells with ST strains. The ΔkatG mutant upregulated levels of pro-inflammatory cytokines up to 12 h post-infection in both the cell lines (Figure 6b). The levels of IL-2, IL-6, and TNF-α increased more than 80-fold upon infection with the ΔkatG mutant. A similar observation was made for cells receiving the Δlon mutant. On the other hand, there was a decrease in pro-inflammatory cytokine mRNA levels in cells receiving the ΔkatG mutant 24 and 48 h post-infection. A reduction in the mRNA levels of pro-inflammatory cytokines was evident in cells infected with the Δlon mutant (Figure 6b). The double deletion mutant ΔkatGΔlon elicited a much lesser inflammatory response compared to that of ΔkatG and Δlon mutants wherein the host cells showed a <2-fold increase in the expression of pro-inflammatory cytokines.

katG and lon help bacteria colonize the spleen after intraperitoneal infection

Based on in vitro assays, we hypothesized that the inability of the ΔkatG mutant to adhere and invade the macrophage-like cells could be linked to poor spleen colonization. In turn, we infected 10 Salmonella-susceptible BALB/c mice per ST strain intraperitoneally (i.p.). The deletion of katG led to poor colonization of the spleen, wherein <3log CFU/g of bacteria was recorded day 6 post-infection (Figure 7b). On the other hand, wild-type exhibited enhanced colonization, with >5log CFU/g. The Δlon and ΔkatGΔlon mutants also exhibited lesser spleen colonizing abilities compared to the wild-type. The colonization-mediated tissue damage in the spleen was evaluated by H&E staining (Figure 7a). The ΔkatG mutant caused minimum damage to the pulp architecture of the mice spleen; the wild-type caused damage associated with bacterial infection, including infiltration of inflammatory cells.

Discussion

The objective of this study was to further our understanding of how the Salmonella proteome is affected by lon gene deletion. The inactivation of cytosolic Lon protease appears to impair the stress response mechanism and causes the accumulation of partially folded proteins related to Salmonella virulence. To address this possibility with respect to lon gene deletion, we used the combination of 2-D gel analysis of proteins to evaluate the global expression pattern. We compared the proteome of wild-type (WT) Salmonella to that of lon deletion mutant using 2-D gel electrophoresis. Due to high-resolution protein separation, two proteomes could be compared to recognize differentially expressed proteins (Figure 1). The availability of a highly curated Salmonella genome is very helpful for the proteomic identification and characterization of candidate proteins essential for bacterial survival. The proteins overexpressed in the lon deletion mutant; spots 1, 2, 3, and 4, were recognized to be enzymes involved in regulating bacterial survival in oxidative and nitrosative stress conditions. Spot 5 corresponds to an enzyme involved in the metabolism of amino sugar whereas spot 6 represents the ABC transporter involved in the transport of ribose sugars essential for bacterial chemotaxis. These observations further confirm the role of Lon protease in Salmonella stress regulation and metabolism. Among five candidate proteins, spot 1, represented by the Catalase-peroxidase enzyme (KatG) and was
selected for further studies due to the potential connection of this protein with oxidative stress management.\textsuperscript{10,36} The \textit{katG} gene in ST encodes the KatG protein of approximately 79 kDa. We have established that Lon as a potential protease responsible for KatG degradation, whereas the other cytoplasmic proteases are unlikely to degrade this H\textsubscript{2}O\textsubscript{2}-dismutating enzyme (Figure 2). Additionally, this indicates that KatG most likely constitutes a substrate of the lon protease. For any proteolytic reaction, a physical interaction between the substrate and proteolytic domain is necessary.\textsuperscript{37} B2H assay and immunoprecipitation revealed that the Lon PD interacts with KatG (Figure 3a).
Further B2H assays revealed that the amino acid arginine at position 271 of the KatG was crucial for its recognition by the Lon protease for proteolysis\(^\text{38}\) (Figure 3b).

To characterize KatG activity in relation to Salmonella virulence, we constructed both individual and double deletion mutants of lon and katG open reading frames. When the two proteins are intact, they coordinate to regulate the response to intracellular oxidative stress and the expression of virulence genes, ensuring the systemic establishment of an infection.\(^\text{7}\) Here, we establish that Lon protease and KatG cooperate to regulate the virulence factors and are required for ST pathogenicity. The ability of ST to adhere, invade and proliferate inside the macrophages is associated with virulence and colonization of the host.\(^\text{39,40}\) The deletion of katG impaired the ability of the bacteria to adhere and invade macrophage-like cells, the double deletion mutant ΔlonΔkatG exhibited a further reduction in the adhesion and invasion abilities (Figure 4a, b). This followed by a diminished intracellular survival and replication of the ΔkatG and ΔlonΔkatG could be linked to the production of reactive oxygen species, ROS\(^\text{27}\) (Figure 4c). The ROS are formed intracellularly as a by-product of normal aerobic metabolism in the bacteria\(^\text{41}\) and are capable of causing bacterial lysis if the accumulation of ROS is not controlled. Here, the accumulation of fluorescent DCF in the Δlon, ΔkatG and ΔlonΔkatG compared to the parental strain shows that the expression of these genes is required to mitigate the ROS-mediated damage\(^\text{42}\) and the deletion of katG and lon confers hypersensitivity to H\(_2\)O\(_2\).\(^\text{43,44}\) Apart from the endogenous ROS, the phagocytic H\(_2\)O\(_2\) that diffuses into the bacterial cytoplasm can kill the bacteria by damaging the DNA.\(^\text{45}\)

Taking this into account we evaluated the ability of the ST wild-type and the mutants to resist the exogenously supplemented H\(_2\)O\(_2\). The outcomes revealed that the inactivation of lon or katG restricted bacterial growth to a similar degree and completely blocked the growth of double deletion mutant, ΔlonΔkatG when exposed to H\(_2\)O\(_2\) (Figure 5a). The overexpression of KatG in Lon mutants may be a compensatory mechanism to counteract the increased oxidative stress, where the KatG is activated as an attempting to degrade H\(_2\)O\(_2\) to ensure survival of the mutant Salmonella.

However, lon mutant with higher catalase activity had increased intracellular ROS that may be due to the dysregulated oxidative stress response. Earlier we have shown that the deletion of lon causes an increased accumulation of hydroxyl radicals in the Salmonella.\(^\text{7}\) In support, the expression levels of katE, katN, and tsaA were downregulated in lon mutant. It should be noted that the primary hydrogen peroxide scavenging role of tsaA (ahpC) over katE, katN, katG, and ahpF has been reported earlier.\(^\text{46}\) Therefore, intracellular ROS accumulation in lon mutant may be attributed to the dysregulated oxidative stress response, despite an increase in catalase activity. Further, increased oxidative stress cannot be fully counteracted by overexpressed catalase such as KatG due to the fact that H\(_2\)O\(_2\) is highly detrimental to phagocytosed Salmonella especially for an attenuated one. Our results suggest that the collective coordination of Lon and catalases, in this case, KatG appeared to be essential during the early infection phase that is vital to establish a successful infection where both virulence regulation and timely responses against oxidative stress exerted by the host cell via an interplay between Lon and KatG proteins. ST injects an array of proteins into the macrophage cytoplasm via the SPI-1 and-2 T3SS system leading to invasion and intracellular replication. Our results show that katG regulates the SPI-1 genes that are involved in regulating the initial bacterial invasion of host cells\(^\text{47}\) as well as SPI-2 genes that are essential for intracellular survival.\(^\text{48}\) The downregulation of SPI-1 factors invA and sopE genes in the ΔkatG mutant shows the catalase-peroxidase enzyme influences the ST entry into the host cells (Figure 6a). The downregulation of SPI-2 effectors sseJ and sifA suggests that KatG of ST is involved in impairing the macrophage antimicrobial mechanism and assists bacterial replication intracellularly. Unlike Lon and PhoP/PhoQ regulatory system, KatG may modulate two major virulence pathways, invasion and survival in macrophages in the same direction for ST pathogenesis.

The dysregulation of bacterial virulence factors in the absence of katG may impact the host inflammatory response.\(^\text{49}\) The evaluation of the transcriptional levels of pro-inflammatory cytokines revealed that the mRNA expression of IL-2, IL-6 and TNF-α increased by ≥50 folds in RAW264.7
and Caco-2 cells receiving ΔkatG mutant 12 h post-infection. However, the levels of pro-inflammatory cytokines dropped to <10 folds at 24 h post-infection (Figure 6b). The initial upregulation of inflammatory response can be attributed to the presence of other H₂O₂ scavenging enzymes, as bacterial antioxidants are shown to use the host cellular H₂O₂ to modulate Ca²⁺ signaling and limit host immune function. The later reduction in the host response may be attributed to the rapid elimination of the ΔkatG mutant from the cells due to disruption in the virulence mechanism. Also, intramacrophage survival and replication are associated with the ST colonization of the mouse spleen. The in vivo colonization studies corroborated with the aforementioned finding wherein the ΔkatG mutant exhibited meager colonization in the mouse spleen from day 3 to 12 post-infection.

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Figure 8. Proposed interaction mechanism of Lon and KatG in Salmonella. The expression of KatG is potentially regulated by Lon protease. When both Lon and KatG are intact, Salmonella’s virulence and oxidative stress tolerance are ideally maintained for maximum intracellular survival. When lon is deleted, overexpressed virulence factors enhance the oxidative damage; to mitigate the stress imparted by the host cell, levels of KatG increases in the bacteria for the efficient detoxification of H₂O₂. Hydrogen peroxide is converted into H₂O and molecular oxygen, relieving the harmful effect of ROS on cellular components. When both lon and katG are eliminated, the bacterium is extremely susceptible to ROS due to the absence of an oxidative stress response mechanism.
compared to the parental strain (Figure 7b). The inability of ΔkatG mutant to colonize the spleen led to minimal or no observable damage to the tissue architecture (Figure 7a) suggests that KatG plays a vital role in intramacrophage survival that is key to systemic infection in the host. The present study demonstrates the ability of proteome analysis to discover the role of global regulator Lon protease in regulating protein expression. Our observation that katG, a gene encoding catalase-peroxidase is controlled by Lon protease is further evidence that is involved in regulating stress response by controlling the production of other bacterial antioxidants. Further, our study showed that katG and lon are essential to combat exogenous and endogenous ROS. ST survival and proliferation in macrophages is regulated by katG. Although SPI-2 effectors are regulated differently by lon and katG, SPI-1 is positively regulated by these enzymes. Based on the collective findings (Figure 8), it can be concluded that both lon and katG contribute to robust oxidative stress defense mechanism and virulence of ST and that the simultaneous loss is detrimental to the virulence of the bacterium.

Materials and Methods

Ethics statement

Specific pathogen-free female mice aged five weeks were obtained from Koatech in Pyeongtaek, Korea. All animals were housed in a temperature- and humidity-controlled room, in which a 12-h light/12-h dark cycle was maintained. Experiments with animals were approved by the Jeonbuk National University Animal Ethics Committee (CBNU2015-00085) following the guidelines of the Korean Council on Animal Care and Korean Animal Protection Law, Article 13 (2007).

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 2. All mutant Salmonella strains were derived from WT Salmonella Typhimurium strain JOL401 using the lambda red recombination method.50 Salmonella strains were routinely grown in Luria Bertani (LB; BD, USA) at 37°C with or without agitation. High levels of aeration were achieved by culturing in Erlenmeyer flasks subjected to vigorous shaking. To enhance aeration, we reduced the culture volume from 10 ml to 4 ml to increase headspace and increased tilt angle during shaking at 220 rpm. In contrast, low oxygen levels were maintained using cultures grown in screw cap tubes without agitation. Ampicillin, chloramphenicol, and kanamycin were used at 100 μg/ml, 25 μg/ml, and 50 μg/ml, respectively. Arabinose and IPTG (isopropyl-β-d-thiogalactopyranoside) were used to induce the gene expression from the plasmid constructs.

Construction of gene deletion Salmonella mutants

The genes encoding KatG, Lon, ClpP, ftsH, and hsIUV were deleted from the genome of Salmonella Typhimurium following the previously described protocols.7,50 The primers (catR) used in the gene deletion are listed in Table 2. The successful gene deletion was screened by using primers pairs for the flanking regions (flanking primers) and coding sequence of the gene (inner primers).

Plasmid construction

The katG open reading frame (ORF) from Salmonella Typhimurium was amplified by PCR and cloned into a pBAD expression vector using EcoRI and HindIII restriction enzymes. The respective primer pairs used in amplification are described in Table 3.

2-D gel electrophoresis and analysis

Proteome isolation was conducted in Salmonella wild-type strain JOL401 and Lon protease mutant strain JOL909. Overnight cultures of both strains were freshly inoculated into LB broth (2% inoculum) and allowed to grow until the late log phase. When the OD$_{600}$ reached 0.8–1.0, cells were harvested by centrifugation at 13000 × g for 30 min. Cells were washed with phosphate-buffered saline (PBS) and subjected to osmotic lysis using the hypotonic solution with 1% lysozyme. Purified proteins were quantified using the Bradford method. 2-D gel electrophoresis was conducted as described in a previous report.54 Resolved gels were stained
Table 2. List of bacterial strains and plasmids used in this study.

| Bacteria/ Plasmid | Genotypic characteristics | Reference |
|--------------------|---------------------------|-----------|
| S.Typhimurium      |                           |           |
| JOL 401            | Salmonella Typhimurium wild type, SPI-1 invAε hilaA avrA; SPI-2, amino acid permease; SPI-3, mgcC; SPI4, ABC transporter; Lab stock |           |
| JOL909             | JOL 401 Δlon             | Lab stock |
| JOL2469            | JOL 401 ΔkatG            | This study|
| JOL2469::katG      | JOL 2469 carrying pWSK29+ katG gene | This study|
| JOL2506            | JOL 2469 ΔlpP (ΔkatGΔlpP) | This study|
| JOL2508            | JOL 2469 Δfsh (ΔkatGΔfsh) | This study|
| JOL2510            | JOL 2469 ΔhisUV (ΔkatGΔhisUV) | This study|
| JOL2755            | JOL 909 ΔkatG (ΔkatGΔlon) | This study|
| JOL2823            | JOL 2755 carrying pWSK29+ katG gene | This study|
| E. coli            |                           |           |
| DH5α               | E.coli F- Φ80dlacZΔM15Δ (lacZYA-argF)U169 recA1 endA1 hsdR17(k-) mcrA phoA supE44 thi-1 gyrA96 relA1 λ- | Invitrogen|
| BTH101             | F-cya-854, recA1, endA1, gyrA96 (Nalr); thi1, hsdR17, spoT1, rfbD1, glnV44(AS) | N/A       |
| JOL2558            | KatG::pET28(a) in DH5α   | This study|
| JOL2583            | Lon PD::pET28(a) in DH5α | This study|
| JOL2799            | pUT18::katG in DH5α      | This study|
| JOL2800            | pKT25::lon PD in DH5α    | This study|
| JOL2802            | pUT18::katG(1–206) in DH5α | This study|
| JOL2803            | pUT18::katG(1–207) in DH5α | This study|
| JOL2804            | pUT18::katG(1–208) in DH5α | This study|
| JOL2805            | pUT18::katG(1–209) in DH5α | This study|
| JOL2806            | pUT18::katG(1–210) in DH5α | This study|
| JOL2807            | pUT18::katG(1–211) in DH5α | This study|
| JOL2808            | pUT18::katG(1–379) in DH5α | This study|
| JOL2809            | pUT18::katG(1–380) in DH5α | This study|
| JOL2810            | pUT18::katG(1–381) in DH5α | This study|
| JOL2811            | pUT18::katG(1–382) in DH5α | This study|
| JOL2812            | pUT18::katG(1–383) in DH5α | This study|
| JOL2813            | pUT18::katG(1–384) in DH5α | This study|
| Plasmid            |                           |           |
| pWSK29             | Low copy cloning vector, AmpR | Lab stock |
| pKD46              | oriR101-repA101ts; encodes lambda red genes (exo, bet, gam); native terminator (tl3); arabinose-inducible promoter for expression (ParAB); bla, AmpR | Lab stock |
| pKD3               | oriR6Kgamma, bla (ampRI), rgnB (Ter), catR, FRT | Lab stock |
| pBAD               | L-arabinose inducible, araBAD promoter, pBR322 AmpR | Lab stock |
| pUT18              | Plac ColEori AmpR | N/A       |
| pKT25              | Plac ColEori KmR | N/A       |

with Coomassie blue. Proteins of interest were further characterized using amino-acid N-terminus sequencing after blotting onto a polyvinylidene difluoride membrane. The obtained sequences were used as a query in a BLAST analysis of the ST genome through the National Center for Biotechnology Information.55

Immunoblot analysis

The expression of katG in ST strains was confirmed by western blot analysis. Strains were grown to 0.6 OD at OD<sub>600</sub>. Four-milliliter cell samples were adjusted to have the same concentration (1 × 10<sup>8</sup> cells/ml), and all cells were collected by centrifugation. Cells were washed with PBS once and suspended in 0.5 ml of PBS. Cell lysis was carried out under denatured conditions using 8 M urea and brief sonication. The whole-cell lysate was filtered, and 20 μl of lysate was resolved in 12% SDS PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% BSA and incubated with anti-KatG polyclonal antibodies at 1:500 dilution. After two hours of incubation at 37°C, membranes were washed four times with 0.01% PBS-T and incubated with HRP-tagged anti-rabbit IgG as the secondary
Table 3. List of primers used in this study.

| Gene          | Sequence (5’-3’)                                                                 | Reference   |
|---------------|---------------------------------------------------------------------------------|-------------|
| katG<sup>catR</sup> | F:ACTTCCCGTGCCAGGCCCTTTCCATTAAAACCCGTGTATTATGAAGTGAAGCTGGCTGTGCTTC  
                                      R:AGCATGCTTGGCTCAATCCCGGAATGAGGGAGGTTGCCAATGGGAATTAGCCATGGTCC    | This study  |
| katG-Flanking | F: ACTTCCCGTGCCAGGCCCTTTCCATTAAAACCCGTGTATTATGAAGTGAAGCTGGCTGTGCTTC  
                                      R: AGCATGCTTGGCTCAATCCCGGAATGAGGGAGGTTGCCAATGGGAATTAGCCATGGTCC    | This study  |
| katG-Inner    | F: GACGGTAACCCCGGTACGTTCAGACT  
                                      R: ACTCCCGGTACGTTCAGACT    | This study  |
| clpP<sup>catR</sup> | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| clpP- Flanking | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Flanking | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Inner   | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Flanking | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Inner   | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| LonPD<sup>catR</sup> | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Flanking | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Inner   | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| LonPD<sup>catR</sup> | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| clpP- Flanking | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Flanking | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Inner   | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| LonPD<sup>catR</sup> | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Flanking | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Inner   | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| LonPD<sup>catR</sup> | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Flanking | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Inner   | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| LonPD<sup>catR</sup> | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Flanking | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Inner   | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| LonPD<sup>catR</sup> | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Flanking | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| FtsH- Inner   | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |
| LonPD<sup>catR</sup> | F:TTGAGGGATGACCTCATTTAATCTCCAGTAGCAATTTTGACCTGTTATGGGTGTAGGCTGGAGCTGCTTC  
                                      R:TTTTAAACAAAAACGAGCCCGTCAGGGCCCGTTTTATTCAAATTTGTGACATGGGAATTAGCCATGGTCC  | This study  |

<sup>(Continued)</sup>
antibody (Southern Biotech, USA). Color development was achieved by adding 3, 3’-diaminobenzidine (DAB; Sigma Aldrich, USA) substrate.

**Bacterial two-hybrid assay**

Potential interactions between Lon protease and KatG were evaluated using the bacterial two-hybrid (B2H) assay.\(^{56,57}\) The *E. coli* BTH101 (cyaA) strain was co-transformed with derivatives of the pUT18 and pKT25 plasmids. The *E. coli* host and plasmids for the B2H assay were a kind gift from Professor Eun-Jin Lee, School of Life Sciences and Biotechnology, Korea University. Transformants were grown in LB medium supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin, at 37°C overnight. Then, 2 μl from broth cultures were withdrawn and spotted on solid MacConkey-maltose agar containing 100 μM IPTG, 100 μg/ml ampicillin, and 50 μg/ml kanamycin. Plates were incubated at 30°C for 40 h. In addition, the results were validated by quantitative β-galactosidase assay, as described in a previous study.\(^{58}\) Bacteria co-transformed with empty or zip vectors served as negative and positive controls, respectively.

**Adhesion and invasion assays**

The adhesion and invasion potentials of the *Salmonella* mutants were evaluated in mice macrophage cell line RAW 264.7.\(^{7}\) Macrophage cells were cultured in 24-well plates and
subjected to infection at a multiplicity of infection (MOI) of 20. After 45 min of incubation, cells were washed three times with PBS. Cell lysis was conducted by adding 1 ml of PBS with 0.1% Triton X-100 to each well for 10 min. Lysed cells were collected, and 100 μl was pipetted out and plated on LB agar for colony counting. Regarding invasion assays, bacteria were allowed to interact with the cell monolayer for 2 h and were subsequently treated with gentamycin (100 μg/ml) for 1.5 h to remove any extracellular bacteria. Then, the cells were lysed with 0.1% Triton X-100 for 10 min. Invaded cells were enumerated by plating on LB agar using decimal dilutions. The data from three independent experiments with standard deviation are presented as CFU/mL.

**Macrophage survival assay**

ST mutant macrophage survival was conducted in mice macrophage cell line RAW264.7. Cells were seeded in a 24-well plate at a density of 5 X 10^6 cells per well in Dulbecco’s modified Eagle’s medium (DMEM; Lonza, Switzerland) supplemented with 10% fetal bovine serum (FBS; Serana, Germany) and cultured at 37°C and 5% CO2 in a humidified atmosphere. At confluence, Bacteria grown to mid-log phase were added onto macrophage cells at 0.1 MOI. Plates were centrifuged at 1000 rpm for 5 min at room temperature and continued to incubate for 20 min. Extracellular bacteria were eliminated by washing with PBS three times. Then wells were replenished by complete medium, DMEM, 10% FBS containing 120 μg/ml gentamycin, and incubated for 1 h. Bacterial survival was measured at 1- and 18-h time intervals by cell lysis after adding PBS and 0.1% Triton X-100 as mentioned earlier. Percent survival was calculated for the 18-h time point in relation to the number of bacteria recovered at the 1-h time point. This experiment was conducted in triplicate.

**Hydrogen peroxide sensitivity assay**

The effect of H2O2 on the growth of ST wild-type and mutants was evaluated using a previously described protocol. Briefly, a triplicate of overnight bacterial cultures were diluted to 1:1000 into 10 ml LB in 50 ml conical tubes and were grown to the mid-exponential phase. Cultures were treated with various concentrations of H2O2 (1, 2, and 4 mM, prepared from 30% H2O2, 9.8 M). The oxidative stress response was evaluated at 3 and 6 hours after H2O2 treatment. At each time point, 10 μl of culture was collected, serially diluted, and plated onto LB agar supplemented with catalase (Sigma Aldrich, USA) for CFU enumeration. Furthermore, a disk diffusion assay was performed to test H2O2 sensitivity, as described previously. The ST strains were cultured under near-anaerobic conditions to the mid-log phase, and 100 μl aliquots were spread on LB plates. A sterile 5-mm diameter filter disk (Sigma Aldrich, USA) containing 4 μl of 2 or 4 mM H2O2 was placed on the surface of the ST-containing LB plate. After incubation at 37°C for 12 h, the size of the area cleared of bacteria (inhibition zone) was measured using a Vernier caliper.

**ROS detection in bacteria**

The role of *lon* and *katG* in the accumulation of ROS in the ST strains was determined by analyzing the detection of 2,7-dichlorofluorescein diacetate (DCFH2-DA) fluorescence (Sigma Aldrich, USA). A stock solution of 10 mM (w/v) DCFH-DA in DMSO was prepared and kept at −20°C in the dark for further use. A 10 μM working solution was prepared in PBS. The bacteria grown to mid-log growth were collected and washed 3 times with PBS buffer to detect the production of ROS. First, 10 μM of DCFH-DA was added to the bacterial cultures and incubated on a shaker at room temperature in the dark for 30 minutes. Then, the bacterial culture was placed on a glass slide with a coverslip and visualized under a fluorescent microscope with a filter for fluorescein isothiocyanate (FITC) (Zeiss, Germany). Additionally, the production of DCF was measured immediately on a fluorescent plate reader at 485-nm excitation and 535-nm emission in the endpoint mode.

**Quantification of catalase activities**

The catalase activity in the wild-type and the mutant strains was quantified using a previously published method. Briefly, bacterial suspension of 1 × 10^8 CFUs in 100 μL volume was added in a Pyrex tube (13 mm diameter × 100 mm height, borosilicate glass; Corning, USA). Subsequently, 100 μL of 1% Triton...
X-100 and 100 μL of 30% hydrogen peroxide were added to the solutions and mixed thoroughly, and the solutions were then incubated at room temperature for 15 min. No reagents were used to stop the reaction, as the generation of oxygen stops naturally within 5 min. Following completion of the reaction, the height of O₂-forming foam, which remained constant for 15 min, in the test tube was finally measured using a Vernier caliper.

qRT-PCR for the evaluation of gene expression

Cytokine gene expression in Caco-2 and RAW 294.7 cells in response to ST infection was quantified, with IL-2, IL-6, and TNF-α having been investigated. Caco2 and RAW 294.7 cells were infected with ST WT and mutant strains at an MOI of 20 and incubated for 12, 24, and 48 h. Cells were harvested at each time interval, and the total RNA was isolated; the corresponding cDNA was then synthesized (Elpis Biotech, Korea). The expression levels of cytokine genes were quantitatively analyzed using qRT-PCR (Table 3). The expression of cytokine genes was normalized against GAPDH expression, and the changes in the relative expression of cytokine genes were determined using the 2^−ΔΔCT method. The mRNA levels of ST virulence-associated genes were compared in the wild-type and mutant strains. The SPI 1 and SPI 2 gene expression was analyzed in Salmonella recovered from cells at 20 min and 3 h post-infection, respectively. The time was chosen to specifically reflect the activation of SPI 1 and SPI 2 genes as they play a role in cell adhesion and intracellular survival, respectively. The expression levels of bacterial genes were normalized against the ST rrsG housekeeping gene.

Mouse virulence assay

Six-week-old female BALB/c mice (n = 10/group) were inoculated with ST strains at 1 × 10⁵ CFU/mouse/100 μl PBS. Mice were monitored daily for ST infection and associated clinical signs. On days 1, 3, 6, 9, and 12 post-infection, mice were euthanized, and the spleens were aseptically collected. Whole spleens were homogenized in 3 ml PBS using a mechanical homogenizer (IKA T 10 basic ULTRATURRAX, Germany). A hundred microliters of homogenate were serially diluted and plated on Brilliant Green Agar (BGA) for bacterial enumeration. Furthermore, the bacterial colonization associated with tissue damage in the spleen was assessed by H&E staining according to standard protocols.

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 corresponding p-values. P-values ≤0.05 were considered to demonstrate a statistically significant comparison.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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