Supporting Information

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SI Materials and Methods

Bacterial Strains. roGFP2 from the pRSETB-roGFP2 vector was cloned into pET28a for inducible expression in the *Escherichia coli* BL21(DE3) background. Additionally, roGFP2 was cloned into the pfpv25 vector for constitutive expression of roGFP2. The pfpv25-roGFP2 vector was then transformed into *Salmonella enterica* Typhimurium (*S.* Typhimurium) SL1344, and 50 μg/mL carbenicillin was added to the growth medium for maintenance of the plasmid. All mutants were made in the SL1344 background. The making of the *ssaR* and *sifA* mutants has been described previously (1). The clean deletion of *sseJ* was made by allele exchange in vector pRE112 as described previously (2). Deletions were confirmed by PCR and sequencing.

Protein Purification. roGFP2 was expressed and purified as previously reported (3).

Fluorescent Protein Assays. For complete reduction, purified roGFP2 was preincubated with 10 mM DTT. Excess DTT was removed from the solution using Centri-Spin 20 columns (Princeton Separations). Reactions were carried out with 10 μg of protein in 200 μL of PBS, pH 7.4, in a black, clear-bottom 96-well plate (Corning). Excitation scans were carried out in a Tecan plate reader with excitation wavelengths covering 350 to 500 nm while emission was measured at 510 nm. Signals for fully oxidized and fully reduced roGFP2 were obtained by adding 100 mM H_2O_2 and 10 mM DTT 30 min before the experiment. Data were analyzed in Excel, and all values were normalized to the values obtained for maximally oxidized and for fully reduced protein.

In Vitro Real-Time Measurement of Intrabacterial Redox Potential. In vitro analysis of the intrabacterial redox potential was done at 37 °C in a Tecan plate reader with excitation at 405 and 480 nm while emission was measured at 510 nm. Log phase bacterial cultures were resuspended in PBS or low-phosphate medium (LPM) (pH 5.8) at OD 1.0, and 200 μL per well were loaded in a black, clear-bottom 96-well plate. Background signals from the nonfluorescent corresponding strain were obtained in the same experiment. Additionally, the signals for fully oxidized and fully reduced bacteria were obtained by adding 50 mM H_2O_2 and 10 mM DTT to the bacteria culture at the start of the experiment. All values were normalized to the values obtained for maximally oxidized and for fully reduced bacterial cultures. Images were analyzed by ImageJ as described previously (4).

Fluorescence Microscopy. For real-time fluorescence analysis of SL1344 by microscopy, bacteria were grown overnight and diluted in PBS. Images were taken every 20 s for a period of 20 min with an Olympus IX81 microscope at a multitude of magnification of 600. Infected cells were fixed with 3% (vol/vol) PFA after incubation with 50 mM NEM. Coverslips were mounted on slides using antifade reagent and analyzed by microscopy. In every experiment, fully oxidized and fully reduced ratio values were obtained, and all data were normalized to fully oxidized and fully reduced ratios. Images were analyzed by ImageJ as was described previously (4).

Culturing Bone Marrow-Derived Macrophages. Bone marrow was collected from the tibias and femurs of 6- to 8-wk-old WT C57BL/6J, iNOS$^{-/-}$ (B6.129P2-Nos2tm1Lau/J), and gp91phox$^{-/-}$ (B6.129S6-Cybbtm1Din/J) male mice (all purchased from The Jackson Laboratory). Each well of a six-well plate was seeded with 1×10^6 cells [RPMI 1640 containing L-glutamine; Life Technologies; supplemented with 10% (vol/vol) heat-inactivated FBS; Life Technologies; 100 U/mL penicillin and 100 μg/mL streptomycin; Life Technologies; and 20 ng/mL M-CSF; Peprotech]. Cells were incubated at 37 °C with 5% (vol/vol) CO_2. On days 4 and 6 of culture, 2 mL of medium were removed from each well and replaced with 2 mL of fresh medium. On day 7 of culture, culture supernatant containing nonadherent cells was removed before performing assays. The purity of adherent cells was confirmed by flow cytometry for all genotypes on day 7 of culture, and >97% of cells were $CD45^+CD11b^+F4/80^+$.

Infection Experiments. HeLa cells were grown in DMEM containing 10% heat-inactivated FBS, 1% GlutaMAX, and 1% nonessential amino acids. Cells were seeded at 3.0×10^5 in six-well plates 48 h before infection. THP-1 cells were grown in RPMI supplemented with 10% (vol/vol) inactivated FBS, 1% GlutaMAX, and 1% nonessential amino acids. Seventy-two hours before infection, THP-1 cells were seeded at 3.6×10^6 in six-well plates, and for the first 24 h 100 nM PMA was added for differentiation into macrophage-like cells. After 24 h, the medium was replaced, and the cells were incubated for another 48 h before infection was started. Overnight cultures of *Salmonella* were used for infection (stationary-phase bacteria) or diluted 1:33 in LB and subcultured for 3 h at 37 °C to obtain log phase bacteria. The bacteria were resuspended in PBS and used at a multiplicity of infection of 50. THP-1 cells were spun down at 500 × *g* for 10 min to synchronize infection. Inhibitors diphenylene iodonium chloride (DPI) (Sigma) and L-NMMA (Cedarlane) were added at concentrations of 5 μM and 2 mM, respectively, throughout the duration of the infection experiment. At 30 min postinfection (p.i.), cells were washed with PBS and further incubated in medium supplemented with 50 μg/mL gentamicin. At 2 h p.i., cells were washed with PBS and incubated in medium supplemented with 12 μg/mL gentamicin. For CFU counts, cells were lysed in PBS supplemented with 1% Triton X-100 and 0.1% SDS. After a serial dilution, the samples were plated on 50 μg/mL streptomycin plates after which colony-forming units were counted manually. For AMNIS analysis, 5 min before sampling time, 50 mM NEM was added to the media. The cells were washed and detached from the surface with Cell Dissociation Buffer enzyme-free Hanks' based (Gibco). Immediately after detachment, 3% (vol/vol) PFA was added to fix the infected cells. Cells were stained the next day.

Staining Before AMNIS Analysis. After fixation the cells were permeabilized with 0.1% Triton X-100 in PBS. The cells were then incubated with a primary antibody against the *Salmonella* O antigen (RbαSalm O Lot2098504) for 3 h and subsequently incubated with a secondary Alexa 660 goat α-rabbit antibody (Invitrogen).

AMNIS ImageStream and IDEAS/ImageJ Analysis. Samples were analyzed by the AMNIS ImageStream. The laser intensities for 405, 488, 658, and 785 nm were 100, 120, 20, and 3.8, respectively. The data files were further analyzed with the IDEAS software, version 6.0.129.0, which is supplied by AMNIS. Infected cells were selected based on fluorescence at 660 nm. Every image of an infected cell was then selected by the program based on fluorescent intensity at 660 nm. Based on this selection, a mask was created called "bacteria" that was used for further analysis of the 405/480-nm ratio of intracellular bacteria. The background fluorescence of

each individual cell was obtained by a separate mask that excluded the bacterial mask but encapsulated the rest of the cell. This mask was named "background" and the 405- and 480-nm fluorescent background intensities for each individual image were subtracted from the 405- and 480-nm fluorescent signal of the bacterial mask. The resulting 405/480 ratio signals were plotted in a histogram. Reduced and oxidized controls were obtained within each experiment, and all values were normalized to oxidized and reduced ratio values. Pseudocolored ratio images were made through analysis by ImageJ as was described previously (4).

Griess Assay and Amplex Red Assay. The Griess assay (Sigma) and the Amplex red hydrogen peroxide assay (Molecular Probes; A22188) were done according to the manufacturer's instructions. Samples were analyzed immediately after the samples were obtained.

Calculation of E_{roGFP2}. To calculate the E_{roGFP2} of *S.* Typhimurium, we used the method as described by Gutscher et al. (5). In short, we measured the R (ratio 405/480 nm) of *S.* Typhimurium in LPM, pH 5.8. Additionally, we obtained the R_{red} (ratio 405/480 nm of reduced control), the R_{ox} (ratio 405/480 nm of oxidized control), and the fluorescence intensity at 480 nm under reduced or oxidized conditions (I480max and I480min, respectively). With these values, we calculated the degree of roGFP2 oxidation (OxD_{roGFP2}) given by the following formula:

$$OxD_{roGFP2} = \frac{R - R_{red}}{(I480min/I480max)(R_{ox} - R) + (R - R_{red})}.$$

The degree of oxidation could then be used to calculate the intracellular sensor redox potential E_{roGFP2} by using the Nernst equation as follows:

$$E_{roGFP2} = E^{O'}_{roGFP2} - \left(\frac{RT}{zF}\right) * \ln\left(\frac{(1 - OxD_{roGFP2})}{OxD_{roGFP2}}\right),$$

in which R is the gas constant (8.315 $J \cdot K^{-1} \cdot mol^{-1}$), T is the absolute temperature (310.15 K), z is the number of transferred electrons (2), and F is the Faraday constant (96,485 $C \cdot mol^{-1}$). The midpoint potential of roGFP2 ($E^{O'}_{roGFP2}$) is −280 mV (6, 7).

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2. Edwards RA, Keller LH, Schifferli DM (1998) Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. *Gene* 207(2):149–157.
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4. Morgan B, Sobotta MC, Dick TP (2011) Measuring E(GSH) and H_2O_2 with roGFP2-based redox probes. *Free Radic Biol Med* 51(11):1943–1951.
5. Gutscher M, et al. (2008) Real-time imaging of the intracellular glutathione redox potential. *Nat Methods* 5(6):553–559.
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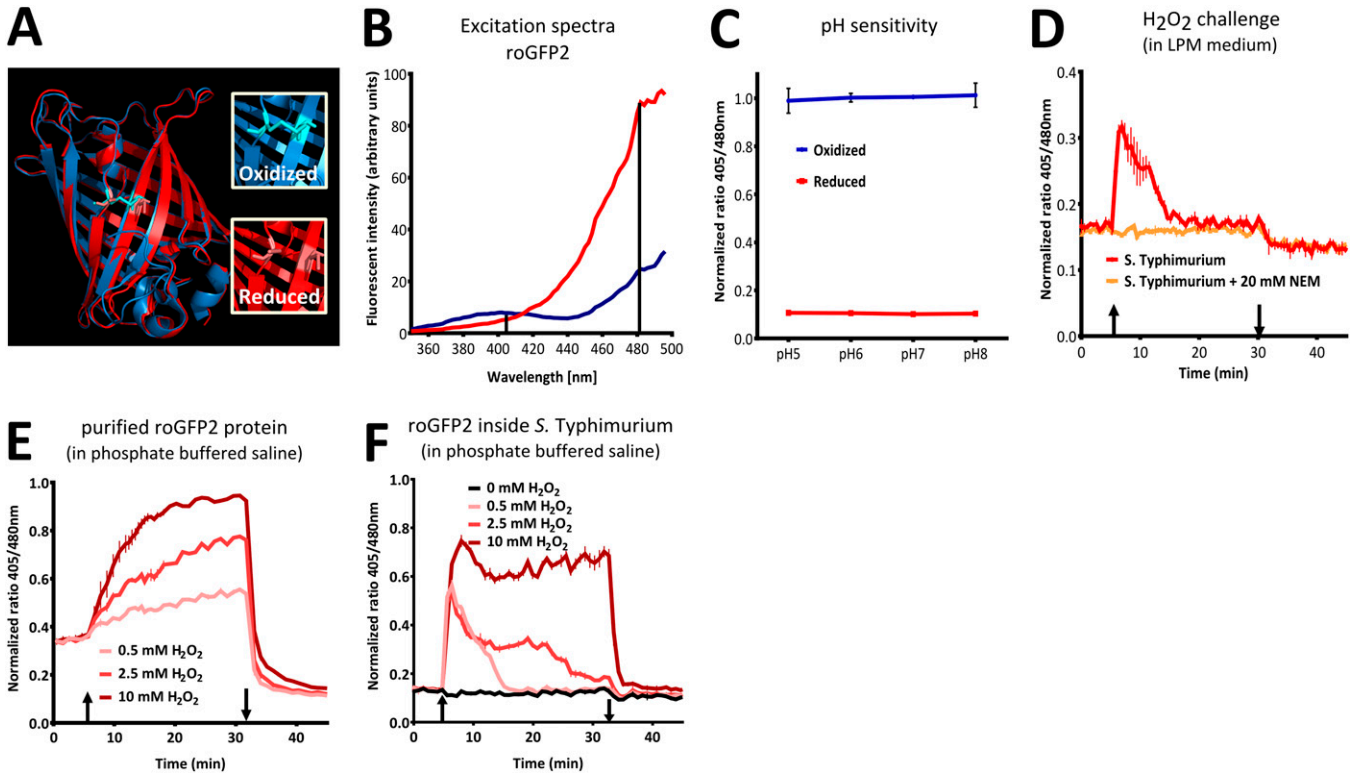


Fig. S1. Characterization of roGFP2. (*A*) Structure of roGFP2 obtained from pdb.org after processing in PyMOL in oxidized and reduced conformation. (*B*) Oxidized and reduced fluorescent excitation spectra with purified roGFP2 after addition of 100 mM H_2O_2 for maximum oxidation or 10 mM DTT for maximum reduction. (*C*) The maximum and minimum ratio value of roGFP2 in *S.* Typhimurium at different pH values. Each value was normalized to the maximum and minimum ratio obtained in PBS at pH 7.4. (*D*) Real-time intrabacterial redox potential after a challenge with 2.5 mM H_2O_2 (upward arrow) and subsequent challenge with 10 mM DTT (downward arrow) in LPM, pH 5.8. *S.* Typhimurium is resuspended in PBS, and 20 mM NEM is added 5 min before the challenge to block oxidation of roGFP2 (see description in text). (*E*) Challenge of purified roGFP2 protein with varying concentrations of H_2O_2 (upward arrow) and subsequent challenge with 10 mM DTT (downward arrow). (*F*) Challenge of intrabacterial roGFP2 protein with varying concentrations of H_2O_2 (upward arrow) and subsequent challenge with 10 mM DTT (downward arrow).

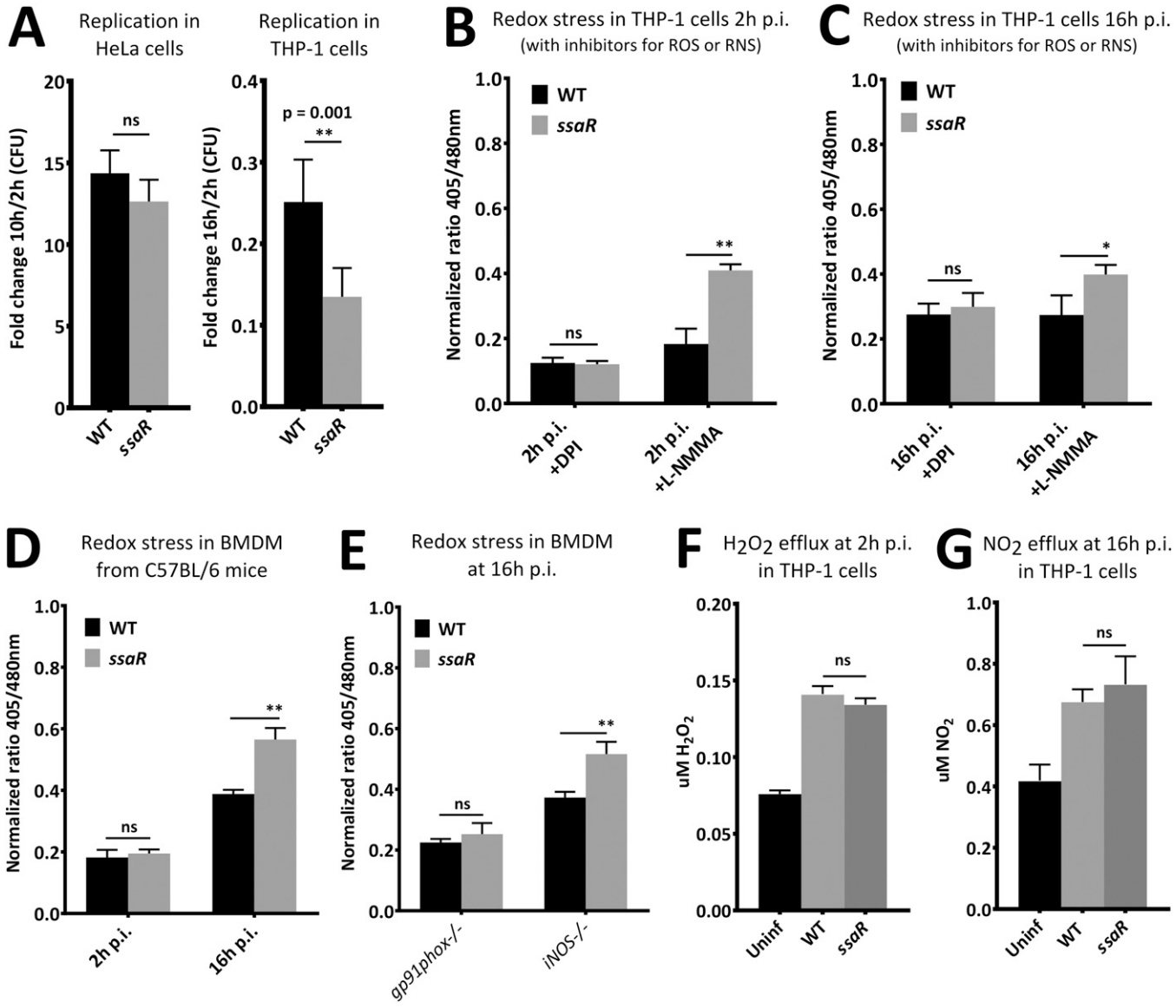


Fig. S2. Infection dynamics of WT and *ssaR S*. Typhimurium. (*A*) Intracellular survival of intracellular WT and *ssaR S*. Typhimurium in HeLa cells and THP-1 cells determined by CFU counts taken at 10/16 and 2 h p.i. Error bars indicate the SD. Each experiment was repeated at least 12 times. (*B* and *C*) Intrabacterial redox stress in WT and *ssaR S*. Typhimurium in THP-1 cells with the addition of inhibitors for oxidative stress (DPI) or nitrosative stress (L-NMMA) at 2 and 16 h p.i. Error bars indicate the SD. (*D*) Intrabacterial redox stress in WT and *ssaR S*. Typhimurium in BMDMs. (*E*) Intrabacterial redox stress in WT and *ssaR S*. Typhimurium in *gp91phox*$^{-/-}$ and *iNOS*$^{-/-}$ BMDMs. Each value represents analysis of at least 15,000 pictures. Error bars indicate the SD over three separate experiments. (*F*) Overall H_2O_2 production of THP-1 cells after 2 h p.i. with WT and *ssaR S*. Typhimurium as measured by the Amplex red assay. (*G*) Overall NO_2 efflux of THP-1 cells after 16 h p.i. with WT and *ssaR S*. Typhimurium as measured by the Griess assay. The NO_2 concentration corresponds with the NO• production. Significance was obtained by a Student *t* test.

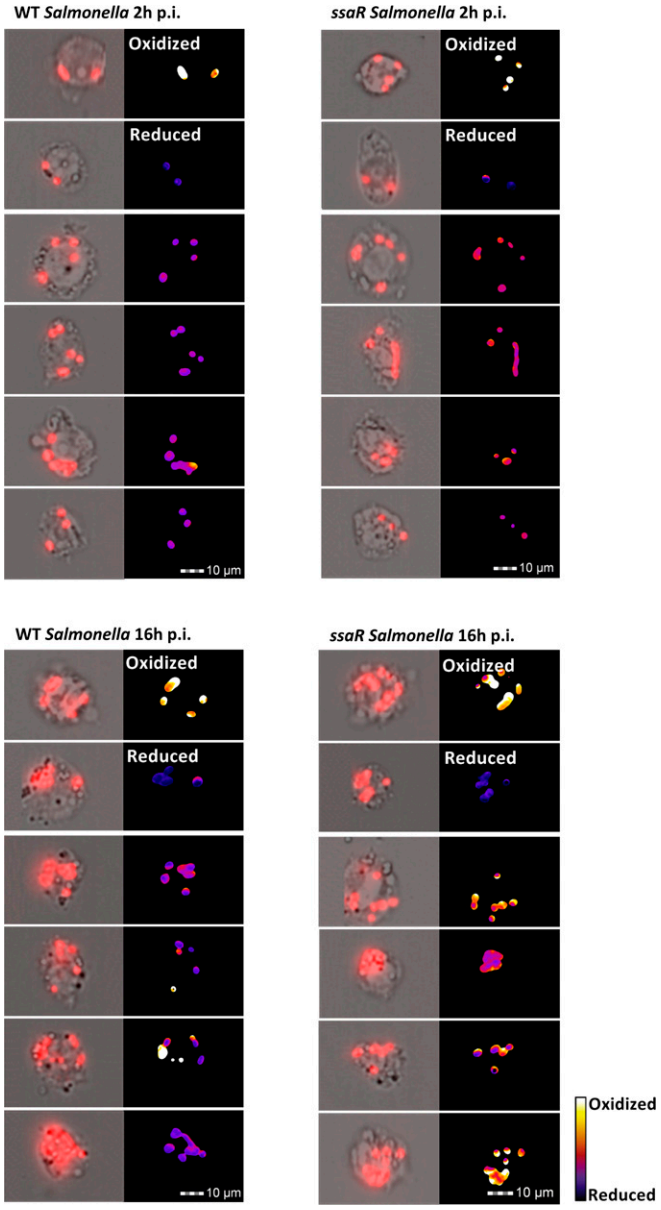


Fig. S3. Representative images of infected THP-1 cells with WT and *ssaR Salmonella*. Representative images of infected THP-1 cells that contain multiple intracellular bacteria from the AMNIS ImageStream. Images were taken at 2 and 16 h p.i. The corresponding pseudocolored ratio images were obtained after analysis with ImageJ. Heterogeneity between intracellular bacteria is observed at 16 h p.i.

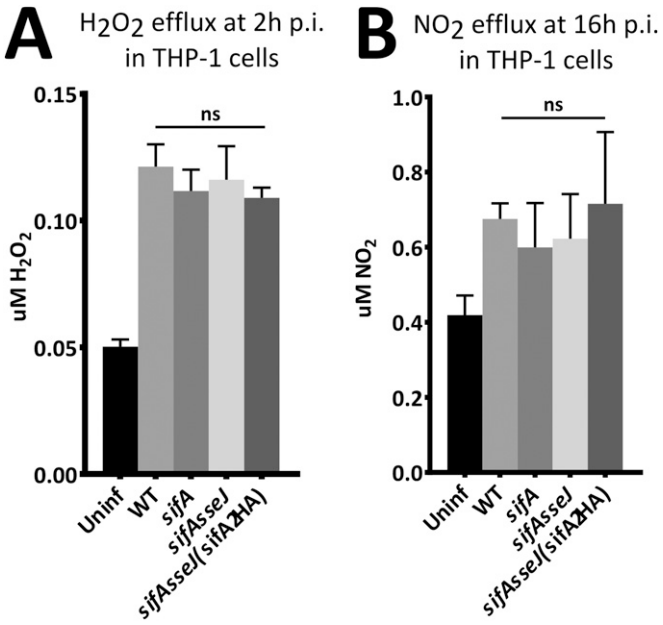


Fig. S4. Measurement of overall H_2O_2 and NO efflux after infection of THP-1 cells with WT, *sifA*, *sifAsseJ*, and *sifAsseJ*2HAsifA *Salmonella*. (*A*) H_2O_2 production at 2 h p.i. with WT *sifA*, *sifAsseJ*, and *sifAsseJ*2HAsifA *S*. Typhimurium in THP-1 cells as obtained by the Amplex red assay. (*B*) NO_2 concentration at 16 h p.i. with WT *sifA*, *sifAsseJ*, and *sifAsseJ*2HAsifA *S*. Typhimurium in THP-1 cells as obtained by the Griess assay. The NO_2 concentration corresponds with the NO• production. Error bars indicate the SD. Significance was obtained by a Student *t* test.

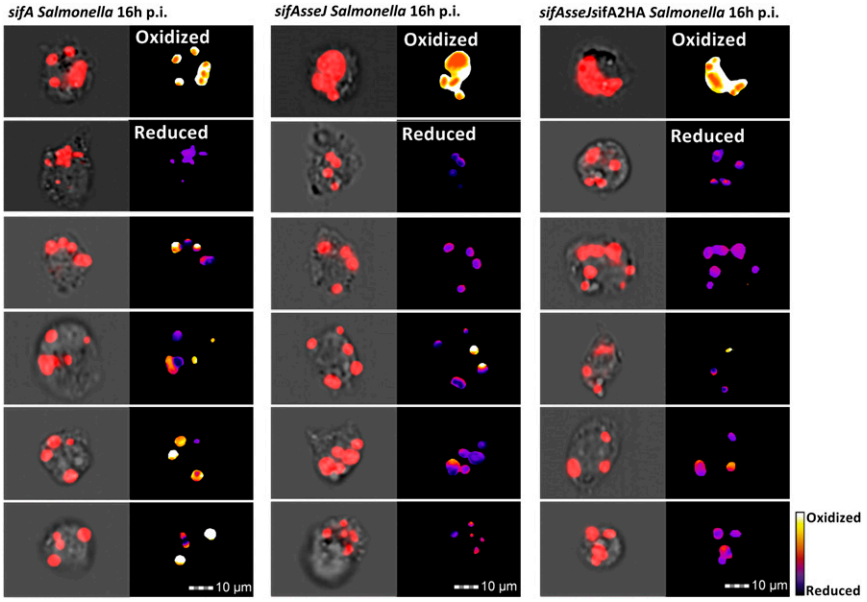


Fig. S5. Representative images of infected THP-1 cells with WT, *sifA*, *sifAsseJ*, and *sifAsseJ*2HAsifA *Salmonella*. Similar to our analysis in Fig. S3, we selected representative images of infected THP-1 cells that contain multiple intracellular bacteria from the AMNIS ImageStream. Images were taken at 16 h p.i. The corresponding pseudocolored ratio images were obtained after analysis with ImageJ. More pronounced heterogeneity between intracellular *sifA* bacteria is observed at 16 h p.i.

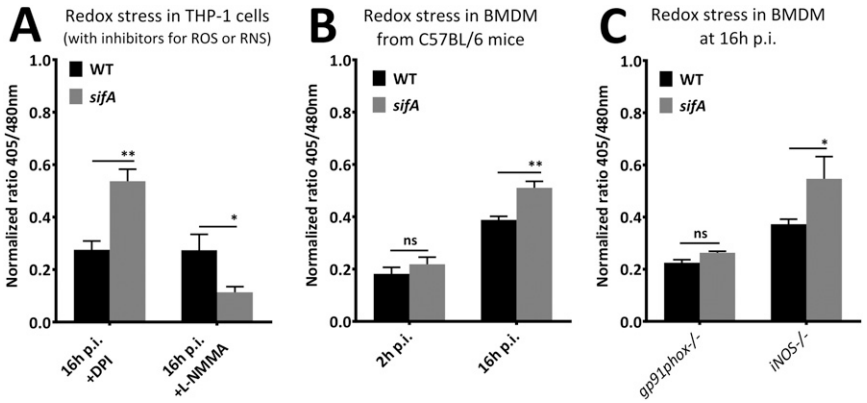
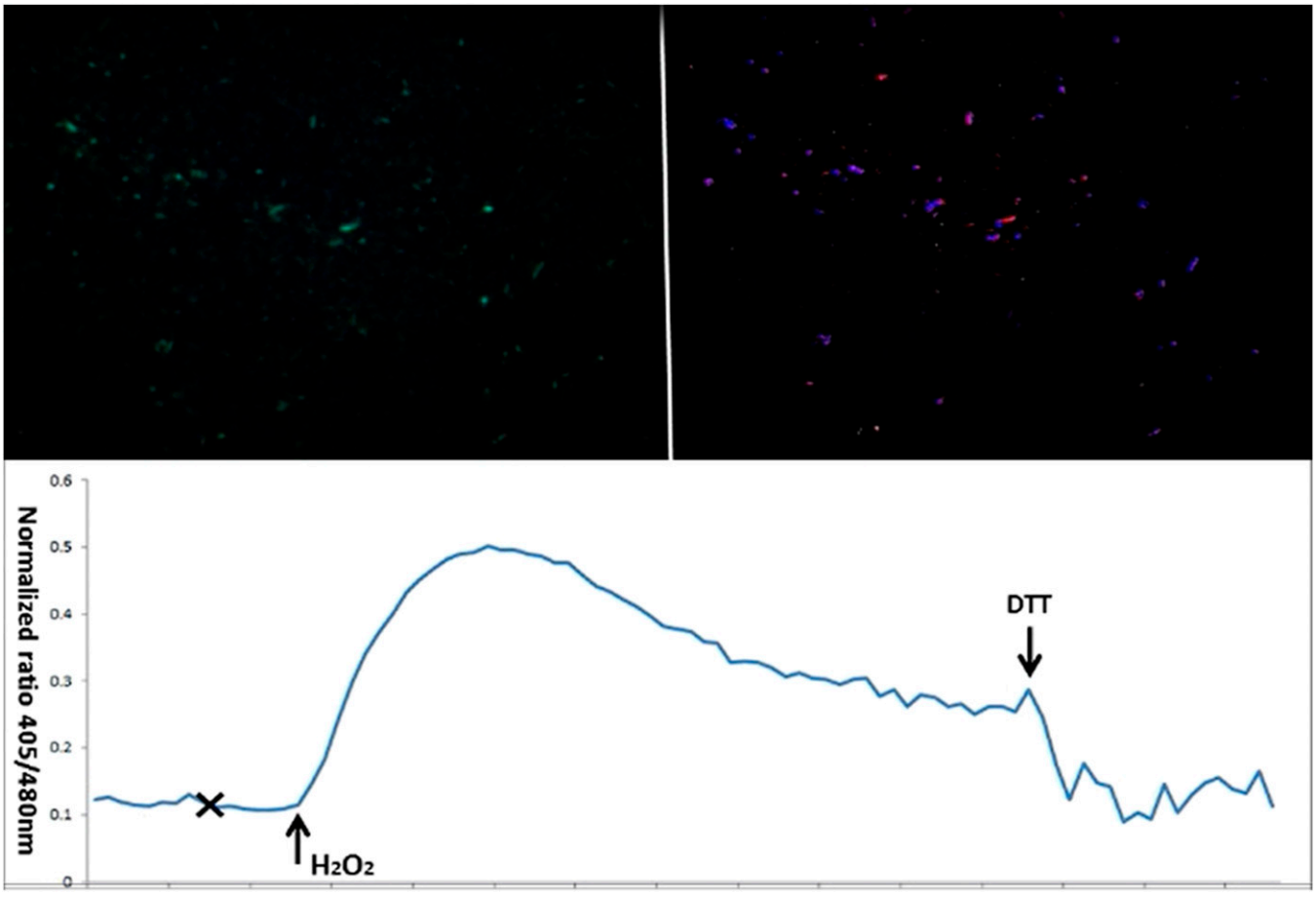


Fig. S6. Redox stress in cytosolic *S*. Typhimurium in THP-1 cells and mouse BMDMs. (*A*) Intrabacterial redox stress in WT and *sifA S*. Typhimurium in THP-1 cells with the addition of inhibitors for oxidative stress (DPI) or nitrosative stress (L-NMMA) at 16 h p.i. Error bars indicate the SD. (*B*) Intrabacterial redox stress in WT and *sifA S*. Typhimurium in BMDMs. (*C*) Intrabacterial redox stress in WT and *sifA S*. Typhimurium in *gp91phox*$^{-/-}$ and *iNOS*$^{-/-}$ BMDMs. Each value represents analysis of at least 15,000 pictures. Error bars indicate the SD over three separate experiments.



Movie S1. Real-time in vitro analysis of intrabacterial redox potential. The upper left panel shows the fluorescent intensities (405 nm in blue and 488 nm in green) after a challenge with 2.5 mM H_2O_2. The upper right panel shows the pseudocolored ratio movie of the same challenge, and the lower panel shows the corresponding graph with the normalized 405/480 ratio values.

Movie S1