



OPEN Pan *msr* gene deleted strain of *Salmonella* Typhimurium suffers oxidative stress, depicts macromolecular damage and attenuated virulence

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Salmonella encounters but survives host inflammatory response. To defend host-generated oxidants, *Salmonella* encodes primary antioxidants and protein repair enzymes. Methionine (Met) residues are highly prone to oxidation and convert into methionine sulfoxide (Met-SO) which compromises protein functions and subsequently cellular survival. However, by reducing Met-SO to Met, methionine sulfoxide reductases (Msrs) enhance cellular survival under stress conditions. *Salmonella* encodes five Msrs which are specific for particular Met-SO (free/protein bound), and 'R'/S' types. Earlier studies assessed the effect of deletions of one or two *msrs* on the stress physiology of *S. Typhimurium*. We generated a pan *msr* gene deletion ($\Delta 5msr$) strain in *S. Typhimurium*. The $\Delta 5msr$ mutant strain shows an initial lag in in vitro growth. However, the $\Delta 5msr$ mutant strain depicts very high sensitivity ($p < 0.0001$) to hypochlorous acid (HOCl), chloramine T (ChT) and superoxide-generating oxidant paraquat. Further, the $\Delta 5msr$ mutant strain shows high levels of malondialdehyde (MDA), protein carbonyls, and protein aggregation. On the other side, the $\Delta 5msr$ mutant strain exhibits lower levels of free amines. Further, the $\Delta 5msr$ mutant strain is highly susceptible to neutrophils and shows defective fitness in the spleen and liver of mice. The results of the current study suggest that the deletions of all *msrs* render *S. Typhimurium* highly prone to oxidative stress and attenuate its virulence.

Non-typhoidal *Salmonella* (NTS) are estimated to infect over 94 million people every year¹. NTS infection manifests in a broad spectrum of diseases, including mild to modern gastroenteritis and fever. However, NTS can cause severe gastroenteritis, septicemia, and death in its fatal form^{2,3}. The emergence of invasive^{4,5} and multidrug resistance⁶ strains impart additional challenges to the current treatment/ prophylaxis regimens.

Host defense against *Salmonella* infections involves a complex interplay of immune responses. Phagocytic cells, such as macrophages and neutrophils, play a very important role in controlling *Salmonella* infection. Initial inflammatory responses include the production of anti-microbial peptides (AMPs), reactive oxygen species (ROS), reactive nitrogen species (RNS), cytokines and chemokines. These immune mediators coordinate with each other not only to eliminate *Salmonella* but also to facilitate other immune cells in the elicitation of adaptive immune responses. Following activation of the phagocytes, NADPH oxidase (NOX) assembles on the phagosomal membrane and facilitates the transfer of electrons from NADPH to oxygen, leading to the generation of superoxide anions (O_2^-) anions⁷. The O_2^- then metabolizes into other ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot OH$) and hypochlorous acid (HOCl)⁸. The ROS can damage various bacterial macromolecules like proteins, lipids and DNA⁹. ROS-mediated damage to proteins includes covalent modifications to amino acids and protein unfolding. Unfolded proteins are prone to aggregation, and subsequently show compromised function¹⁰. Out of twenty constituent amino acids, the sulfur-containing amino acids, cysteine and methionine, are highly prone to oxidation¹¹.

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To counter host-generated oxidants, *Salmonella* harbors the T3SS system, antioxidants and repair enzymes⁸. T3SS effectors inhibit NADPH assembly and interfere with O_2^- production¹². The antioxidant enzymes, superoxide dismutases, catalases and peroxiredoxins catalytically degrade O_2^- and H_2O_2 ^{13,14}. The protein repair enzymes, Msrs reduce Met-SO back to Met^{10,15}, whereas protein isospartyl methyl transferase repair of isoaspartate to aspartate⁸.

Msrs apparently serve two functions in the cell. First, by repairing Met-SO, Msrs modulate the activity of oxidized proteins. Second, Met residues (free or protein-bound) act as a sink to quench excess oxidants generated during host inflammatory response and get converted into Met-SO, which later get repaired by Msrs. Met-SO formation and their subsequent repair prevents ROS accumulation thereby ROS-mediated macromolecular damage in the cell and thus enhances cellular survival under oxidative stress.

S. Typhimurium encodes four cytoplasmic Msrs (MsrA, MsrB, MsrC and BisC) which are specific for particular types of Met-SO (free/ protein bound and "R"/ "S" types). MsrA reduces the "S" form (free or protein-bound) of Met-SO. MsrB and MsrC repair protein-bound and free "R" Met-SO respectively. BisC repairs biotin sulfoxides and free "S" Met-SO. The contribution of MsrA, MsrC, and BisC in stress survival and virulence of *S. Typhimurium* is well documented^{10,16–19}. However, MsrB does not play a vital role in this bacterium¹⁶.

Periplasmic proteins are involved in various physiological functions. However, due to location, they are highly prone to oxidation. *S. Typhimurium* expresses one dedicated Msr (MsrP) to repair proteins in this compartment²⁰. A recent study demonstrated the role of MsrP in mitigating N-chlorotaurine induced envelope stress¹⁵. Further, the same study established the role of the CpxRA two-component system in sensing N-chlorotaurine and regulating MsrP expression. Subsequently, we observed a moderate role of MsrP against ChT-induced oxidative stress, neutrophil-mediated killing, and virulence in mice²¹.

As each Msr contributes (to some extent) to oxidative stress survival of *S. Typhimurium*, we hypothesized that the pan *msr* gene deletion strain might show very high sensitivity to oxidants, depict macromolecular damage and attenuated virulence.

Results

Confirmation of $\Delta 5msr$ mutant strain

Deletions of the *msrA*, *msrB*, *msrC*, *msrP*, and *bisC* genes in *S. Typhimurium* were confirmed by PCR. The test primers designed in the flanking regions to the various genes yielded smaller amplicons in the mutant strains. However, bigger size PCR products were observed in *S. Typhimurium* (Fig. 1).

Deletion of all the *msrs* extends initial growth in lag and log phases of *S. Typhimurium*

The growth of the $\Delta 5msr$ and *S. Typhimurium* strains was assessed for a period of 11 h. Sigmoidal growth curves were observed for both strains. As compared to *S. Typhimurium*, the $\Delta 5msr$ mutant strain showed a considerable lag in the growth phase for the initial six hours, however, both strains grew similarly from 7 h onwards (Fig. 2).

$\Delta 5msr$ mutant strain shows hypersusceptibility to oxidants in vitro

Met residues are highly prone to oxidation. However, by repairing Met-SO, Msrs enhance the survival of bacterial pathogens under oxidative stress. We assessed the susceptibility of exponentially growing $\Delta 5msr$ mutant strain in LB media to various oxidants in vitro. Compared to *S. Typhimurium*, the $\Delta 5msr$ mutant strain was slightly

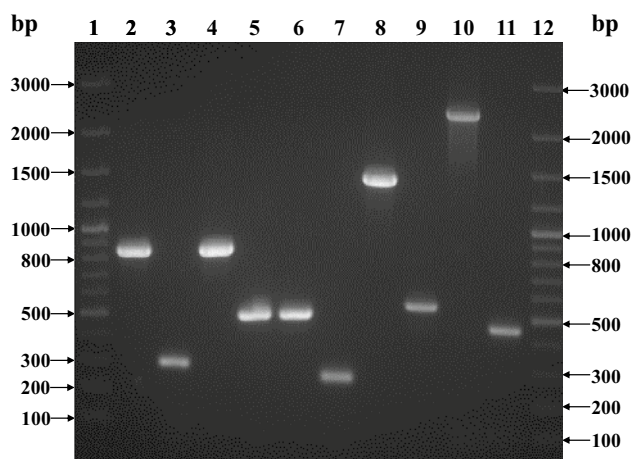


Figure 1. Agarose gel analysis of *msrA*, *msrB*, *msrC*, *msrP* and *bisC* gene deletions in *S. Typhimurium*. The deletions of above-mentioned genes were confirmed by PCR using test primers (as mentioned in Table 1) and genomic DNA from either *S. Typhimurium* (L2, L4, L6, L8, L10 respectively) or $\Delta 5msr$ (L3, L5, L7, L9, L11 respectively) strain as templates. Lane 1 and Lane 12 are 100 bp DNA ladder. Sizes of PCR products are mentioned in the figure itself. The image displayed is cropped from the full-length gel. The full-length gel is presented in supplementary figure S1.

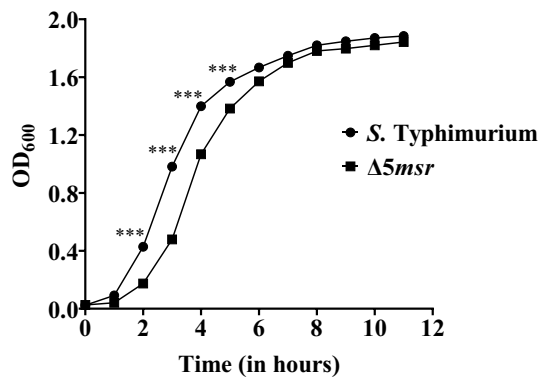


Figure 2. $\Delta 5msr$ mutant strain shows extended lag and log phases of in vitro growth. The $\Delta 5msr$ mutant and *S. Typhimurium* strains were grown in LB broth for 11 h. Aliquots were taken at hourly intervals and absorbance were recorded at 600 nm. Data are presented as mean \pm SD ($n = 3$) and analyzed by two-way ANOVA. *** $p < 0.001$.

(~ 2), but not significantly, susceptible to 1 mM HOCl. However, the mutant strain showed ~ 14 -fold more susceptibility ($p < 0.0001$) to 2 mM HOCl. Furthermore, the mutant strain was extremely susceptible (~ 1200 fold more; $p < 0.0001$) to 3 mM HOCl (Fig. 3a).

Similarly, when the cultures were suspended in PBS and exposed to HOCl, the $\Delta 5msr$ mutant strain was ~ 900 -fold more susceptible to 50 μ M HOCl than the *S. Typhimurium* strain. We did not observe any colonies following the exposure of the $\Delta 5msr$ mutant strain to 100 μ M HOCl. However, *S. Typhimurium* exposed to 100 μ M HOCl showed (\log_{10} , mean \pm SE) 4.03 ± 1.03 CFUs/ml (Fig. 3b).

In comparison to *S. Typhimurium*, the $\Delta 5msr$ mutant strain showed ~ 76 fold more susceptibility to 2.5 mM ChT ($p < 0.0001$) in LB media. However, both strains did not survive when exposed to 5 mM ChT (Fig. 3c). Interestingly, the $\Delta 5msr$ mutant strain showed ~ 25 and ~ 300 fold more susceptibility than *S. Typhimurium* when cultures were suspended in PBS and exposed to 125 μ M and 250 μ M of ChT respectively ($p < 0.0001$). We did not recover any viable colonies following the incubation of the $\Delta 5msr$ mutant strain with 500 μ M ChT (Fig. 3d). However, the recovered numbers (\log_{10} , mean \pm SE) in the case of *S. Typhimurium* exposed to 500 μ M ChT were 5.75 ± 0.64 CFUs/ml.

Surprisingly, in LB media both strains were not susceptible when exposed to 5% paraquat. However, the $\Delta 5msr$ mutant strain was susceptible to 10% paraquat exposure, but it was not significantly different from that of *S. Typhimurium*.

In separate experiments where the cultures were suspended in PBS and exposed to 10% paraquat, the $\Delta 5msr$ mutant strain showed \sim fourfold more susceptibility ($p < 0.0001$) in comparison to *S. Typhimurium* (Fig. 4b). Taken together, our data suggest that the Msrs contribute to the survival of *S. Typhimurium* against oxidants.

$\Delta 5msr$ mutant strain depicts higher MDA levels

Lipids are highly prone to oxidation. MDA is a marker of lipid peroxidation²². We next assessed the levels of MDA in both strains. MDA levels (mean \pm SE; micromoles per mg of proteins) in 0 mM HOCl exposed $\Delta 5msr$ mutant and *S. Typhimurium* strains were 2.47 ± 0.21 and 1.46 ± 0.17 respectively. However, upon 3 mM HOCl exposure, the levels were 3.21 ± 0.48 and 1.77 ± 0.32 in $\Delta 5msr$ mutant and *S. Typhimurium* strains respectively. The MDA levels were significantly higher ($p < 0.0001$) in the $\Delta 5msr$ mutant strain than in *S. Typhimurium* (Fig. 5).

$\Delta 5msr$ mutant strain accumulates higher levels of protein carbonyls

Carbonyls are considered stable markers of protein oxidation. We next determined the levels of total protein carbonyls in the $\Delta 5msr$ mutant and *S. Typhimurium* strains. The carbonyls were assessed spectrophotometrically as well as by oxyblot. Total protein carbonyl levels (mean \pm SE; per mg of proteins) were 3.35 ± 0.21 and 2.61 ± 0.15 in $\Delta 5msr$ mutant and *S. Typhimurium* strains, respectively. The carbonyl levels following exposure to 3 mM HOCl were 5.45 ± 0.15 and 3.85 ± 0.18 in $\Delta 5msr$ mutant and *S. Typhimurium* strains respectively (Fig. 6a). The levels were significantly ($p < 0.0001$) higher in the mutant strain.

In oxyblot analysis, several bands (as indicated by arrows) were darker in $\Delta 5msr$ mutant strain-loaded lanes as compared to the lanes loaded with *S. Typhimurium* lysates. Quantitation of the integrated densities of bands using ImageJ software also revealed darker areas in $\Delta 5msr$ mutant strain-loaded lanes (Fig. 6b.2 and Supplementary Table T1).

$\Delta 5msr$ mutant strain shows less free amines

Higher levels of ROS are associated with lower levels of free amines²³. We estimated the free amine levels in the lysates of the $\Delta 5msr$ mutant strain and *S. Typhimurium*. The $\Delta 5msr$ mutant strain showed 1.3-fold ($p < 0.0001$) less free amines than *S. Typhimurium* (Fig. 7).

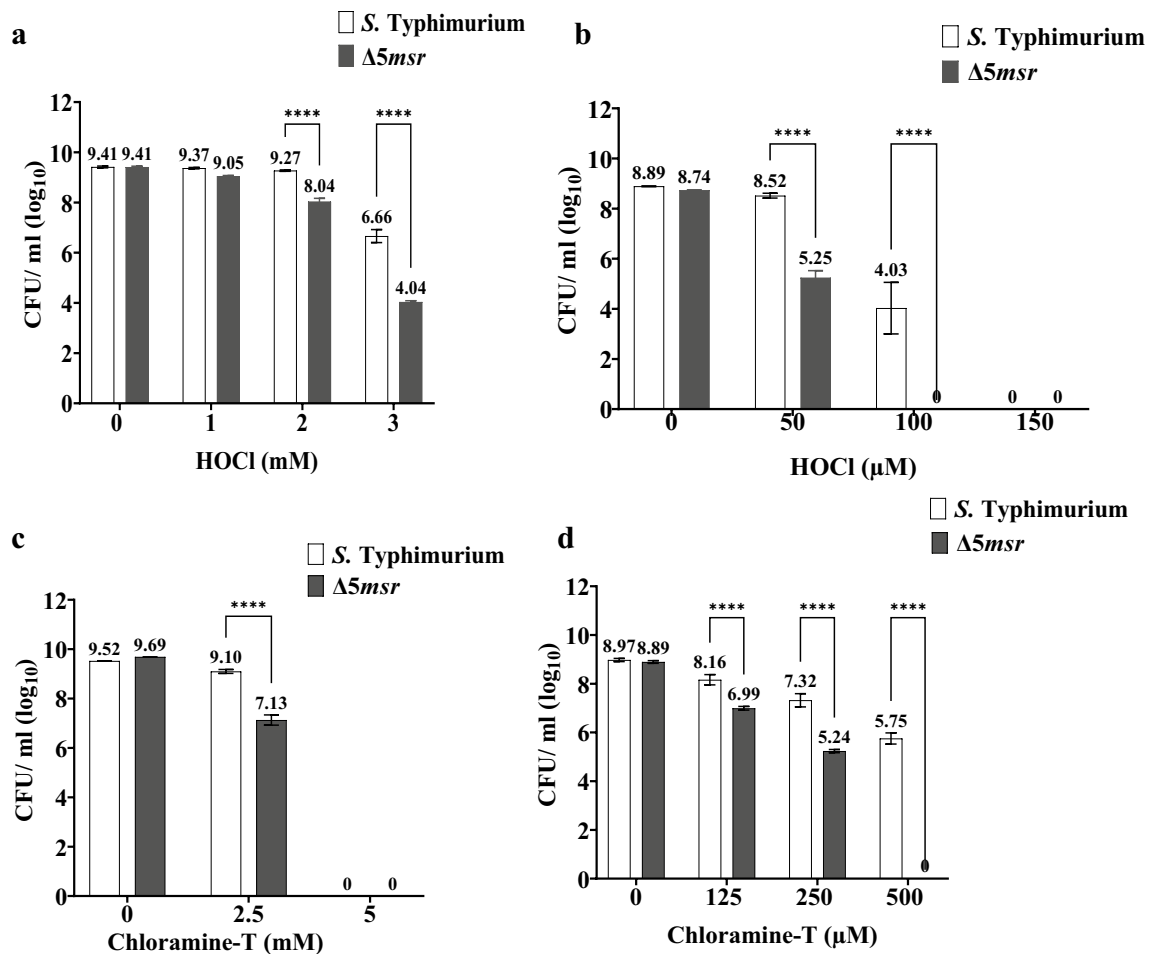


Figure 3. $\Delta 5msr$ mutant is highly susceptible to HOCl and ChT. $\Delta 5msr$ mutant and *S. Typhimurium* strains were grown up to mid-log phase in LB broth and incubated with the indicated concentrations of HOCl or ChT, either directly adding to culture media (a, c), or after pelleting and suspending cultures in PBS (b, d). Following incubation of two hours (a, c) or 30 min (b, d), the cultures were serially diluted and plated on agar media. CFUs were enumerated following overnight incubation of the plates. Data are presented as mean \pm SE (n = 8) for (a), (n = 9) for (b), (n = 3) for (c) and (n = 8) for (d) Data were analyzed by two-way ANOVA. **** $p < 0.0001$.

$\Delta 5msr$ mutant strain accumulates higher orders of oligomers:

Met-oxidation is linked with oligomerization of the proteins²⁴. Native gel analysis revealed varying sizes of protein oligomers. These oligomers were retained in the stacking gels in both strains. However, the degree of migration of higher-order oligomers was much slower in the $\Delta 5msr$ mutant strain (Fig. 8, lanes 2 and 4) than in *S. Typhimurium* (Fig. 8, lanes 1 and 3). This suggests that $\Delta 5msr$ strain accumulates oligomers of much higher molecular weight.

$\Delta 5msr$ mutant exhibits increased neutrophil sensitivity

The role of *msr*s in survival against neutrophil-mediated killing was determined. The $\Delta 5msr$ mutant and *S. Typhimurium* strains were incubated with neutrophils. Recovery of $\Delta 5msr$ mutant and *S. Typhimurium* strains (mean CFUs/ml; thousands \pm SE) after 30 min of incubation were 120 ± 47 and 320 ± 35 respectively. The $\Delta 5msr$ mutant strain showed 2.67 fold more ($p < 0.0001$) susceptibility to neutrophils (Fig. 9). Treatment with Triton X-100 had no significant effects on bacterial counts (Supplementary Fig. S5).

$\Delta 5msr$ mutant strain shows defective survival in mice spleen and liver

$\Delta msrA$, $\Delta msrP$, $\Delta bisC$, $\Delta msrA\Delta msrB$, $\Delta msrA\Delta msrC$ and $\Delta msrA\Delta msrB$ mutants depicted mild to moderate defect in colonization in mice tissues^{16,19}. We assessed the effect of deletions of all *msr*s on the virulence of *S. Typhimurium* in mice. The $\Delta 5msr$ mutant strain has been highly defective ($p < 0.0001$) in colonization in mice spleen and liver (Table 1).

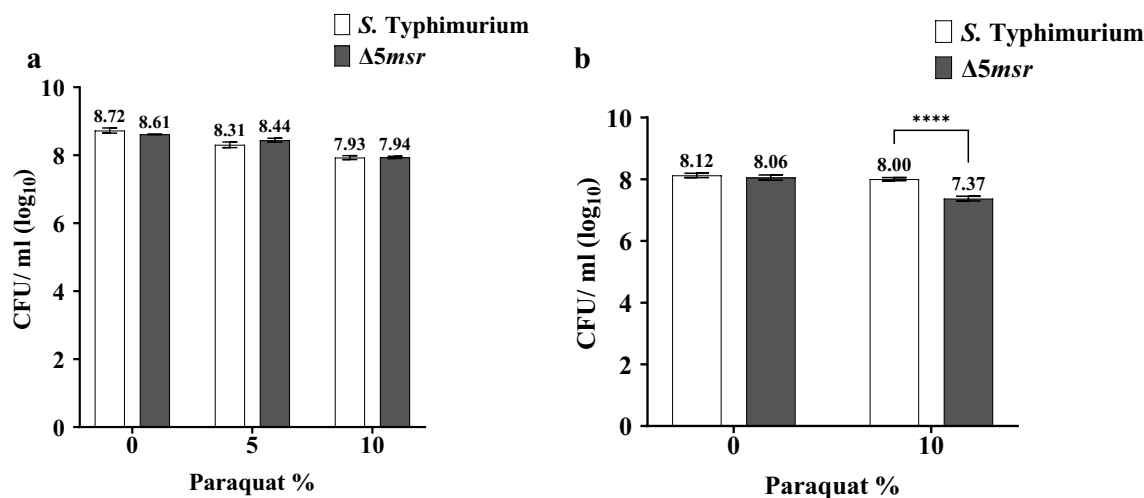


Figure 4. $\Delta 5msr$ is highly susceptible to paraquat under non-growing conditions but not under growing conditions. Mid-log grown cultures of $\Delta 5msr$ mutant and *S. Typhimurium* strains were incubated with the indicated concentrations of paraquat, either directly adding to culture media (a), or after pelleting and suspending cultures in PBS (b). Following 2 h of incubation, the cultures were serially diluted and plated on agar media. CFUs were enumerated following overnight incubation of the plates. Data are presented as mean \pm SE (n = 3) for (a) and (n = 6) for (b). Data were analyzed using two-way ANOVA. **** $p < 0.0001$.

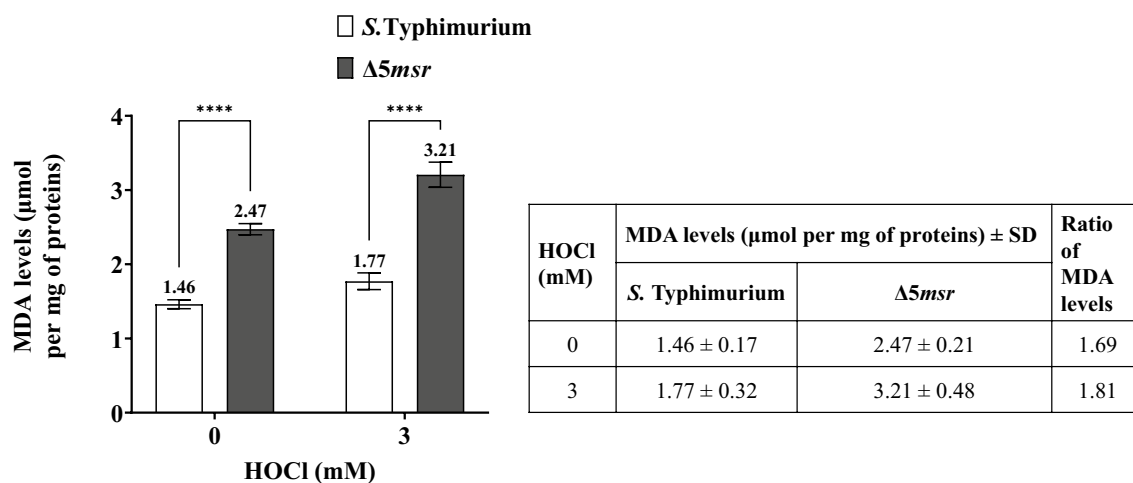


Figure 5. $\Delta 5msr$ mutant strain depicts higher MDA levels. Cell-free lysates of 0 and 3 mM HOCl exposed cultures of $\Delta 5msr$ mutant and *S. Typhimurium* strains were incubated with TBA. MDA levels were estimated as described in materials and methods. Ratios of MDA levels are depicted alongside figure. The data is presented as mean \pm SE (n = 3) and analyzed by two-way ANOVA. **** $p < 0.0001$.

Discussion

Survival of *Salmonella* inside the host, at least in part, depends upon its ability to quench oxidants generated by the host's inflammatory response. Primary antioxidant enzymes play a crucial role in defending host-generated oxidants^{14,25–28}. The scavenging abilities of SODs, catalases, etc., generally fall short of the quantity of oxidants generated during a respiratory burst. This leads to macromolecular damage. Owing to location and reactivity, proteins (Met residues in particular) are highly prone to oxidation which leads to compromised protein function. The functional protein pool in the cell can be maintained by protease-mediated degradation of oxidized proteins followed by ribosomal synthesis²⁹. However, Msr-mediated repair is energetically cheaper and a relatively rapid way to maintain an active protein pool in the cell. This not only helps to thrive bacterial pathogens under stress conditions but might also aid in the infection process. Therefore, Msrs might contribute to the virulence of bacterial pathogens including *S. Typhimurium*. Indeed, *msr* gene deletion strains of several bacterial pathogens showed defective survival under oxidative stress conditions^{15,16,18,19,30,31} and attenuated virulence^{10,16,21,32,33}. As stated above, *S. Typhimurium* encodes five *msrs*. The above studies assessed the roles of single or two Msrs together in the stress survival of *S. Typhimurium*. To determine the importance of Met-SO repair in whole cell physiology and stress survival of *S. Typhimurium*, first, we deleted all five known *msrs* from *S. Typhimurium*. The pan *msr* deletion ($\Delta 5msr$) strain might not be able to repair Met-SO. The deletions were confirmed by PCR.

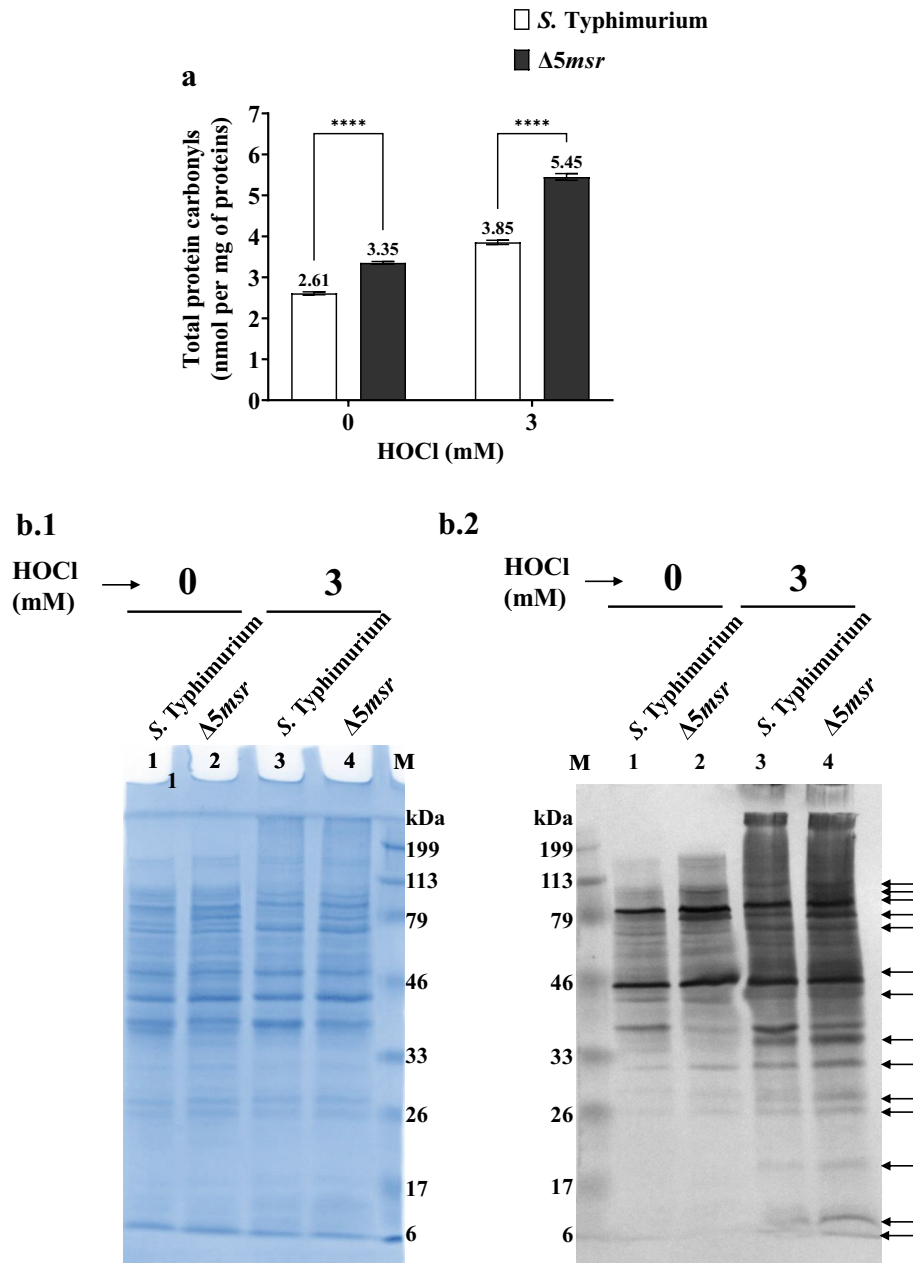


Figure 6. (a) $\Delta 5msr$ mutant accumulates more protein carbonyls. Cell-free lysates of $\Delta 5msr$ mutant and *S. Typhimurium* strains were derivatized with DNPH. Total protein carbonyl levels were estimated as described in methods. The data is presented as mean \pm SE ($n=3$) and analyzed by two-way ANOVA. **** $p < 0.0001$. (b) Oxyblot analysis of $\Delta 5msr$ mutant and *S. Typhimurium* strains. Cell-free lysates of 0 mM and 3 mM HOCl exposed cultures of $\Delta 5msr$ mutant and *S. Typhimurium* strains were derivatized with 2, 4-DNPH. 20 μ g of proteins were resolved on SDS gels. Separated proteins were electroblotted to PVDF membrane. Following blocking, the membranes were incubated in anti-DNPH antibodies and secondary antibodies conjugated with alkaline phosphatase. Blots were developed using NBT and BCIP as substrates. Arrows indicate darker bands in $\Delta 5msr$ lysate loaded lane (6 b.2). Coomassie stained gel served as loading control (6 b.1). The images displayed are cropped from the full-length gels. The full-length gels are presented in supplementary figures S1 (6 b.1) & S2 (6 b.2). ImageJ analysis of the blots is presented in supplementary Table T1.

The primers localized in flanking regions to target genes amplified bigger size amplicon in *S. Typhimurium* while smaller in $\Delta 5msr$ mutant strain (Fig. 1). The $\Delta 5msr$ mutant strain showed a slightly reduced growth in the lag and early log phases as compared to *S. Typhimurium* (Fig. 2). O_2^- generated due to leaky respiratory chain and subsequently other ROS formed might have oxidized Met residues, and due to lack of active repair, the $\Delta 5msr$ mutant strain showed moderate in vitro growth defect.

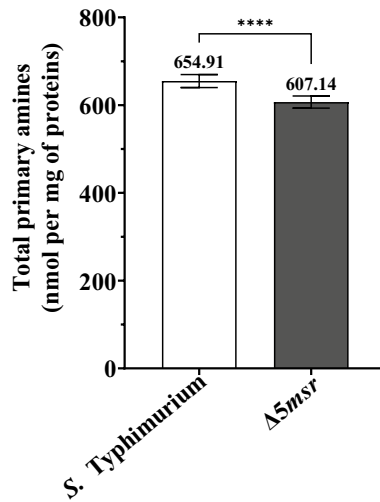


Figure 7. $\Delta 5msr$ mutant depicts fewer primary amines. The lysates of mid-log grown cultures of $\Delta 5msr$ mutant and *S. Typhimurium* strains were incubated with 0.01% TNBS. Total free amines were calculated as described in methods. The data is presented as mean \pm SE (n = 18) and analyzed by students' *t*-test. *****p* < 0.0001.

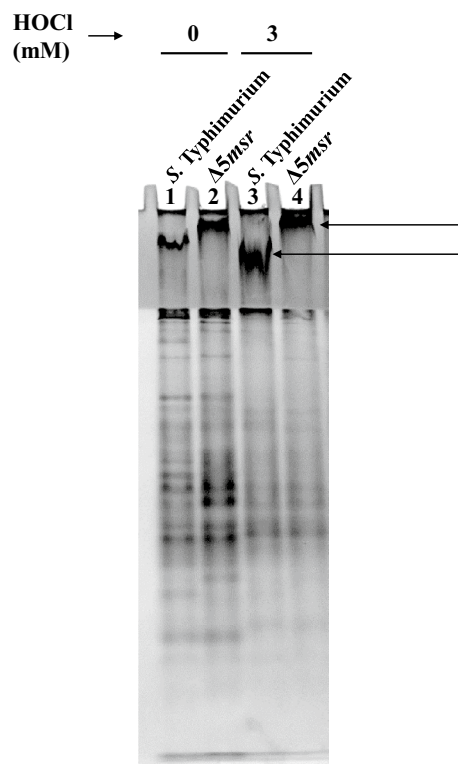


Figure 8. $\Delta 5msr$ shows higher order multimers. $\Delta 5msr$ mutant and *S. Typhimurium* strains were grown up to mid-log phase and exposed to indicated concentrations of HOCl. Following 2 h of exposure, the cultures were lysed. Cell-free lysates were resolved on native-PAGE. Arrows show migration of higher order multimers. The image displayed is cropped from the full-length gel. The full-length gel is presented in supplementary figure S4.

ROS/ RNS-mediated oxidation of bacterial macromolecules is the primary way by which phagocytic cells kill invading bacterial pathogens. *Salmonella* must have evolved the mechanism(s) to counter ROS/ RNS-mediated damage. Primary antioxidants and protein repair enzymes are a few important mechanisms employed by *Salmonella* to survive against ROS/ RNS-mediated damage. Msr mediated repair of Met-SO acts as a dual-edged sword. First, Msr restores the function of Met-SO containing proteins¹⁰. Second, Met residues act as a sink for oxidants⁹. Cyclic oxidation and reduction of Met (free or in proteins) residues reduces overall cellular oxidant

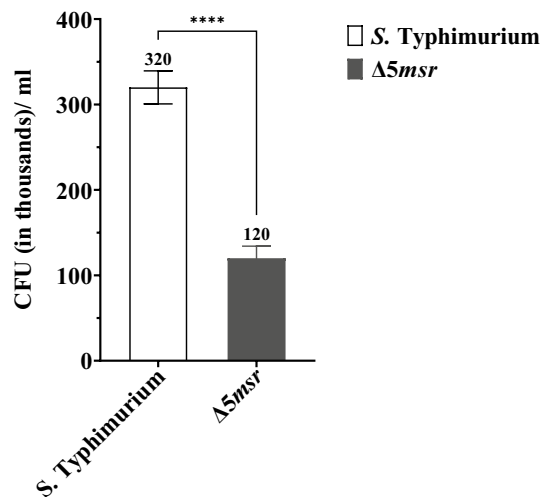


Figure 9. $\Delta 5msr$ mutant strain shows high susceptibility to neutrophils. $\Delta 5msr$ mutant strain and *S. Typhimurium* were incubated with neutrophils. Following incubation, the mix was centrifuged and neutrophils were lysed with 0.1% TritonX-100. The lysates were then serially diluted and plated on agar media. CFUs were counted following incubation of the plates. The data is presented as mean \pm SE (n = 3) and analyzed by students' *t*-test. *****p* < 0.0001.

Days post infection	CI (mean \pm SE)	
	Spleen	Liver
3	0.032 \pm 0.017 (n = 8)	0.016 \pm 0.009 (n = 8)
5	0.111 \pm 0.088 (n = 8)	0.018 \pm 0.012 (n = 8)

Table 1. $\Delta 5msr$ mutant strain shows defective fitness in mice spleen and liver. The mice were inoculated with a mixture of $\Delta 5msr$ mutant and *S. Typhimurium* strains (in a ratio of 1: 1). Mice (at each time point, n = 8) were dissected on days 3 and 5. Bacterial burdens of $\Delta 5msr$ mutant and *S. Typhimurium* strains were determined in spleen and liver and CIs were calculated as described in materials and methods.

levels³⁴. We hypothesized that deletion of all *msrs* might render *S. Typhimurium* highly susceptible to oxidants. In comparison to *S. Typhimurium*, the $\Delta 5msr$ mutant strain has been manyfold susceptible to HOCl (*p* < 0.0001) and ChT (*p* < 0.0001) under growing and non-growing conditions (Fig. 3), but to paraquat (*p* < 0.0001) under non-growing condition (Fig. 4). Interestingly, neither of the strains exhibited sensitivity to 5% paraquat exposure under growing conditions. Although the $\Delta 5msr$ mutant and *S. Typhimurium* strains exhibited slight sensitivity to 10% paraquat under growing conditions, however, there was no significant difference between the sensitivities of the two strains. This could be attributed to the oxidant-quenching ability of the growth medium³⁵. Furthermore, the $\Delta 5msr$ mutant strain expresses higher levels of SODs, potentially aiding the mitigation of superoxide radicals generated upon the addition of paraquat. Consequently, higher paraquat concentrations might be necessary to render the $\Delta 5msr$ mutant strain susceptible to its oxidative effects. In a previous study, the $\Delta msrP$ strain of *S. Typhimurium* showed mild sensitivity (about 7.7 fold) to 100 μ M HOCl exposure²¹. However, the $\Delta msrA$ and $\Delta msrA\Delta msrC$ strains of *S. Typhimurium* displayed significantly higher susceptibilities (~ 93 fold and ~ 3000 fold respectively) to the same concentration of HOCl¹⁹. Likewise, in our study, the $\Delta 5msr$ mutant strain showed extreme sensitivity to HOCl. Notably, no viable colonies were observed following exposure of the $\Delta 5msr$ mutant strain to 100 μ M HOCl (Fig. 3b).

During the respiratory burst, taurine gets oxidized by HOCl and converted into chlorotaurine. Chlorotaurine is a relatively mild but stable oxidant that acts as a sustained source of oxidative stress^{36,37}. ChT is a synthetic analog of chlorotaurine which preferentially oxidizes Met residues³⁸. The $\Delta msrP$ strain of *S. Typhimurium* showed similar sensitivity to 200 μ M ChT exposure²¹. However, the $\Delta msrA$ and $\Delta msrA\Delta msrC$ strains have been ~ 49 and ~ 112 fold more susceptible to 200 μ M ChT (our unpublished observations). Interestingly, the $\Delta 5msr$ mutant strain showed ~ 1600 fold more sensitivity to 250 μ M ChT as compared to *S. Typhimurium* (Fig. 4a). These observations suggest that while each *Msr* contributes to *S. Typhimurium* in resisting oxidative stress, the cumulative effects of deletion of all *Msr*s renders *S. Typhimurium* extremely sensitive to oxidants.

Lipids are highly susceptible to ROS-mediated oxidation. We sought to explore if deletion of *msrs* increases lipid peroxidation in *S. Typhimurium*. MDA is a well-known marker of lipid peroxidation²². Indeed, higher MDA levels (*p* < 0.0001) were observed in $\Delta 5msr$ mutant strain as compared to *S. Typhimurium*, which further increased (*p* < 0.0001) upon HOCl exposure (Fig. 5). This further substantiates that *Msr*s play an important role in maintaining ROS homeostasis and resisting oxidative stress in *S. Typhimurium*.

Carbonyls are considered as stable markers of protein oxidation¹¹. Therefore, carbonyls have been emphasized as hallmarks of oxidative stress³⁹. We investigated whether the deletion of *msrs* affects carbonyl levels. Higher carbonyl levels ($p < 0.0001$) were observed in the $\Delta 5msr$ mutant strain as compared to *S. Typhimurium* (Fig. 6).

Levels of free amines are inversely proportional to protein oxidation²³. We observed lower levels of free amines in the $\Delta 5msr$ mutant strain as compared to *S. Typhimurium* ($p < 0.0001$, Fig. 7).

Met oxidation can change the surface hydrophobicity of the proteins⁴⁰, causing exposure of the buried hydrophobic residues which leads to unfolding and aggregation⁴¹. Oxidation of purified malate synthase of *S. Typhimurium*¹⁰, catalase of *H. pylori*²⁴ and the *E. coli* chaperone GroEL⁴² leads to their aggregation. We explored the effect of deletion of all *msrs* on protein aggregation in *S. Typhimurium*. Native gel analysis suggests the accumulation of higher molecular weight oligomers in the $\Delta 5msr$ mutant strain (Fig. 8).

Neutrophils play a very important role in host defense against *Salmonella* infection⁴³. Due to the presence of NOX (source of superoxide ion) and MPO (source of HOCl) systems, the neutrophils produce copious amounts of ROS and RCS⁴⁴, which directly kill the invading bacteria. *Salmonella* has evolved several strategies to survive neutrophil attack^{45,46}. One such strategy is Msr mediated repair of Met-SO. The MsrP plays a minimal role in the survival of *S. Typhimurium* against neutrophils²¹. On the other hand, the $\Delta msrA$ ¹⁸ and $\Delta msrA\Delta msrC$ strains (our unpublished observations) of *S. Typhimurium* showed hypersensitivity to neutrophils. Our findings indicate that the $\Delta 5msr$ mutant strain is significantly more ($p < 0.0001$) sensitive to neutrophils as compared to *S. Typhimurium* (Fig. 9). Furthermore, the observed sensitivity was a direct result of the interaction of neutrophils with the bacteria and the addition of Triton X-100 did not affect CFU/ml of both the strains (Supplementary Fig. S5), suggesting that deletion of *msrs* doesn't enhance the sensitivity of *S. Typhimurium* to 0.1% Triton X-100.

The contributions of Msrs in the virulence of *S. Typhimurium* have been assessed by using single or double gene deletion strains^{16,17,21}. The $\Delta msrA$ and $\Delta bisC$ mutant strains of *S. Typhimurium* exhibited fitness defects in the spleen and liver of mice. The CI for $\Delta msrA$ mutant in mice spleen and liver were 0.24 ± 0.07 and 0.16 ± 0.03 respectively¹⁶. Similarly, the CI for $\Delta bisC$ mutant in mice spleen and liver were 0.2 and 0.16 respectively¹⁷. However, the $\Delta msrP$ strain of *S. Typhimurium* was moderately defective in mice tissues²¹. Whereas the $\Delta msrB$ and $\Delta msrC$ mutant strains of *S. Typhimurium* were not attenuated in mice tissues¹⁶.

In other studies, double gene deletion mutants of *S. Typhimurium* were generated to investigate the additive effect of *msr* deletions on the virulence of *S. Typhimurium*. The $\Delta msrA\Delta msrB$ and $\Delta msrA\Delta msrC$ strains were highly defective in fitness in mice spleen and liver. The CI values of $\Delta msrA\Delta msrB$ ¹⁶ and $\Delta msrA\Delta msrC$ (our unpublished observations) strains were 0.13 ± 0.05 and 0.21 ± 0.07 respectively in mice spleen, and 0.23 ± 0.09 and 0.11 ± 0.02 respectively in mice liver. However, the $\Delta msrB\Delta msrC$ double mutant strain showed a moderate defect in mice tissues. The $\Delta 5msr$ mutant strain exhibited severe defects in the spleen and liver of mice. CI values in mice spleen and liver were 0.032 ± 0.017 and 0.016 ± 0.009 respectively on day 3 post-infection (Table 1). The lower CI values of the $\Delta 5msr$ mutant strain (in mice tissues) as compared to single or double *msr* deletion strains is likely due to the combined effects of deletion of all five Msrs. These findings highlight the essential roles of Msrs in *Salmonella* survival and virulence in the host.

By repairing Met-SO to Met, Msrs maintain overall ROS homeostasis in cells and prevent ROS-mediated macromolecular damage. Our data suggests that Msrs not only contribute to the survival of *S. Typhimurium* against oxidative stress but also aid in the colonization of this bacterium in animal tissues. Earlier studies suggested mild to moderate attenuation of single/ double *msr* deletion(s) strains. The highly attenuated phenotype observed in the current study might be due to the combined effect of deletions of all *msrs*. The current study might pave the way to develop novel therapeutic/ prophylactic strategies against this bacterium of zoonotic importance.

Methods

Construction and confirmation of pan *msr* gene deletion strain ($\Delta 5msr$) in *S. Typhimurium*

The $\Delta msrA\Delta msrC$ mutant strain (available in our lab) was revived and confirmed by PCR¹⁹. Further deletions of *msrP*, *msrB* and *bisC* genes were carried out by the 1-step gene inactivation method⁴⁷. Briefly, the plasmid pKD46 was introduced into the $\Delta msrA\Delta msrC$ strain¹⁹ by electroporation. The antibiotic cassette along with homologous regions to the flanking genes were amplified from plasmid pKD3 using primers as detailed in Table 2. The cassette was purified and electroporated to λ -red recombinase expressing $\Delta msrA\Delta msrC$ strain. The positive recombinants were selected on antibiotic containing media. Following PCR based screening, the antibiotic cassette was removed by pCP20 encoded FLP recombinase. All three genes were deleted one by one by similar method. All images were visualized using MultiImage™ Light Cabinet (Alpha Innotech Corp.) and acquired using AlphaImager EC software 3.2.2.

In vitro growth analysis

The in vitro growths of the $\Delta 5msr$ mutant and *S. Typhimurium* strains were analyzed as described previously⁴⁸. Isolated colonies were inoculated in 10 ml of LB broth. Following overnight incubation, the cultures were diluted 1: 100 in fresh medium. The cultures were then grown in a shaker incubator at 37 °C at 180 rpm. The optical densities (OD) were measured at 600 nm at an interval of 1 h.

In vitro oxidant susceptibility assays

Overnight grown cultures of the $\Delta 5msr$ mutant and *S. Typhimurium* strains were diluted in LB broth (at 1: 100 ratio) and incubated at 37 °C in a shaker incubator to an OD₆₀₀ of 0.8. The cultures were then exposed to various concentrations of HOCl (NaOCl, Sigma), chloramine T (ChT) (SRL, India), or methyl viologen (paraquat, Sigma) for 2 h. In parallel experiments, the mid-log phase grown cultures were pelleted, washed and suspended in PBS to an OD₆₀₀ of 1.0. The suspensions were then exposed to various concentrations of HOCl, ChT, or paraquat. Following 30 min (for HOCl and ChT) or 2 h (for paraquat) of incubation at room temperature in the dark, the

Serial No	Primer Name	Sequence	Binding location of primer in genome of <i>S. Typhimurium</i> LT2	Amplicon size	Purpose	PCR conditions	References
1	MsrA deletion Test Forward	5' AGATACATTAAT GTTGTIATT 3'	4,645,918 to 4,645,938	300 bp in $\Delta 5msr$ and 850 bp in <i>S. Typhimurium</i>	Confirmation of <i>msrA</i> deletion	95 °C 5 min 95 °C 30 s 48 °C 40 s × 35 72 °C 90 s 72 °C 10 min	19
2	MsrA deletion Test Reverse	5' GTAACGTTTAAT GAAAACCG 3'	4,645,089 to 4,645,108				
3	MsrB deletion Forward	5'AGTTGGTACTGA GGTGTAAATGTT TTGTAGAAATCGGTC AGGCAATGTAGG CTG 3'	–	1096 bp	To amplify FRT flanked Chloramphenicol cassette	95 °C 5 min 95 °C 30 s 42 °C 30 s × 35 72 °C 90 s 72 °C 10 min	Current study
4	MsrB deletion Reverse	5'CATGATGTGTCC CCTCCTGTGGAATAA TTTGCTGAATCGTTT TTTATGAATA 3'	–				
5	MsrB deletion Test Forward	5' ATTCGCCTCACT CTTCCTTTCG 3'	1,369,809 to 1,369,830	498 bp in $\Delta 5msr$ and 869 bp in <i>S. Typhimurium</i>	Confirmation of <i>msrB</i> deletion	95 °C 5 min 95 °C 30 s 62 °C 40 s × 40 72 °C 100 s 72 °C 10 min	Current study
6	MsrB deletion Test Reverse	5' CCATCTGACCGT GAGTATCGA 3'	1,370,657 to 1,370,677				
7	MsrC deletion Test Forward	5' GAGCAAGAACGC ATTTAATGC 3'	1,944,530 to 1,944,550	280 bp in $\Delta 5msr$ and 500 bp in <i>S. Typhimurium</i>	Confirmation of <i>msrC</i> deletion	95 °C 5 min 95 °C 30 s 48 °C 40 s × 40 72 °C 90 s 72 °C 10 min	19
8	MsrC deletion Test Reverse	5' GCGTACGCAGGC CGTGTT 3'	1,944,054 to 1,944,071				
9	MsrP deletion Forward	5'GACCGGGAGTCT GTGATGAAAAAGATA CGTCCATTAACAGAA GCCGTGTAGGCT GGAGCTGCTTC 3'	–	1092 bp	To amplify FRT flanked Chloramphenicol cassette	95 °C 5 min 95 °C 30 s 62 °C 40 s × 35 72 °C 100 s 72 °C 10 min	21
10	MsrP deletion Reverse	5'TGCTGTCAGACG CACTTAAAAATTCTC CCGCAAATTGAG ACCGCGCATATGAAT ATCCTCCTTAG 3'	–				
11	MsrP ST test Forward	5'AGGGCCGTACGC TGGTAAGAT 3'	3,547,964 to 3,547,985	550 bp in $\Delta 5msr$ and 1401 bp in <i>S. Typhimurium</i>	Confirmation of <i>msrP</i> deletion	95 °C 5 min 95 °C 30 s 62 °C 40 s × 40 72 °C 100 s 72 °C 10 min	21
12	MsrP ST test Reverse	5'GAAACACCATAA TCCTAACAGGCG 3'	3,549,341 to 3,549,364				
13	BisC deletion Forward	5'CTCCCTGCAAAC CGTTGTGCATGA CCAGGTGCACAG TAAAACGCGGTA GGTGG 3'	–	1103 bp	To amplify FRT flanked Chloramphenicol cassette	95 °C 5 min 95 °C 30 s 42 °C 30 s × 35 72 °C 90 s 72 °C 10 min	Current study
4	BisC deletion Reverse	5'CGCCTGCGGTAG GTTCAGGGTCCG GCCATGCGCCTT CGTGAATACAATGAA TAT 3'	–				
15	BisC ST test Forward	5'TTGACCACGCA CCATCGCGT 3'	3,832,645 to 3,832,665	445 bp in $\Delta 5msr$ and 2335 bp in <i>S. Typhimurium</i>	To amplify FRT flanked Chloramphenicol cassette	95 °C 5 min 95 °C 30 s 64 °C 40 s × 40 72 °C 150 s 72 °C 10 min	Current study
16	BisC ST test Reverse	5'TTATGAGTTGGC AGGCGGATCAA 3'	3,830,332 to 3,830,354				

Table 2. Primers and PCR conditions used for deletion and confirmation of various genes in *S. Typhimurium* LT2 (NCBI accession no. NC_003197.2).

excess oxidants were quenched by the addition of L-methionine (10 mM final) for 15 min. Following incubation, the suspensions were ten-fold serially diluted and plated on agar media. CFUs/ ml were calculated after overnight incubation of the plates at 37 °C.

Preparation of cell-free lysates

Mid-log phase grown cultures of $\Delta 5msr$ mutant or *S. Typhimurium* strains were exposed to PBS or HOCl, harvested and washed twice with PBS by centrifugation at $4500\times g$ at $4\text{ }^{\circ}\text{C}$ for 10 min. Pellets were lysed by sonication. Unbroken cells and debris were removed by centrifugation at $15,000\times g$ for 30 min at $4\text{ }^{\circ}\text{C}$. Supernatants were collected, aliquoted and stored at $-80\text{ }^{\circ}\text{C}$. Total proteins in such lysates were estimated using the Pierce™ BCA Protein Assay Kit (Thermo Scientific).

Quantitation of malondialdehyde (MDA) levels

MDA levels were estimated using TBARS assays as described elsewhere⁴⁹ with minor modifications. Cell-free lysates of $\Delta 5msr$ mutant and *S. Typhimurium* strains were diluted to a concentration of 5 mg/ml. 500 μg of proteins (in 100 μl) were incubated with an equal volume of 10% TCA prepared in 0.25 N HCl (5% TCA final concentration) for 5 min. The mixtures were centrifuged at $15,000\times g$ for 10 min, supernatants were collected and incubated with 100 μl of 0.67% TBA (0.375% final concentration) for 10 min in a boiling water bath. Absorbance was recorded at 535 nm against a blank containing all the reagents except the sample. MDA levels were calculated using the formula $c = A_{535}/(\epsilon l)$, where $\epsilon = 1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (molar absorption coefficient of TBA-MDA adduct is $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$), c is the concentration of MDA in moles/ liter (M), and A = absorbance at 535 nm and expressed as micromoles per mg proteins⁵⁰.

Estimation of primary amines

Primary amines were estimated by TNBSA assays, as described previously (Habeeb, 1966) with minor modifications. Briefly, cell-free lysates were diluted with 0.1 M sodium bicarbonate (pH 8.5) to a concentration of 100 μg /ml of proteins. Fifty micrograms of samples were incubated with 0.25 ml of freshly prepared 0.01% TNBS (Sigma) in 0.1 M sodium bicarbonate (pH 8.5) for 2 h at $37\text{ }^{\circ}\text{C}$. The reaction was stopped by the addition of 0.25 ml of 10% SDS and 0.125 ml of 1 N HCl. Absorbance was recorded at 335 nm. Primary amines in the lysates were quantified using a standard curve generated using L-methionine standards.

Estimation of total protein carbonyls

Total protein carbonyls were estimated using 2, 4 dinitrophenyl hydrazine (2, 4 DNPH) assays⁵¹. 150 μl of cell-free lysates (a total of 750 μg of proteins) were incubated with 600 μl of 2, 4 DNPH solution (10 mM in 2.5 M HCl) for an hour in the dark. The mixtures were briefly vortexed every 15 min. Proteins were recovered by the addition of 750 μl of TCA (10% final concentration) on ice and centrifugation at $15,000\times g$ for 20 min at $4\text{ }^{\circ}\text{C}$. The pellets were then washed twice with 10% TCA. Free DNPH was removed by washing with ethanol and ethyl acetate (1:1). The final pellets were air-dried, dissolved in 6 M guanidine hydrochloride, and incubated at $37\text{ }^{\circ}\text{C}$ for 30 min with intermittent vortexing. The absorbance was recorded at 355 nm using 6 M guanidine hydrochloride as a blank. Carbonyl levels were calculated using the formula $c = A_{355}/(\epsilon l)$, where $\epsilon = 22,000 \text{ M}^{-1} \text{ cm}^{-1}$ (molar absorption coefficient of hydrazone is $22,000 \text{ M}^{-1} \text{ cm}^{-1}$), c is the concentration of carbonyl groups in moles/ liter (M), and A = absorbance at 355 nm⁵².

Oxyblot analysis

Oxyblot analysis was performed using an OxyBlot™ Protein Oxidation Detection kit (EMD Millipore), as described previously⁵¹. One hundred micrograms of cell-free lysates were denatured with SDS (6% final) and derivatized with 2, 4 DNPH for 15 min at room temperature. Following derivatization, the samples were incubated in a neutralization solution. 20 μg of derivatized proteins were resolved on 10% SDS gel and electroblotted onto a PVDF membrane. Free sites on the membrane were blocked by 2% skimmed milk prepared in PBS-T (0.05% Tween 20). After six washes with PBST, the membrane was incubated with anti-DNPH antibodies (1:150 dilution) for 3 h at $37\text{ }^{\circ}\text{C}$. After washing, the membrane was washed with anti-rabbit goat IgG conjugated with alkaline phosphatase (1:15,000; Sigma) for 4 h at $37\text{ }^{\circ}\text{C}$. NBT and BCIP were used as substrates for the development of the blots⁵¹. Gels and their blots were visualized in the Bio-Rad GelDoc™ imaging system and images were acquired using the Image Lab software ver. 3.0. Quantification of the densities of the bands in various lanes was done using ImageJ software.

Native polyacrylamide gel electrophoresis

Accumulation of protein aggregates was determined using native polyacrylamide gel electrophoresis¹⁰. 100 μg of cell-free lysates were resolved in native gels (4% stacking, 8% separating). The gels were stained with CBB-R250 and visualized using the Bio-Rad GelDoc™ imaging system and images of the gels were acquired using the Image Lab software ver. 3.0.

Neutrophil sensitivity assays

The sensitivity of $\Delta 5msr$ mutant and *S. Typhimurium* strains to neutrophils was evaluated as previously described¹⁸ with minor modifications. Briefly, anticoagulant mixed goat blood was diluted with sterile PBS in 1:1 ratio. A double-density gradient was prepared by layering equal volumes of Histopaque 1077 over Histopaque 1119 (Sigma). The diluted blood was layered over the Histopaque gradient. Following centrifugation at $750\times g$ for 45 min at room temperature, the neutrophils were collected from the lower Histopaque 1077/ 1119 interface. The cells were washed twice with RPMI-1640 (complete, without phenol red) at $250\times g$ for 10 min, resuspended in the same medium and kept on ice. The total number of viable cells was counted by the trypan blue dye exclusion method. Additionally, morphological identification of neutrophils was done by Giemsa staining. Neutrophils were adjusted to 2×10^6 cells/ ml. 250 μl of cells (0.5×10^6 cells) per well were seeded into 24-well tissue culture

plates. Mid-log phase grown cultures of $\Delta 5msr$ mutant and *S. Typhimurium* strains were pelleted, washed and resuspended in RPMI-1640 medium. Neutrophils and bacterial cultures were mixed in a multiplicity of infection (MOI) of 1: 1 (neutrophils: bacteria). The mix was then incubated in a CO₂ incubator at 37 °C for 30 min. Following incubation, the mix was centrifuged at 13,000 rpm for 3 min. The supernatant was discarded and the neutrophils were lysed by 0.1 percent Triton X-100 for 5 min. In a different experiment, neutrophil isolation was excluded and only the resuspended cultures of $\Delta 5msr$ mutant and *S. Typhimurium* strains ($\sim 0.5 \times 10^6$ cells) were incubated with either 0.1 percent Triton X-100 or PBS for 5 min. The detergent action of Triton X-100 was neutralized by the addition of an equal volume of 1 X PBS to the mix. Following this, lysates were tenfold serially diluted and plated on agar plates. The colonies were counted following overnight incubation of the plates.

Assessment of virulence in mice

Animal experiments were conducted with the approval of the Institutional Animal Ethics Committee (IAEC) at the Indian Council of Agricultural Research-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, India, under the approval file No. F.26–1/2022–23/J.D.(R)/IAEC MEETING. All animal procedures strictly adhered to the guidelines and regulations set forth by IAEC, ICAR-IVRI, Izatnagar, India. The experimental protocols were conducted in accordance with the ARRIVE guidelines. The effect of deletion of all *msr* genes in the virulence of *S. Typhimurium* was evaluated in a mouse model^{16,48}. In brief, six to seven weeks old Swiss Albino mice were intraperitoneally infected with a mixture of the $\Delta 5msr$ strain carrying a chloramphenicol cassette ($\Delta 5msr:: Cm$) and *S. Typhimurium* strain (8×10^4 bacteria in 100 μ l in 1: 1 ratio). The actual inoculated numbers were determined by retrospective plating. Eight mice were sacrificed on days 3 and 5 post-infection, and their spleens and livers were harvested. The whole spleen and 100 mg of liver tissues were homogenized in PBS, and the homogenates were tenfold serially diluted and plated on HE agar as well as HE agar supplemented with chloramphenicol. The colonies observed on chloramphenicol-supplemented with HE agar plates were considered $\Delta 5msr$ mutant strain colonies. The number of *S. Typhimurium* colonies was calculated by subtracting the number of colonies that appeared on chloramphenicol-supplemented HE agar plates from the total number of colonies that appeared on unsupplemented HE agar plates. Competitive indices (CI) were calculated as described previously^{16,48}. The CI is the ratio of the number of $\Delta 5msr$ strain CFU to *S. Typhimurium* CFU recovered divided by the ratio of the numbers of $\Delta 5msr$ CFU strain to *S. Typhimurium* CFU inoculated.

Statistical analysis

Data were analyzed by GraphPad Prism version 9.2 (Trial). Comparisons between multiple groups were done using either two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or students' *t*-test. $p < 0.01$ was considered significant among different test groups.

Data availability

The data generated and analyzed in the current study are available with the corresponding author.

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Author contributions

R.S., T.K.S.C. and M.M. designed the experiments. R.S., T.K.S.C., L.L. and E.S. performed the experiments and analyzed the data. R.S. and M.M. prepared and edited the manuscript. S.Q. read the manuscript and provided critical inputs. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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