Comparative roles of clpA and clpB in the survival of S. Typhimurium under stress and virulence in poultry

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By assisting in the proteolysis, disaggregation and refolding of the aggregated proteins, Caseinolytic proteases (Clps) enhance the cellular survival under stress conditions. In the current study, comparative roles of two such Clps, ClpA (involved in proteolysis) and ClpB (involved in protein disaggregation and refolding) in the survival of Salmonella Typhimurium (S. Typhimurium) under different stresses and in virulence have been investigated. clpA and clpB gene deletion mutant strains (∆clpA and ∆clpB) of S. Typhimurium have been hypersensitive to 42 °C, HOCl and paraquat. However, the ∆clpB strain was comparatively much more susceptible (p < 0.001) to the above stresses than ∆clpA strain. ∆clpB strain also showed reduced survival (p < 0.001) in poultry macrophages. The hypersusceptibilities of ∆clpB strain to oxidants and macrophages were restored in plasmid based complemented (∆clpB + pclpB) strain. Further, the ∆clpB strain was defective for colonization in the poultry caecum and showed decreased dissemination to the spleen and liver. Our findings suggest that the role of ClpB is more important than the role of ClpA for the survival of S. Typhimurium under stress and colonization in chickens.

Food borne-infections account for about 86% of human cases of non-typhoidal salmonellosis1. Salmonella enterica serovar Typhimurium (S. Typhimurium) is one of the most frequently isolated serovars of Salmonella from food-borne infections2. S. Typhimurium causes mild to moderate gastroenteritis in healthy individuals3, however, it is associated with fatal infections in young, old and immunocompromised people4,5. In chickens, S. Typhimurium provokes very mild gastroenteritis. However, infected hens serve as chronic carriers without showing any clinical signs of infection but lay contaminated eggs6. Amongst the various food sources entailed in human Salmonella infection, poultry products have been reported to be the foremost complicit in outbreaks across the world (CDC, 2013)7. After entering via the oral route, S. Typhimurium reaches into the intestine of the host and penetrates epithelial linings. This is brought about with the aid of T3SS encoded by 40 kb region located in the Salmonella genome called Salmonella Pathogenecity Island (SPI) 1. The T3SS effector molecules stimulate the engulfment of bacteria into the epithelial membrane by mediating rearrangement of actin filaments and causing formation of membrane ruffles around bacteria8. Macrophages phagocytose the bacteria once Salmonella reaches sub-mucosa. T3SS2 effector molecules secreted by SPI2 helps in the survival and multiplication of Salmonella inside Salmonella containing vacuole (SCV) present within macrophages. Finally, localization occurs through blood circulation and disseminating macrophages into target organs of predilection especially lymph organs (spleen and caeca) and liver9. Different studies reveal differences in the extent of colonization of organs depending on the host infected. In poultry, major colonization is usually seen in caeca while spleen and liver harbour four orders of magnitude of bacteria lower than that of caeca10.

Inside the host S. Typhimurium encounters several stresses, including oxidants generated by the phagocytes and higher (42 °C) body temperature of poultry11. Superoxide anion (O2−), hydrogen peroxide (H2O2) and hypochlorous acid (HOCl) are some of the important reactive oxygen species (ROS) generated by phagocytic cells12.
The exposure of *S. Typhimurium* to high temperature and oxidants results in unfolding and aggregation of proteins. These aggregates contain higher amounts of intermolecular β-sheet structures and are functionally inactive. The accumulation of protein aggregates hampers the cellular survival therefore they need to be taken care of at any cost.

To withstand such assaults, bacteria have evolved several mechanisms. These systems are composed of primary antioxidants, protein repair enzymes and various families of proteases and molecular chaperones. Proteases and molecular chaperones are categorized under heat shock proteins (Hsps) which specifically identify aggregated/misfolded proteins. Subsequently, Hsps cause either proteolysis or disaggregation and refolding of protein aggregates. Expression of molecular chaperones are found to be induced under stress conditions.

In *S. Typhimurium*, three types of energy dependant proteases, including Clp family (viz. ClpA, ClpB, ClpX, ClpP), Lon and HslVU are present. Clps are classified under the Hsp100 family proteins and function as both proteases and chaperones. The ClpA and ClpB/Hsp104 chaperones belong to class 1 AAA+ (ATPases associated with various cellular activities) protein superfamily. ClpA is a part of the two-component protease (ClpAP) that binds protein substrates and presents them to the protease (ClpP) for degradation. Recognition of substrate proteins is undertaken by the N-terminal domain of ClpA which also simultaneously unfolds the proteins. This unfolding aids the proteolytic degradation occurring in the main proteolytic site of ClpP which receives these unfolded proteins via translocation through the hexameric body of ClpA.

ClpB is an essential protein of heat shock response. Structurally, ClpB possesses a longer middle region known as ClpB/Hsp 104-linker which is essential for the chaperone activity. Although sharing a 42% sequence identity and 64% sequence similarity with ClpA, ClpB functions differently than ClpA. Instead of degradation, ClpB causes disaggregation and refolding of protein complexes in cooperation with DnaK, DnaJ and GrpE (KJE) chaperones. ClpB first acts as a molecular chaperone and catalyses fragmentation of large protein aggregates into small fragments. Subsequently, the activities of KJE resolubilise and refold these smaller fragments rendering their conformation more or less akin to their native forms. Another thought suggests that ClpB/KJE act as a bichaperone in which the aggregated proteins are threaded into the ClpB/KJE complex and then unfolded proteins are extracted by the translocation activity of ClpB.

ClpA and ClpB ATPases are implicated in stress tolerances of many organisms and are reported to protect vital cellular proteins during stress conditions. Further, the ClpA and ClpB ATPases are found to play very important roles in the virulence of several bacterial pathogens. However, the role of ClpA and the comparative importance of ClpA and ClpB in the stress survival and virulence of *S. Typhimurium* are not known. Here we evaluated and compared the roles of ClpA and ClpB in the survival of *S. Typhimurium* under stress and virulence in poultry. To accomplish this, we have generated *clpA* and *clpB* gene deletion mutants and complemented strains. Then their sensitivities to high temperature, oxidative stresses and intramacrophage survival were assessed. Further, we have investigated the effect of *clpA* and *clpB* gene deletions in the colonization of *S. Typhimurium* in chickens.

**Results**

**Construction and confirmation of clpA and clpB gene deletion and complementation strains in *S. Typhimurium*.

The PCR based analyses of *clpA* and *clpB* gene deletion mutants are shown in Supplementary Fig. S1A, A and B. Test primers c located in the flanking regions of *clpA* gene amplified 296 bp in ∆*clpA* and 2.8 kilobase pair (kb) in wild type (WT) strains, respectively (Supplementary Fig. S1A, ∆*clpA* and WT lanes). Similarly, the test primers d designed in flanking regions of *clpB* gene amplified 566 base pair (bp) in ∆*clpB* and 3 kb in WT strains respectively (Supplementary Fig. S1B, ∆*clpB* and WT lanes).

Complementations of ∆*clpA* and ∆*clpB* strains were confirmed by RT-PCR. *clpA* specific primers j amplified 159 bp product in WT strain (Supplementary Fig. S2, lane WT). This ampliscp was absent in ∆*clpA* strain (Supplementary Fig. S2, lane ∆*clpA*) and reappeared in ∆*clpA* + *pclpA* (complemented) strain (Supplementary Fig. S2, lane ∆*clpA* + *pclpA*). Similarly, by using *clpB* gene specific primers k, WT and complemented (∆*clpB* + *pclpB*) strains gave amplifications of 168 bp (Supplementary Fig. S2, WT and ∆*clpB* + *pclpB* lanes) which was absent in ∆*clpB* strain (Supplementary Fig. S2, ∆*clpB* lane).

It is important to analyze the effect of gene deletion on *in vitro* growth of bacteria. ∆*clpA* and ∆*clpB* mutant strains did not exhibit any defective growth in LB broth (Fig. 1). ∆*clpA* and ∆*clpB* mutants and WT strains exhibited sigmoidal growth curvess.

**Contribution of clpA and clpB in the survival of *S. Typhimurium* at 42 °C and oxidative stress.

The body temperature of poultry is 42 °C, hence *S. Typhimurium* experience constant thermal stress inside birds. Therefore, the contributions of *clpA* and *clpB* genes in the *in vitro* survival of *S. Typhimurium* at 42 °C have been evaluated. As compared to WT, ∆*clpA* and ∆*clpB* strains did not show any sensitivity to 37 °C (Fig. 2A). However, ∆*clpA* and ∆*clpB* strains were hypersusceptible (*p < 0.001*) to 42 °C exposure (Fig. 2B). In comparison to ∆*clpA* strain, ∆*clpB* strain showed hypersusceptibility to 42 °C (Fig. 2B). The numbers of bacteria recovered following 120 h of incubations [log₁₀ colony forming unit(s) (CFUs)/ml as mean ± standard deviation (S.D.)] were 8.43 ± 0.032, 7.48 ± 0.008 and 6.15 ± 0.075 for WT, ∆*clpA* and ∆*clpB* strains respectively.

Oxidative burst is an important part of the host immune response and proteins are primary targets of such responses. Next, susceptibilities of WT, ∆*clpA* and ∆*clpB* strains to various oxidants were evaluated. In comparison to ∆*clpA* and WT strains, ∆*clpB* strain was highly susceptible (*p < 0.001*) to paraquat (Fig. 3). Following two h of incubation with paraquat, the recovered viable numbers were (log₁₀ CFUs/ml as mean ± S.D.) 8.05 ± 0.04, 8.35 ± 0.04 and 7.22 ± 0.12 in WT, ∆*clpA* and ∆*clpB* strains. Complemented (∆*clpB* + *pclpB*) strain exhibited intermediate susceptibility to paraquat with a recovery of 7.61 ± 0.06 (log₁₀ CFUs/ml as mean ± S.D.) (Fig. 3).

Next, sensitivities of ∆*clpA* and ∆*clpB* strains to H₂O₂ were assessed. The exposure of ∆*clpA* and ∆*clpB* strains to 5 mM H₂O₂ did not show any hypersensitivity (*p > 0.05*) as compared to WT strain (Supplementary Fig. S3).
Following exposure to H$_2$O$_2$, we recovered (log$_{10}$ CFUs/ml as mean ± S.D.) 9.26 ± 0.05, 9.15 ± 0.04 and 9.19 ± 0.16 viable bacteria in case of WT, ΔclpA and ΔclpB strains respectively.

HOCl is one of the most potent oxidants generated by neutrophils and is a part of the oxidative burst encountered by S. Typhimurium upon internalization by phagocytic cells. In comparison to WT strain, the ΔclpB strain was highly susceptible ($p < 0.001$) to 1.5 and 3 mM concentrations of HOCl. However, ΔclpA strain showed susceptibility ($p < 0.001$) to 3 mM but not to 1.5 mM HOCl (Fig. 4). Bacterial numbers recovered following 3 mM HOCl exposure (log$_{10}$ CFUs/ml as mean ± S.D.) were 5.49 ± 0.27, 3.32 ± 0.04 and 0.82 ± 1.43 for WT, ΔclpA and ΔclpB strains respectively. Hypersusceptibilities of ΔclpA and ΔclpB strains to HOCl were restored in plasmid based complemented (ΔclpA + pclpA and ΔclpB + pclpB) strains. Following incubation with 3 mM HOCl,
the numbers of viable bacteria recovered (log_{10} CFUs/ml as mean ± S.D.) were 6.76 ± 0.68 and 6.94 ± 0.35 for ∆clpA + pclpA and ∆clpB + pclpB strains respectively.

**∆clpB strain is highly susceptible to monocyte derived macrophages (MDM).** The numbers of WT bacteria recovered (log_{10} as CFUs/ml as mean ± S.D.) following 24 and 48 h post infection were 4.037 ± 0.031 and 3.413 ± 0.043 respectively. The numbers of bacteria recovered from ∆clpA strain infected macrophages were 3.600 ± 0.048 and 3.355 ± 0.090 following 24 and 48 h of incubation. However, the ∆clpB strain showed defective intramacrophage survival (p < 0.001) as compared to WT and ∆clpA strains. We recovered 3.574 ± 0.040 and 2.460 ± 0.151 CFUs of ∆clpB mutant bacteria following 24 and 48 h of incubation. The function of clpB was partly restored in complemented (∆clpB + pclpB) strain which showed increased viability over ∆clpB strain. Numbers of complemented strains recovered following 24 and 48 h of incubation were 3.654 ± 0.052 and 3.078 ± 0.036 respectively (Fig. 5).

**∆clpA and ∆clpB strains accumulate more aggregated proteins.** As ClpA and ClpB inhibit protein aggregations, we hypothesized higher levels of protein aggregates in ∆clpA and ∆clpB strains. Following incubations with PBS or 1.5 mM HOCl, we observed more amounts of protein aggregates in ∆clpA and ∆clpB strains as compared to WT counterpart. The amount of loading was normalized in terms of CFUs and was equivalent to 45 × 10^7 CFUs/lane. Interestingly, the amount of aggregates was higher in ∆clpA strain as compared to ∆clpB strain (Fig. 6). Following 3 mM of HOCl exposure, we observed higher levels of aggregates in WT, ∆clpA and ∆clpB strains of S. Typhimurium. The amount of loading per lane was equivalent to aggregates isolated from 3 × 10^7 CFUs. Some of these aggregates failed to enter in the gel (marked by arrow in the figure). WT strain did not show much increase in protein aggregates following HOCl treatment. We observed HOCl-dose dependent increase in amount of aggregates in ∆clpB strain. ∆clpA strain showed more amounts of aggregates in 0 and 1.5 mM HOCl treated samples than WT and ∆clpB strains.
clpB contributes to the colonization in poultry caecum and dissemination of S. Typhimurium to spleen and liver. Our in vitro analyses suggest that clpB plays more important role than clpA in the survival of S. Typhimurium under heat and oxidative stresses (Figs 2, 3 and 4). This prompted us to compare the roles of clpA and clpB in the virulence of S. Typhimurium. Salmonella free birds were orally infected with WT or ΔclpA or ΔclpB strains of S. Typhimurium and their caecal colonizations were evaluated. We recovered Salmonella from caeca of all (4/4) chicks infected with WT strain of S. Typhimurium at all times post infection (Table 1). In birds infected with ΔclpA strain, 4/4 (100%) caecae were positive for Salmonella on 7 and 14 days post infection. After

Table 1. Contribution of clpA and clpB in the caecal colonization of S. Typhimurium. Six days old chicks were orally inoculated with different strains of S. Typhimurium (as indicated in Table). At different times post infection, the birds were dissected and presence of S. Typhimurium and mutant strains were confirmed by PCR (as described in materials and methods).
21 days post infection, ∆clpA strain was recovered from 75% of the chicks (3 positive out of 4). However, ∆clpB strain was able to colonize initially in only 2 out of 4 (50%) chicks on 7 and 14 days post infection and eventually got cleared on 21 days (Table 1).

After concluding the role of clpB in the caecal colonization of S. Typhimurium, next, we analyzed the contribution of clpB in the dissemination of S. Typhimurium to poultry spleen and liver. We determined the bacterial loads in the spleen and liver on 7, 14 and 21 days post infection. In the spleen of WT strain infected birds, we obtained bacteria at all times post infection (Fig. 7A). The numbers of Salmonella recovered on 7, 14 and 21 days (log_{10} CFUs/spleen as mean ± S.D.) were 2.57 ± 0.56, 1.46 ± 1.69 and 0.40 ± 0.80 respectively. Similarly, bacteria were recovered on all times post infection from the spleen of ∆clpA strain infected chickens. The counts were (log_{10} CFUs/spleen as mean ± S.D.) 1.35 ± 1.57, 1.31 ± 1.52 and 0.40 ± 0.80 on 7, 14 and 21 days post infection (Fig. 7A). Interestingly as compared to that in WT strain infected birds, the bacterial loads in the spleen of ∆clpB strain inoculated chicks reduced significantly (p < 0.01) on 7 and 14 days post infection. On 21 days post infection, we did not recover any bacteria in the spleen of ∆clpB strain infected birds (Fig. 7A). The recovered numbers of bacteria (log_{10} CFUs/spleen as mean ± S.D.) on 7 and 14 days post infection were 0.96 ± 1.22 and 0.40 ± 0.80 respectively.

In liver, we recovered bacteria from WT strain infected birds at all times post infection (Fig. 7B). The numbers of WT bacteria recovered (log_{10} CFUs/gm of liver as mean ± S.D.) were 3.02 ± 0.68, 1.88 ± 1.36 and 0.5 ± 1.0 on 7, 14 and 21 days post infection respectively. In ∆clpA strain infected birds, we recovered 2.20 ± 1.49 and 0.75 ± 1.5 bacteria (log_{10} CFUs/gm of liver as mean ± S.D.) on 7 and 14 days post infection (Fig. 7B). However, the bacterial loads in the liver of ∆clpB strain infected birds were significantly (p < 0.01) less on 7 days post infection (0.75 ± 1.5). Following 14 and 21 days post infection; we did not recover any bacteria from the liver of ∆clpB strain infected birds (Fig. 7B).

Discussion

S. Typhimurium encounters numerous stresses inside the host which primarily affect the integrity of cellular proteins. Molecular chaperones and proteases can refold or remove these abnormal proteins, thus play very important roles in maintaining the cellular homeostasis. Clp proteases belong to AAA + class of proteins which are classified in to Class I and Class II types. Class I protease which include ClpA, ClpB and ClpC have two AAA + domains and degrade/disaggregate larger substrates while class II AAA + proteases like ClpX have only one AAA + domain and can only deal with smaller substrates. These proteins require various accessory proteins...
to execute their functions. ClpB complexes with DnaK, DnaJ and GrpE. ClpA, ClpX and ClpC associate with ClpP but each require a different set of adapter proteins. Like ClpS assists ClpA; MecA and YphH coordinate with ClpC while ClpX complexes with SspB, RssB and UmuD<sup>Q</sup>. Out of the known Clp proteases, we sought to analyse the relative importance of degradation versus disaggregation of protein aggregates in the survival of S. Typhimurium. In the current study, we have evaluated the comparative roles of ClpA (protein degradation chaperone) and ClpB (protein disaggregation chaperone) in the survival of S. Typhimurium under in vitro stress and in virulence.

First, we generated and confirmed clpA and clpB mutants and complemented (ΔclpA + pclpA and ΔclpB + pclpB) strains (Supplementary Figs S1 and S2). ΔclpA and ΔclpB strains grew comparable to WT strain at 37 °C but were highly susceptible (p < 0.001) to 42 °C exposure (Fig. 2A and B). Following 120 h of exposure at 42 °C, (the log<sub>10</sub> CFUs/ml; mean ± S.D. values for WT, ΔclpA and ΔclpB strain were 8.43 ± 0.032, 7.48 ± 0.008 and 6.15 ± 0.075 respectively), ΔclpA strain was more than 8 folds susceptible (p < 0.001) than WT strain (Fig. 2B). However, ΔclpB strain was more than 18 and 21 folds more susceptible (p < 0.001) than WT and ΔclpA strains respectively at 42 °C (Fig. 2B). Our experiments suggest that both ClpA and ClpB contribute to the survival of S. Typhimurium at 42 °C, however, ClpB plays a more crucial role in defending the temperature stress in this bacterium. Thomas and Banexy observed defective recovery of E. coli ΔclpB mutant strain following 42 °C exposures<sup>56</sup>. ΔclpA<sup>57</sup> or ΔclpB<sup>58</sup> mutant strains in B. suis suffered temperature stress and showed reduced growth at 42 °C. Similarly, clpB is reported to play very important role in the adaptation or survival of E. coli, H. pylori and Pseudomonas putida to thermal stresses<sup>53,55</sup>. Interestingly, clpA and clpB genes get induced in E. coli, S. Typhimurium and Myxococcus xanthus following incubation at 42 °C<sup>54,58</sup>.

To eliminate the invading bacteria, phagocytes generate a battery of ROS including O<sub>2</sub> -, H<sub>2</sub>O<sub>2</sub>, highly toxic hydroxyl radicals and HOCl. Paraquat (methyl viologen) is a superoxide generating compound<sup>59</sup>. ΔclpB strain was about 8 folds more susceptible (p < 0.001) than WT strain to parauquat (recovered viable numbers were 7.22 ± 0.12 and 8.05 ± 0.04 for ΔclpB and WT strains, respectively; Fig. 3). Complemented ΔclpB strain (ΔclpB + pclpB strain) showed intermediate sensitivity to paraquat (Fig. 3). Next, we evaluated the susceptibilities of ΔclpA and ΔclpB strains to H<sub>2</sub>O<sub>2</sub>. ΔclpA and ΔclpB strains did not show hypersusceptibility (p > 0.05) to H<sub>2</sub>O<sub>2</sub> (Supplementary Fig. S3). HOCl is reported to be 100-folds more toxic than H<sub>2</sub>O<sub>2</sub><sup>60</sup>. We next analyzed the sensitivities of WT, ΔclpA and ΔclpB strains to HOCl. ΔclpB strain was much more susceptible to HOCl than ΔclpA strain. At 3 mM HOCl concentration, ΔclpB strain was 173 folds more susceptible (p < 0.001) than WT strain (Fig. 4). The susceptibility of ΔclpB strain (in comparison to WT strain) was more than 4792 folds (p < 0.001) at 1.5 mM and 3633 folds (p < 0.001) at 3 mM HOCl (following 1.5 mM HOCl treatment viable numbers for WT and ΔclpB were 8.53 ± 0.06 and 4.85 ± 0.03. While after 3 mM HOCl exposure, recovered viable numbers for WT and ΔclpB (log<sub>10</sub> CFUs/ml; mean ± S.D.) were 5.49 ± 0.27 and 0.82 ± 1.43 respectively). Interestingly in comparison to ΔclpA strain, ΔclpB strain was highly susceptible to HOCl (4423 folds at 1.5 mM and 21 folds at 3 mM HOCl). The recovered CFUs/ml following incubation of ΔclpA at 1.5 and 3 mM HOCl were 8.49 ± 0.11 and 3.32 ± 0.04 respectively. Taken together, our in vitro experiments suggest that clpB is more important than clpA in the survival of S. Typhimurium under superoxides and HOCl induced oxidative stress.

Lourdault et al. reported the high susceptibility of clpB mutant strain of L. interogs to butyl peroxide<sup>61</sup>. However, clpA and clpB gene deletion in B. suis did not affect the sensitivity to H<sub>2</sub>O<sub>2</sub><sup>62</sup>. Conversely, upregulated expression of Ehrlichia chaffeensis clpB gene was observed following infection of macrophages<sup>63</sup>, suggesting an important role of ClpB protein under oxidative stress. S. Typhimurium encodes three catalases, including KatE, KatG and KatN<sup>64</sup> which catalytically degrades H<sub>2</sub>O<sub>2</sub>. Catalases might be active at similar levels in all these three strains which might be the reason we did not observe hypersusceptibility of ΔclpA and ΔclpB strains to H<sub>2</sub>O<sub>2</sub>.

LPS stimulated macrophages generate robust immune response<sup>65</sup>. After 24 h of incubation, the ΔclpA and ΔclpB strains were about 3 folds more susceptible in macrophages than WT strain of S. Typhimurium. Following 48 h of incubation, ΔclpB strain was about nine and eight folds more susceptible in macrophages as compared to WT and ΔclpA strains respectively (Fig. 5). Similarly, defective intramacrophage survival of ΔclpB strains of Francisella and Coxiella burnetii have been observed<sup>65,66</sup>. However, clpA gene was found to be dispensable for intramacrophage growth of Brucella suis<sup>61</sup>. Further, upregulation of P. salmonis and Ehrlichia chaffeensis clpB has been observed following infection of these organisms with SHK-1 cell lines and macrophages<sup>67,68</sup>. These data suggest a crucial role of clpB in the intramacrophage survival of bacterial pathogens.

Our SDS- gel analysis revealed greater amounts of protein aggregates in ΔclpA and ΔclpB strains than that in WT strain of S. Typhimurium. Interestingly, we observed higher amounts of protein aggregates in ΔclpA strain than in ΔclpB strain (Fig. 6). Following incubation of clpB gene deletion strain of E. coli at 42 °C, increased aggregation of pre S2-β-galactosidase was observed<sup>69</sup>. In a separate study, degradation of green fluorescent protein aggregates at 42 °C was observed in ΔclpB and WT strains but not in ΔclpA strain of Brucella suis<sup>70</sup>. ClpB might be actively involved in degrading the protein aggregates in ΔclpB strain that could be the reason we did not observe higher levels of aggregates in ΔclpB strain as compared to ΔclpA strain.

Following exposure of 3 mM HOCl we have observed more protein aggregates in WT, ΔclpA and ΔclpB strains of S. Typhimurium. Some of these aggregates were resistant to SDS and beta-mercaptoethanol (β-ME) and failed to enter in stacking gel (Fig. 6, marked by arrow). Cell has a limited capacity to refold/remove aggregated proteins. The protein aggregates formed under severe stress (3 mM HOCl in our experiment) might be beyond the repair/removal capabilities of cellular chaperone/protease systems<sup>71</sup>. Further, we have observed significant amount of killing of WT as well as of mutant strains following exposure to 3 mM HOCl (Fig. 4).

S. Typhimurium primarily colonizes in the caecum of young chicks<sup>72</sup> and disseminates to spleen and liver. Eventually these birds serve as a carrier and lay contaminated eggs. As compared to WT and ΔclpA strains, ΔclpB strain was highly defective in caecal colonization and dissemination to spleen and liver (Table 1 and Fig. 7). Similarly, clpA deletion did not affect the colonization of B. suis and H. pylori in mice<sup>67,73</sup>. However, following inoculation of a pool of transposon mutants in chickens, defective recovery of a clpB gene insertion mutant was
| Sl. No. | Name of primer | Sequence | Specific Purpose |
|--------|----------------|----------|-----------------|
| a.     | UPclpA Forward | 5′′′AAAAAGCCTGATGGATAAAAATTTGGCGGAGGTGCTGCTGTTGAGGCGAAGTGCTCTT3′′′ | To amplify kanamycin cassette with flanking regions of clpA. |
|        | UPclpA Reverse | 5′′′GGGGGCTGTAAGGGCGCGGCTTCGATGAGCTAAATGACATAGAATATCCTCCTT3′′′ | |
| b.     | UPclpB Forward | 5′′′TAATCTCCAGTCTGAAATTTGACCCCTTATTGTTAGGAGGTGCTGCTGTTGAGGCGAAGTGCTCTT3′′′ | To amplify kanamycin cassette with flanking regions of clpB. |
|        | UPclpB Reverse | 5′′′AAAAAGCCTGATGGATAAAAATTTGGCGGAGGTGCTGCTGTTGAGGCGAAGTGCTCTT3′′′ | |
| c.     | clpA test deletion Forward | 5′′′AGAAGCGTGAAGCGAATTGAT3′′′ | To confirm the deletion of clpA from S. Typhimurium. |
|        | clpA test deletion Reverse | 5′′′GGGTGTTAGACGACTGAAACC3′′′ | |
| d.     | clpB test deletion Forward | 5′′′CTGGCGAATACCCGGCGGT3′′′ | To confirm the deletion of clpB from S. Typhimurium. |
|        | clpB test deletion Reverse | 5′′′ACAGACTCCTAACAAGCTT3′′′ | |
| e.     | clpA amplification Forward | 5′′′ATATATGGATTGCTATGCGTCAATCAAGAACTGGAAC3′′′ | To amplify and clone clpA in pQE60 (for complementation). |
|        | clpA amplification Reverse | 5′′′ATATAAGCTTTTATGTCGGCGGCTTCCG3′′′ | |
| f.     | clpB-pQE60_XhoI Forward | 5′′′ATATAATTCGAGATGCTTCTGGATCTGCTTAC-3′′′ | To amplify and clone clpB in pQE60 (for complementation). |
|        | clpB-pQE60_BamHI Reverse | 5′′′ATATATTGATCCTTACTGCACTGCCACAATAC-3′′′ | |
| g.     | pQE60-clpB Forward | 5′′′AAAGGAGGGCAGTGCTTCCGCG3′′′ | To confirm clpA-pQE60 clone. |
|        | pQE60-clpB Reverse | 5′′′GGCGGCAACCGAGCGTTCT3′′′ | |
| h.     | pQE60-clpA Forward | 5′′′AAAGGAGGGCAGTGCTTCCGCG3′′′ | To confirm clpB-pQE60 clone. |
|        | pQE60-clpA Reverse | 5′′′GGCGGCAACCGAGCGTTCT3′′′ | |
| i.     | typh Forward | 5′′′TTGTTCACCTTTTAAACCGTGA3′′′ | S. Typhimurium specific primer. For confirmation of S. Typhimurium by amplifying typh gene. |
|        | typh Reverse | 5′′′GCCCTGACAGCGGTTAGATT3′′′ | |
| j.     | clpA RT-PCR Forward | 5′′′ATGGCGCGTGTGATTCAGGAT3′′′ | To confirm expression of clpA by RT-PCR. |
|        | clpA RT-PCR Reverse | 5′′′GGTTCGAGCTTCTGGTTGCTT3′′′ | |
| k.     | clpB RT-PCR Forward | 5′′′CGGTTCCGATCTCATTCCAGG3′′′ | To confirm expression of clpB by RT-PCR. |
|        | clpB RT-PCR Reverse | 5′′′TGAGCAATAGAAGCGATGTGTT3′′′ | |

Table 2. List of primers used in this study.

In our experiments we observed that clpA is more important than clpB in the cellular survival under stress conditions. Consistence to this hypothesis, both ClpA and ClpB play important roles in preventing protein aggregations in the cell. As ClpA degrades protein aggregates, protein pool in the cell needs to be replenished via translational synthesis. While ClpB is involved in disaggregation and refolding of existing protein aggregates which would be a rapid and energy efficient way for restoration of protein function(s) in the cell (Supplementary Fig. S4). Therefore, ClpB would play more important role than ClpA in the cellular survival under stress conditions. Consistence to this hypothesis, in our experiments we observed that clpB gene deletion strain of S. Typhimurium was much more susceptible to different stresses in vitro (than ΔclpA strain) and showed defective virulence in chickens.

Methods

Ethical Statement. All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC), Indian Council of Agricultural Research-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, India with the approval file No. I.26-1/2015—2016/J.D.(R). All animal experiments were performed in accordance with the guidelines and regulations of IAEC, ICAR-IVRI, Izatnagar, India.

Bacterial strains and plasmids. S. Typhimurium E-5591 was obtained from National Salmonella Centre (Veterinary type), Division of Bacteriology and Mycology, Indian Veterinary Research Institute (IVRI), Izatnagar, India. The NEB5α strain of E. coli was obtained from New England BioLabs. The plasmid pQE60 was procured from Qiagen, Hilden, Germany. The plasmids pKD4, pKD46 and pCP20 were a kind gift from Dr. Robert J. Maier, Department of Microbiology, University of Georgia, Athens, GA, USA.

Culturing of S. Typhimurium. S. Typhimurium, its isogenic mutants and complemented strains were grown in Luria Bertani (LB) broth or Hektoen Enteric (HE) agar as described earlier. Ampicillin at the concentration of 100 µg/ml was included while culturing the complemented strain.

Construction of clpA and clpB gene deletion mutants and complemented strains. Primers utilized in this study are listed in Table 2. The clpA and clpB gene deletion mutants and complemented strains in S. Typhimurium were inoculated in to chickens, clpB gene deletion strain showed defective fitness. clpB gene deletion strains of Listeria monocytogenes, Francisella tularensis and Leptospira interrogans exhibited attenuated virulence and defective survival in animal models. In brief, our data and previous reports suggest that clpB is more important than clpA for the survival of bacterial pathogens in the host.

Both ClpA and ClpB play important roles in preventing protein aggregations in the cell. As ClpA degrades protein aggregates, protein pool in the cell needs to be replenished via translational synthesis. While ClpB is involved in disaggregation and refolding of existing protein aggregates which would be a rapid and energy efficient way for restoration of protein function(s) in the cell (Supplementary Fig. S4). Therefore, ClpB would play more important role than ClpA in the cellular survival under stress conditions. Consistence to this hypothesis, in our experiments we observed that clpB gene deletion strain of S. Typhimurium was much more susceptible to different stresses in vitro (than ΔclpA strain) and showed defective virulence in chickens.

Regarding the specific purpose of each primer, Table 2 provides a detailed list.
Typhimurium were constructed as described earlier. Briefly, the FRT flanked kanamycin cassettes were amplified using pKD4 plasmid as template. The kanamycin cassettes were electroporated in lambda red recombinase expressing S. Typhimurium. Positive recombinants were selected on kanamycin plates and confirmed by PCR. The kanamycin cassettes were then removed by flp recombinase. The clpA and clpB deletions mutants were confirmed by c and d primers located about 200 bp upstream and downstream from clpA and clpB genes. The gene deletion mutant strains were designated as ΔclpA and ΔclpB.

For complementations, the clpA and clpB genes were amplified by primers e and f and cloned into plasmid pQE60 (clpA at HindIII and BamHI restriction sites and clpB at XhoI and BamHI restriction sites). The positive recombinant plasmids were introduced into the ΔclpA and ΔclpB strains by electroporation. The positive colonies were confirmed by PCR using g and h primers (Table 2). The complemented strains were designated as ΔclpA + pclpA and ΔclpB + pclpB.

**Confirmation of transcription in complemented strains by Reverse Transcriptase (RT) – Polymerase chain reaction (PCR).** Overnight grown cultures of WT, ΔclpA, ΔclpB, ΔclpA + pclpA and ΔclpB + pclpB strains of S. Typhimurium were harvested by centrifugation. RNA samples were treated with RNase free DNase I and then dissolved in nuclease free water. All RNA samples were tested for contamination of DNA using S. Typhimurium clpA and clpB gene specific primers (Table 2 j and k).

RT-PCR was performed according to the protocol as described in Superscript VILO cDNA synthesis kit (Invitrogen). In brief, in 20 µl reactions, 0.5 µg of RNA samples were mixed with 4 µl of 5 × VILO reaction mix and 2 µl of 10 × Superscript enzyme mix. cDNA was synthesized by incubation of the above mix at 25 °C for 10 minutes (min) followed by 42 °C for 60 min and termination at 85 °C for 5 min. Part of clpA and clpB genes were PCR amplified using these cDNA samples as templates and j and k primers (Table 2).

**Growth curve study.** The growth of WT, ΔclpA and ΔclpB strains was analyzed as described earlier. In brief, isolated colonies of WT, ΔclpA and ΔclpB strains were grown overnight in LB broth. Overnight cultures were diluted (1: 100) in fresh LB broth and grown in a shaker incubator at 180 revolutions per minute (rpm), 37 °C. Aliquots were withdrawn at one h of intervals and optical densities (O.D.) were measured at 600 nanometre (O.D. 600 nm).

**Susceptibilities of ΔclpA and ΔclpB strains to 42 °C.** Overnight cultures of WT, ΔclpA and ΔclpB strains were diluted 100 folds in fresh LB broth. The cultures were then incubated either at 37 °C or at 42 °C. Aliquots were withdrawn at 0, 3, 6, 12, 24, 36, 48, 60, 72, 96 and 120 h post incubation, serially diluted with 1 × phosphate buffered saline (PBS) and plated on HE agar plates. The plates were incubated at 37 °C for overnight. CFUs/ml were then calculated.

**Evaluation of in vitro susceptibilities of ΔclpA and ΔclpB mutant strains to different oxidants.** Overnight cultures of different strains were sub-cultured in fresh LB broth (at the ratio of 1:100) and incubated in a shaking incubator at 37 °C. The mid log phase grown cultures were then exposed to different concentrations of paraquat, H₂O₂ and HOCl (sodium hypochlorite, NaOCl, Sigma) for 2 h. Aliquots were withdrawn, serially diluted and plated on HE agar plates for 24 h. Aliquots were withdrawn, serially diluted and plated on HE agar plates. CFUs/ml were calculated following incubation of plates at 37 °C for overnight.

**Susceptibility of WT, ΔclpA and ΔclpB strains to macrophages.** The susceptibilities of WT, ΔclpA, ΔclpB and complemented (ΔclpA + pclpA and ΔclpB + pclpB) strains of S. Typhimurium to monocyte derived macrophages (MDM) were determined as described earlier with minor modifications. Briefly, heparinised poultry blood was layered over equal amount of Histopaque-1077 (Sigma) and mononuclear cells (MNCs) were recovered by centrifugation at 1300 × g for 30 min at 25 °C. MNCs were washed twice with RPMI-1640 medium (HiMedia) supplemented with 2% chicken serum, 8% fetal bovine serum and 1 × antibiotic/antimycotic solution (Gibco). The cells were counted by trypsin blue dye exclusion method. MNCs were adjusted to 2 × 10^6 cells/ml in similar media. Then, the cells were seeded in 24 well cell culture plates at the number of 1 × 10^6 cells/well and incubated for 6 h at 37 °C/5% CO₂. Non-adhered cells were removed by washing. Cells were stimulated with Salmonella enterica serovar Typhimurium LPS (Sigma) at 0.5 μg/ml for 48 h. Following incubation, the cells were washed with antibiotic free media and infected with WT, ΔclpA, ΔclpB or complemented strains of S. Typhimurium at multiplicity of infection (MOI) of 1:50 (macrophages:bacteria). The infection was simulated by centrifugation at 120 × g for 10 min at 25 °C. The cell–bacterial mix was incubated for 2 h. To kill non invaded bacteria the mix was incubated in gentamicin (50 μg/ml) containing media for 90 min. The cells were then incubated in gentamicin (10 μg/ml) containing media. Following 24 or 48 h of incubation, the cells were lysed with 0.1% Triton X-100 and lysates were 10 folds serially diluted and plated on HE agar plates. Plates were incubated at 37 °C and colony forming units (CFUs)/ml were determined.

**Analysis of protein aggregations.** Overnight grown cultures of WT, ΔclpA and ΔclpB strains were diluted in fresh LB broth and incubated at 37 °C and 180 rpm for 3 h. The cultures were then exposed to 0, 1.5 and 3 mM concentrations (final) of HOCl for 30 min. Bacteria were harvested by centrifugation at 7000 rpm for 10 min. Protein aggregates from such exposed cultures were isolated as described earlier. In brief, the bacterial pellets were suspended in 500 μl of 50 mM Tris(hydroxymethyl) aminomethane (Tris) (pH 7.4), 5 mM ethylenediaminetetraacetate (EDTA), 20% sucrose and 1 mg/ml lysozyme. The mixtures were incubated at 25 °C for 10 min and diluted by addition of 5 volumes of 30 mM Tris buffer (pH 7.4). Following 20 seconds of brief sonication, mixtures were supplemented with 10 mM magnesium chloride (MgCl₂) and DNaseI. Unbroken cells were removed by centrifugation at 2,000 × g for 2 min. Supernatants were incubated with 0.5% Triton X-100 for 15 min
at 25 °C. The insoluble cell fractions (protein aggregates) were recovered by centrifugation at 8,000 × g for 15 min at 25 °C. The protein aggregates were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Loading was normalized in terms of CFUs/mL. Relative amounts of protein aggregates in different treatments/groups were analysed by ImageJ software (NIH) bundled with 32 bit Java 1.8.0. Sum of peak area in different lanes were calculated using the software. Total sum of peak area in 0 mM HOCl treated WT sample (lane) is considered as one.

Analysis of virulence in poultry. One day old chicks were procured from ICAR-Central Avian Research Institute (CARI), Izatnagar, India and provided with ad libitum feed and water. The birds were screened for the presence of Salmonella spp. as described earlier. The Salmonella free birds were divided into three groups. At the age of six days (~1 week) they were orally infected with WT or ΔclpA or ΔclpB strains at a dose of 1 × 10⁹ CFUs/ bird. Following 7, 14, and 21 days post-infection, 4 birds were sacrificed from each group. Caecal colonization and bacterial burdens in liver and spleen were assessed as described elsewhere.

Statistical analysis. Data were analyzed by SPSS. Comparisons between multiple groups were done by using one way analysis of variance (ANOVA) followed by Post hoc Tukey alpha test. p < 0.05 was considered significant among different test groups.

Data availability. The data analysed and generated during the current study are available from the corresponding author.

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**Author Contributions**

L.S., S.K.D., T.K.G. and M.M. designed the experiments. L.S., S.K.D., M.K. and M.K. performed the experiments. D.K. helped in performing macrophage experiment. L.S., S.K.D. and M.K. analyzed the data. L.S., S.A. and M.M. wrote the paper. All authors revised the manuscript critically and approved the final version to be published.

**Additional Information**

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