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Supplementary Materials for

Prophage terminase with tRNase activity sensitizes *Salmonella enterica* to oxidative stress

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Materials and methods

Bacterial strains and growth conditions. *Salmonella enterica* serovar Typhimurium strain 14028s (ATCC, Manassas, VA) and its mutant derivatives (**Table S5**) were grown in LB broth or E-salts minimal medium [57.4 mM K_2HPO_4, 1.7 mM $MgSO_4$, 9.5 mM citric acid and 16.7 mM H_5NNaPO_4, pH 7.0, supplemented with 0.1% casamino acids, and 0.4% D-glucose (EGCA)], or MOPS minimal medium [40 mM MOPS buffer, 4 mM Tricine, 2 mM K_2HPO_4, 10 µM $FeSO_4 \cdot 7H_2O$, 9.5 mM NH_4Cl, 276 µM K_2SO_4, 500 nM $CaCl_2$, 50 mM NaCl, 525 µM $MgCl_2$, 2.9 nM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 400 nM H_3BO_3, 30 nM $CoCl_2$, 9.6 nM $CuSO_4$, 80.8 nM $MnCl_2$, and 9.74 nM $ZnSO_4$, pH 7.2] supplemented with 0.4% D- glucose at 37ºC in a shaking incubator. Ampicillin (100 mg/ml), kanamycin (50 mg/ml), chloramphenicol (20 mg/mL) and tetracycline (20 mg/mL) (Sigma, USA) were used where appropriate.

Construction of Salmonella* Δ*rtc,* Δ*Gifsy fragment and* Δ*gpA mutants. Deletion mutants were constructed using the λ-Red homologous recombination system. Specifically, the chloramphenicol cassette from the pKD3 plasmid or kanamycin cassette from pKD13 were PCR amplified using primers with a 5' -end overhang homologous to the bases following the ATG start site and the bases preceding the stop codon of target genes (**Table S6**). The PCR products were gel purified, and electroporated into *Salmonella* expressing the λ-Red recombinase from the plasmid pTP233. Transformants were selected on LB plates containing 10 mg/ml chloramphenicol or 50 mg/ml kanamycin. The mutants were confirmed by PCR and sequencing.

Rationale for the H_2O_2 concentrations used in these investigations.

The phagosome containing *Salmonella* is estimated to contain about 16-17 µM H_2O_2 (*32*). *Salmonella* containing vacuoles are usually occupied by a single or a handful of bacteria. The in vivo conditions are not conductive to perform biochemical assays. We have determined that, under the high dense culture conditions used *in vitro*, higher concentrations of peroxide are needed to elicit responses. The

concentrations of 400 μM and 5 mM peroxide were determined empirically in MOPS-GLC media and LB broth, respectively, by testing the bacterial survival and *rtcB* gene expression (**Fig S1D, S3A**). 400 μM peroxide was used for the SunSET assays conducted with *Salmonella* grown in MOPS-GLC media (**Fig 1D**). We found that the growth of *Salmonella* was similar in untreated and 400 μM peroxide-treated groups (**Fig 1C**); however, 400 μM H_2O_2 inhibited *de novo* translation (**Fig 1A, 1B**) whereas inducing *rtc* gene expression (**Fig S1D**). We tried to recapitulate these conditions to analyze tRNA fragmentation by Northern blots. However, our preliminary studies demonstrated that the quality of total RNA extracted from MOPS-GLC was not satisfactory for Northern blot analysis. Therefore, we grew the bacteria in LB broth, which has shown excellent results in previously published tRNA cleavage work (*10*). The addition of 5 mM H_2O_2 to 10^8 *Salmonella* in LB broth did not affect bacterial viability. Moreover, under these conditions, 5 mM H_2O_2 induced excellent *rtcB* and *rtcR* gene transcription. On the other hand, 0.4-1 mM H_2O_2 did not induce *rtc* gene expression (**Fig S3A**). Taking into account the preliminary work done to optimize the isolation of high quality tRNA specimens under conditions that do not exert killing of *Salmonella* while inducing *rtc* gene transcription, we chose to treat *Salmonella* grown to high densities in LB broth with 5 mM H_2O_2 (**Fig S3A**).

Assessment of de novo protein synthesis. Protein translation was determined by Western blot version of SUnSET as described previously with minor modifications (*8*). Briefly, *Salmonella* stains were grown in MOPS-GLC minimal media pH-7.2 at 37 ºC to an OD_{600} of ~ 0.25 in a shaking incubator. The cells were treated with 400 μM H_2O_2 for 10 min, followed by an additional 40 min incubation in the presence of 250 mg/ml puromycin. Cytoplasmic proteins were extracted and quantified by the BCA method. 30 mg of protein samples were separated on 10% SDS-polyacrylamide gels. The specimens were transferred onto nitrocellulose membranes and immunoblotted using anti-puromycin antibody (Millipore). Protein synthesis was quantified from the immunoblots using the entire molecular weight range of puromycin-incorporated in each lane. The relative puromycin-incorporated levels were quantified with the ImageJ software (NIH). Ponceau stained membrane was used to make sure protein loading

was comparable across samples.

RNA isolation, library preparation and RNA seq. RNA seq analysis was performed on dataset GEO#GSE203342. Briefly, *Salmonella* grown in MOPS-GLC medium at 37ºC to an OD_{600} of 0.25 and treated with or without 400 μM H_2O_2 for 30 min. The cells were harvested by centrifugation and 1 ml of TRIzol reagent (Life Technologies) was added to the cell pellet. Following chloroform extraction, RNA was precipitated from the aqueous phase by the addition of 3 M sodium acetate (1/10, vol/vol), 50 mg/ml glycogen (1/50, vol/vol), and an equal volume of 100% isopropyl alcohol. Precipitated RNA was washed twice with 70% (vol/vol) ethanol, suspended in RNase free dH_2O, and treated with RNase free DNase I, according to the supplier's specifications (Promega). Reactions were terminated by the addition of an equal volume of phenol/chloroform/ isoamyl alcohol solution (25:24:1) (PCI). The aqueous phase was treated with an equal volume of chloroform. RNA in the resulting aqueous phase was precipitated by the addition of 3 M sodium acetate (1/10 vol/vol), 50 mg/mL glycogen (1/50 vol/vol and 3 volumes of 100% ethanol. The quality of the isolated RNA was assessed on an Agilent Bioanalyzer. Ribosomal RNA was removed from the total RNA preparation using the MICROBExpress kit (Life Technologies). Starting with 1 mg purified mRNA, samples were fragmented with the NEB Magnesium Fragmentation module at 94ºC for 5 min. RNA was purified by PCI extraction and ethanol precipitation and sodium acetate, and libraries were prepared for Illumina sequencing by following the protocol accompanying the NEBNext Ultra RNA Library Prep Kit through completion of the second strand synthesis step. Libraries were made by NEBNext Ultra RNA Library Prep Kit protocol for a target insert size of 300 bp. Samples were barcoded using NEBNext Multiplex Oligos (Universal primer, Index Primers Set 1 and Index Primers Set 2), and the resulting indexed libraries were sequenced on an Illumina MiSeq using 300-nt reads. The i7 Illumina adapters were trimmed from raw paired reads by utilizing Cutadapt version 2.10 in the Linux terminal with the sequences AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC and AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTAT CATT for the forward and reverse reads, respectively. Reads were then mapped with

STAR aligner version 2.3.2 using CP001363.1 and CP001362.1 as the reference genome for *S*. Typhimurium 14028s (*33*). Picard version 2.18.27 was then used to remove duplicates and sort the reads. HTseq version 0.13.5 was then leveraged to generate count files by locus for each sample (*34*). Counts for each sample were then statistically analyzed utilizing DEseq2 1.30.1 and edgeR 3.32.1 in R Studio running R version 4.0.4 by using Fisher's exact test on the tagwise dispersion of counts for loci that had at least 80 reads total across all samples be analyzed (*35, 36*). Differentially expressed genes between H_2O_2-treated and untreated samples are given in **Table S1**. Genes categorized following KEGG annotations were imported with pheatmap package in R for graphical representation. ClueGo analysis was performed to identify differentially regulated pathways (**Table S2 and S3**). Volcano plots were generated with GraphPad Prism v. 9.

RNA isolation and quantitative RT-PCR. *Salmonella* strains grown in MOPS-GLC medium or LB medium in a shaking incubator at 37°C to an OD_{600} of 0.25 were centrifuged at 16,000 × g for 10 min at 4°C. The bacterial pellets were saved at -80 °C until further processing. DNA-free RNA was purified using a High Pure RNA isolation kit (Roche) according to the manufacturer's instructions. First-strand cDNA generation from total RNA was generated using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega). Relative mRNA quantitation was done using the SYBR green quantitative real-time PCR (qRT-PCR) master mix (Roche) using the primers described in **Table S6.** Data evaluation of 3 biological replicates done in triplicate was performed using the threshold cycle method. Gene expression was normalized to internal levels of the housekeeping gene *rpoD*.

Northern blots. *Salmonella* strains grown in LB broth in a shaking incubator at 37 °C to an OD_{600} of 0.25 were treated with 5 mM H_2O_2 for indicated times. Treated cells were centrifuged at 16,000 × g for 5 min and immediately, total RNA was purified following Trizol method. Total RNA (1 mg) was electrophoresed on 12% acrylamide TBE 8M urea gels and transferred to nylon membrane (Hybond N+, GE) by electroblotting. Membranes were UV-crosslinked (254 nm, 120 mJ dose) twice,

blocked in ULTRAhyb-Oligo Buffer (Ambion), and incubated with 5′-^{32}P-labeled oligonucleotide probes (**Table S6**) in ULTRAhyb-Oligo buffer at 42°C for 18 h. Membranes were washed with 2X SSC/0.1% SDS washing buffer two times for 10 min each, exposed on a phosphor-imager storage screen, and imaged on a Typhoon 9400 (GE Healthcare).

Animals and animal experiments. C57BL/6J and congenic *Cybb*$^{-/-}$ mice deficient in the gp91*phox* membrane-bound subunit of the phagocyte NADPH oxidase were bred and housed in a specific pathogen-free facility at University of Colorado, Anschutz Medical Campus. Six to 8-week-old, male and female C57BL/6J and *Cybb*$^{-/-}$ mice were inoculated i.p. with ~150 CFU of a *Salmonella* mixture containing equal numbers of wild-type, and Δ*rtcB::km* or Δ*rtcR::cm Salmonella*. The bacterial burden was quantified in livers and spleens 3 days post-infection by plating onto LB agar containing the appropriate antibiotics. Alternatively, in the intestinal infection model, mice were taken off food and water for 4 h prior to p.o. treatment with 20 mg/mouse of streptomycin (Sigma, USA). After 24 h, mice were infected p.o. with equal number of CFUs (2 x 10^8) of both wild-type and mutant *S. enterica*. Animals were euthanized 4 d after oral challenge and livers, spleens, and mesenteric lymph nodes tissues were collected, as well as the contents from cecum, colon and ileum, for quantification of bacterial burden on LB agar plates containing the appropriate antibiotics. Competitive index was calculated as $(\text{strain 1} \div \text{strain 2})_{\text{output}} \div (\text{strain 1} \div \text{strain 2})_{\text{input}}$. All mice were bred according to protocol #00058 approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Colorado School of Medicine. The macroenvironment is electronically controlled to provide 22.2 ± 1 °C, a 1:10 light/dark cycle, and 30–40% humidity with at least 12 air changes per hour.

***Histopathology*.** Livers and spleens were isolated from C57BL/6J and *Cybb*$^{-/-}$ mice 3 days after i.p. inoculation with ~150 CFU of wild-type or Δ*rtcB::km Salmonella.* The specimens were fixed in formalin, paraffin-embedded, 4 μm microtome-sectioned, and hematoxylin & eosin-stained. The treatments of the animals and grouping of samples were blinded to the individual scoring the number of microabscesses and

necrotic foci in a light microscope. The average number of microabscesses and necrotic foci per 200X field of liver and spleen H&E images were quantified.

Intracellular survival. Bone marrow-derived macrophages (BMDM) were cultured in L- cell-conditioned medium for 7 days followed by growing in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Grand Island, NY) and antibiotics. Confluent BMDM cells grown in 96 well plates were infected at an MOI of 2 with *Salmonella* grown overnight in LB broth at 37 °C in a shaker incubator. Intracellular survival was assessed after cell host lysis by the addition of PBS containing 0.1% Triton X-100. The specimens were serially diluted in PBS, and the number of *Salmonella* capable of forming a colony in LB agar plates was enumerated after overnight incubation at 37°C. Fold replication was calculated from the number of bacteria recovered after 2 and 18 h of infection compared to time zero.

Plaque assay: Bacteria grown up to OD_{600} of 0.25 in LB broth were treated with 5 mM H_2O_2 for 3 h and pelleted. The supernatant was filtered through 0.22 μm filter and spotted onto soft agar spread with the recipient ΔGifsy-1 strain. Plates were air dried and incubated overnight at 37 °C.

H_2O_2 cytotoxicity. *Salmonella* grown in LB broth in a shaking incubator at 37°C to an OD_{600} of 0.25 was treated with 5 mM H_2O_2 for 30 or 120 minutes. Untreated and treated cells were serially diluted and plated on LB agar for CFU counts. Where indicated, *Salmonella* grown overnight in LB broth was washed in PBS and resuspended in PBS at 5 x 10^5 CFU/ml. Cells were treated with 400 μM H_2O_2 for 2 h and plated for CFU counts.

Protein purification. The *gpA* gene (STM14_3191) was PCR amplified along with RBS and cloned into pBAD18 between *EcoR*I and *Hind*III restriction sites. The resulting plasmid was transformed into *E. coli* Top10 and log phase cultures were induced with 1% arabinose for 5 h. The *gpA* gene was also cloned into pET22b vector between restriction sites *Nde*I and *Xho*I and the recombinant vector was cloned into

E. coli BL21 DE3. Log phase cultures were induced with 5 mM IPTG for 2.5 h at 30°C. Bacteria were harvested by centrifugation at 10,000 g for 10 minutes, resuspended in 1／100th volume degassed lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and transferred to an anaerobic chamber prior to loading onto Ni^{2+}-NTA slurry column for purification under native conditions. Ni^{2+}-NTA bound protein was washed with 10 column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) and eluted (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) in 1 ml aliquots. Protein purification was assessed by SDS-PAGE analysis and elutes with purified protein were pooled, sealed in the anaerobic chamber, and stored at -80°C until further FPLC purification. The freshly purified protein was anaerobically loaded on a HiPrep 26/60 Sephacryl S-300 High Resolution column (Cytiva, USA) equilibrated in 50 mM Tris-HCl, 150 mM NaCl, pH 8.0. Separation was performed in the same buffer at a flow rate of 0.5 mL/min. Protein elutes corresponding to two main peaks observed in the chromatograph were concentrated, assessed for purity in SDS-PAGE and stored at -80°C.

Site-directed mutagenesis: Five mutations (H432A, R477A, H481A, K497G and E587G) were introduced by site-directed mutagenesis into the wild-type *gpA* gene in the pET22b vector. Briefly, the pET22b-*gpA* template was PCR-amplified with primers corresponding to each mutation. After 18 cycles of PCR, the amplicons were treated with *Dpn*I for 1 h and transformed into *E. coli* BL21 DE3. Expression and purification of mutant proteins was done as described as above.

In vivo ectopic expression of terminase. The *gpA* gene (STM14_3191) was PCR amplified along with RBS and cloned into pBAD18 and transformed into *S. enterica* serovar Typhimurium strain 14028s. Bacteria were grown in LB both to an OD_{600} of 0.25 and induced with 0.5% arabinose. Bacteria were simultaneously treated with PBS or 0.5 mM H_2O_2 for 2 h and total RNA was extracted and processed for Northern blotting of tRNA fragments.

Endoribonuclease activity. *Salmonella* total RNA was extracted from 0.25 OD_{600} cultures as described above. Reaction mixtures were made in 10 ml volumes. 20 pM GpA protein in 20 mM Tris-HCl (pH 7.5) were treated with either 400 µM H2O2 or 500 µM DTT and incubated at 37°C for 30 min. Treated proteins were mixed with 2 pM total RNA containing 20 mM Tris-HCl, pH 7.5, 5 mM MnCl2, 5 mM ZnCl2 and 0.5 mM ATP and incubated at 37°C for 1 h. The reactions were quenched by adding equal volumes of 90% formamide + 50 mM EDTA, heated at 85°C for 1 minute and electrophoresed through 12% 8M Urea PAGE gels. The RNA was blotted onto Nylon membranes. Northern blots with radiolabelled tRNA probe was performed as before.

In vitro cosN cleavage assay

A 239-bp fragment containing the Gifsy-1 *cosN* site was amplified from the genome of *S.* Typhimurium strain 14028s and cloned into *Hind*III-digested pET22b, creating the pET22- CosN plasmid. 200 ng of *cosN*-containing or empty plasmids were incubated with recombinant GpA proteins in a reaction buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 5 mM $MnCl_2$, 5 mM $ZnCl_2$ and 0.5 mM ATP at 37°C for 60 minutes. The *cosN* cleavage reactions were terminated by the addition of loading dye containing 25 mM EDTA, and the samples were electrophoresed on 1 % (w/v) agarose gels. Gel images were captured digitally, and *cosN* cleavage was determined by intensities of linearized and nicked bands over supercoiled form of plasmid.

3' RACE. *S. enterica* serovar Typhimurium strain 14028s grown in LB broth in a shaking incubator at 37°C to an OD_{600} of 0.25 was treated with 5 mM H_2O_2 for 2 h. Treated cells were centrifuged at 16,000 × g for 5 min and total RNA was purified following the Trizol method. To determine the site of tRNA fragmentation, total RNA was incubated with GpA protein oxidized with 1 mM H_2O_2 for 2 h. Total RNA was first incubated with 10 mM HCl for 2 h on ice followed by incubation with 5 U of shrimp alkaline phosphatase (NEB) to remove 3′ cyclic phosphates. The RNA was cleaned-up following phenol-chloroform extraction and then ligated to the RNA oligonucleotide 3'-Ada. After ligation, total RNA was treated with DNase I, reverse-transcribed using SuperScript III and oligonucleotide RT, and amplified with oligonucleotides LeuPF and

RT (Supplemental **Table S6**). PCR products cloned using Zero Blunt PCR cloning kit (Invitrogen) were sequenced.

In vitro RtcB ligase assay. Total RNA was treated with H_2O_2-oxidized, recombinant GpA to generate tRNA fragments. The resulting specimens were cleaned up using high pure RNA extraction kit (Sigma, USA). Purified RNA was treated for 1 h with recombinant RtcB (NEB, USA) in the presence of GTP and Mn^{2+} as per supplier's protocol. The reaction was stopped with 25 mM EDTA. $tRNA^{LeuPQTV}$ was detected by Northern blot as described above.

Bioinformatics. Complete protein sequence of *Salmonella* GpA (STM14_3191) was queried in Jackhmmer for five iterations and 100 representative sequences were retrieved. Similar analysis was done with known tRNA ribonucleases like colicins, Prrc and RNases from *Salmonella* and other proteobacterial members. The sequences were aligned with COBALT aligner and the result clustal alignment file was uploaded to MegaX software to construct maximum-likelihood phylogenetic tree. Structure of Gifsy-1 GpA was retrieved from the AlphaFold2 database. The per-residue model confidence score (pLDDT) for Gifsy-1 GpA is 94.65. PDB files were loaded onto ChimeraX program on biopython to view the structures.

Amino acid composition calculation and heatmap construction. Bio.seq module of biopython was utilized to calculate total protein composition and codon adaptation indices for all amino acids from *S. enterica* serovar Typhimurium 14028s. In brief, parse and seq commands of bio.seqIO were employed to retrieve protein sequences from NCBI entry CP001363. The commands were run on biopython in miniconda environment. The results were extracted as a csv table. Heatmaps were constructed using pheatmap package in R. Proteins with <7.5% leucine content were filtered and gene ontologies were determined by ClueGO analysis (**Table S4**).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5.0 software. One-way and two-way ANOVA, *t*-tests and log-rank tests were used. Data

were considered statistically different when $p < 0.05$.

Supplementary Figure 1

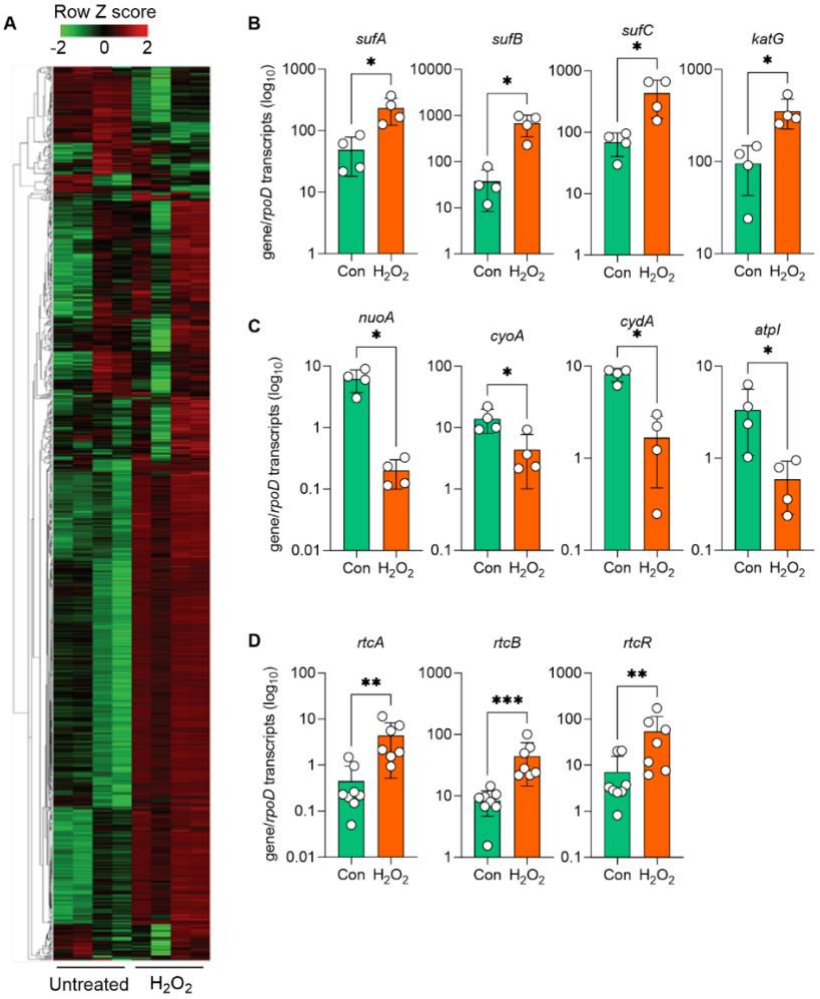

Fig S1: Clustering analysis of the differentially expressed genes (DEGs) in *S. enterica* treated with H_2O_2. (A) Hierarchical clustering graph of the DEGs based on the averaged $\log_{10}$(FPKM+ 1) values of all genes in each cluster. The clustering was made with 4 replicates of control and H_2O_2-treated samples. Heatmap was drawn with pHeatmap package on R. (B, C, D) RT-qPCR of representative gene expression from cDNA prepared from wild-type *S. enterica* grown to an OD_{600} of 0.25 in MOPS-GLC minimal medium and treated with 400 μM H_2O_2 or PBS for 1 h. Total gene transcripts were calculated by comparing Ct values against standard curves. The data, normalized to internal the *rpoD* housekeeping gene, represent the mean ± SD. (n=4). *$p < 0.05$ as determined by unpaired Mann-Whitney test.

Supplementary Fig 2

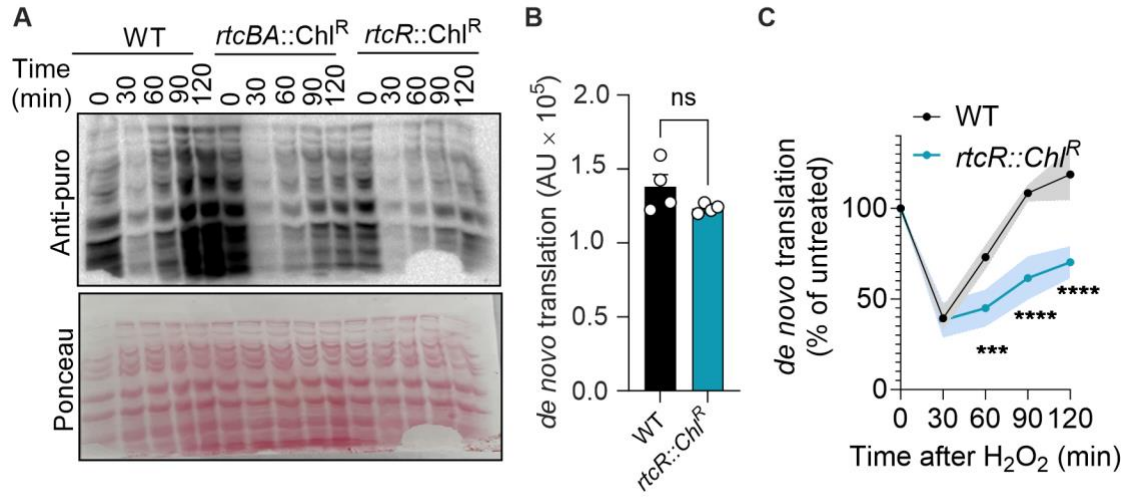

Fig S2. Deletion of RNA repair RtcR stalls *de novo* protein translation in *S. enterica* during oxidative stress. (A) Immunoblot and densitometry (B, C) of the puromycin$^+$ proteome in wild-type (WT), *rtcBA::Chl^R* and *rtcR::Chl^R S. enterica* grown to log phase in MOPS-GLC minimal medium. Where indicated, the specimens were treated with 400 μM H_2O_2. The nascent proteome in B was determined prior to H_2O_2 treatment. The data are mean ± SD (n=4), ****$p \leq 0.0001$ and ***$p \leq 0.001$ were determined by Student's *t*-test (B) or two-way ANOVA with Sidak's multiple comparison test (C).

Supplementary Figure 3

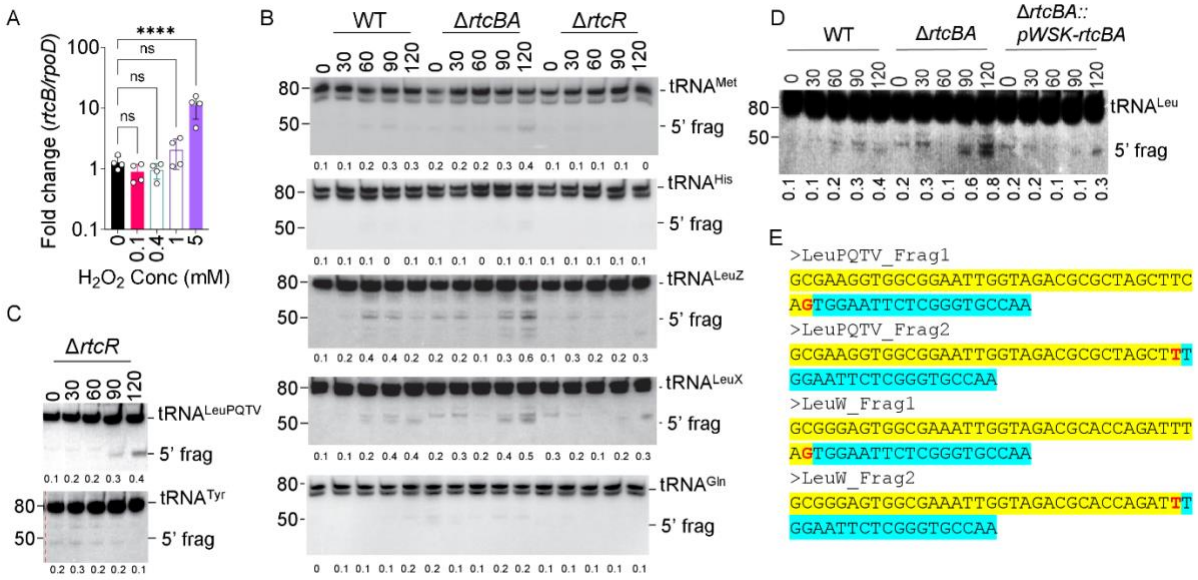

Fig S3. Leucine tRNAs are specifically cleaved in *S. enterica* under oxidative stress. (A) qRT-PCR of *rtcB* transcripts from cDNA prepared from wild-type *S. enterica* grown to an OD_{600} of 0.25 in LB broth and treated with 5 mM H_2O_2 or PBS for 1 h. Fold changes were calculated by the $2^{\Delta\Delta Ct}$ method. The data, normalized to internal the *rpoD* housekeeping gene, represent the mean ± SD. (n=4). ****$p < 0.0001$ was determined by one-way ANOVA with Dunnet's multiple comparison test. (B) Leucine, histidine, glutamine and methionine tRNA fragmentation was visualized by Northern blotting in wild-type (WT), Δ*rtcBA* and Δ*rtcR S. enterica* strains grown to mid-log phase in LB broth and treated for indicated times (in min) with 5 mM H_2O_2. Densitometric ratios of 5' fragment / intact tRNA are shown below each lane (B-D). (C) $tRNA^{LeuPQTV}$ and $tRNA^{Tyr}$ fragmentation was visualized by Northern blotting in Δ*rtcR S. enterica* grown to mid-log phase in LB broth and treated with 5 mM H_2O_2. (D) $tRNA^{LeuPQTV}$ fragmentation was visualized by Northern blotting in WT, Δ*rtcBA and* Δ*rtcBA-pWSK29 rtcBA* complement *S. enterica* grown to mid-log phase in LB broth and treated with 5 mM H_2O_2 for indicated times. (E) Cleavage site in $tRNA^{LeuPQTV}$ isolated from WT *S. enterica* grown as in B was identified by sequencing of 3' RACE products. The adapter sequence is highlighted in blue.

Supplementary Figure 4

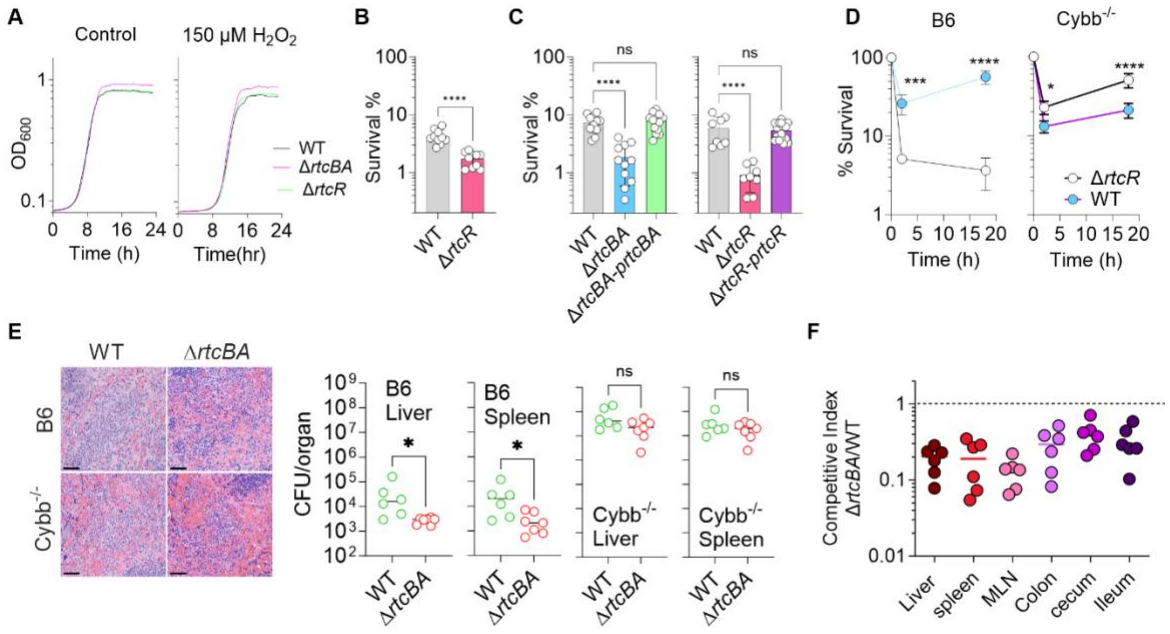

Fig S4. The RNA repair system protects *S. enterica* from oxidative stress. (A) *S. enterica* strains were grown in 96-well plates in EG minimum medium supplemented with glucose in the presence or absence of 150 mM H_2O_2. OD_{600} was read in 30 min intervals in Tecan plate reader at 37°C. (B, C) Killing of indicated *S. enterica* strains grown to mid-log phase in MOPS-GLC minimum medium 2 h after treatment with 400 μM H_2O_2 in PBS. Data are mean ± SD (N = 10). ****$p \leq 0.0001$ was determined by Student's *t*-test (B) or one-way ANOVA with Dunnet's multiple comparison test (C). (D) Intracellular survival of *Salmonella* in bone marrow-derived macrophages from C57BL/6 (B6) and *Cybb*$^{-/-}$ mice. The data are the mean ± SD (n = 6). *$p \leq 0.05$, ***$p \leq 0.001$ and ****$p \leq 0.0001$ were determined by two-way ANOVA with Sidak's multiple comparison test. (E) Histopathology of paraffin-embedded, H&E-stained spleen tissues isolated 3 days post infection of mice with single cultures of WT or Δ*rtcBA S. enterica.* Bars represent 50 mm. Bacterial burden in tissue are plotted. **$p < 0.01$ was assessed by Mann-Whitney Test. (F) Competitive index of *S. enterica* in the indicated tissue collected from streptomycin-treated C57BL/6 (B6) and *Cybb*$^{-/-}$ mice 4 days after oral inoculation with 2 x 10^8 CFU of equal numbers of WT and Δ*rtcBA S. enterica.* Horizontal bars are the median (E, n=6-8; F, n=6).

Supplementary Figure 5

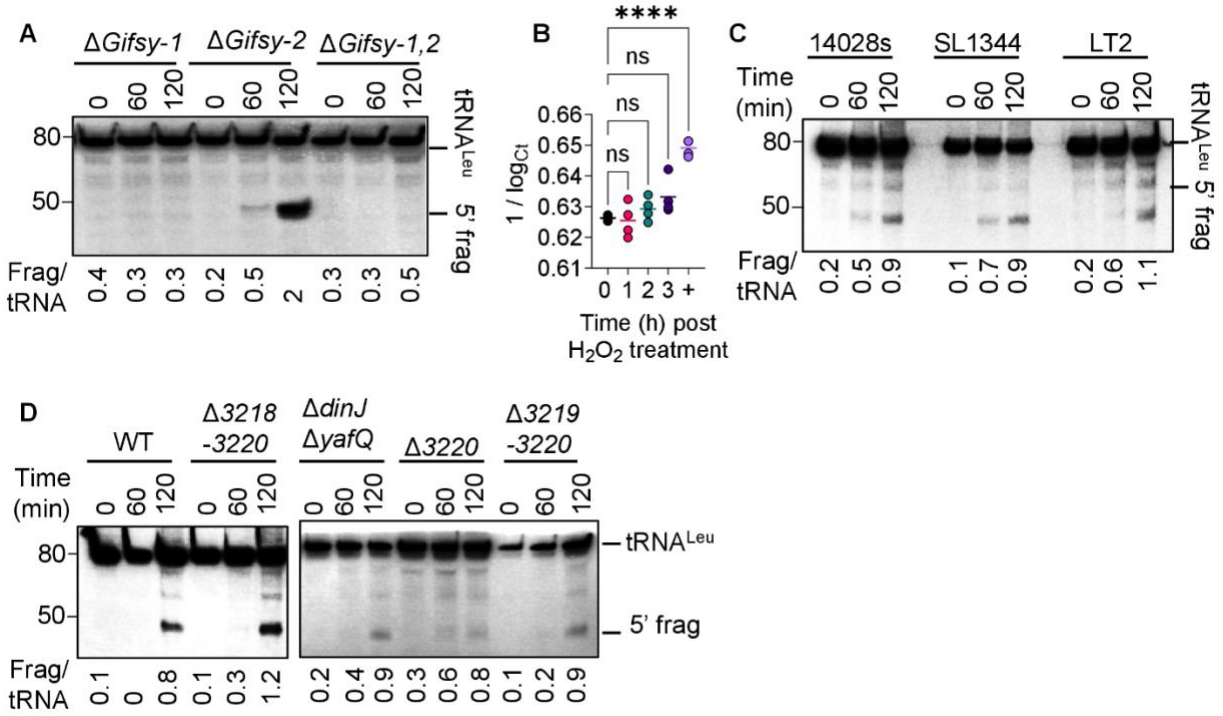

Fig S5. The prophage Gifsy-1 mediates tRNA fragmentation in *S. enterica*. (A, C, D) $tRNA^{LeuPQTV}$ fragmentation in indicated *S. enterica* strains assessed by Northern blotting. Cultures were grown to mid-log phase in LB broth and treated with 5 mM H_2O_2. (B) RT-qPCR probing for prophage circularization in *S. enterica* grown in LB broth until OD_{600} of 0.25 and treated with 5 mM H_2O_2 for indicated times. *S. enterica* grown to OD_{600} of 0.6 and treated with 5 mM H_2O_2 for 3 h served as a positive control (*37*). Inverse of log transformed Ct values were plotted. Data are the mean ± SD. (n=3). ****$p < 0.0001$ was determined by one-way ANOVA with Dunnet's multiple comparison test. Densitometric ratios of 5' fragment / intact tRNA are shown below each lane (A, C, D).

Supplementary Figure 6

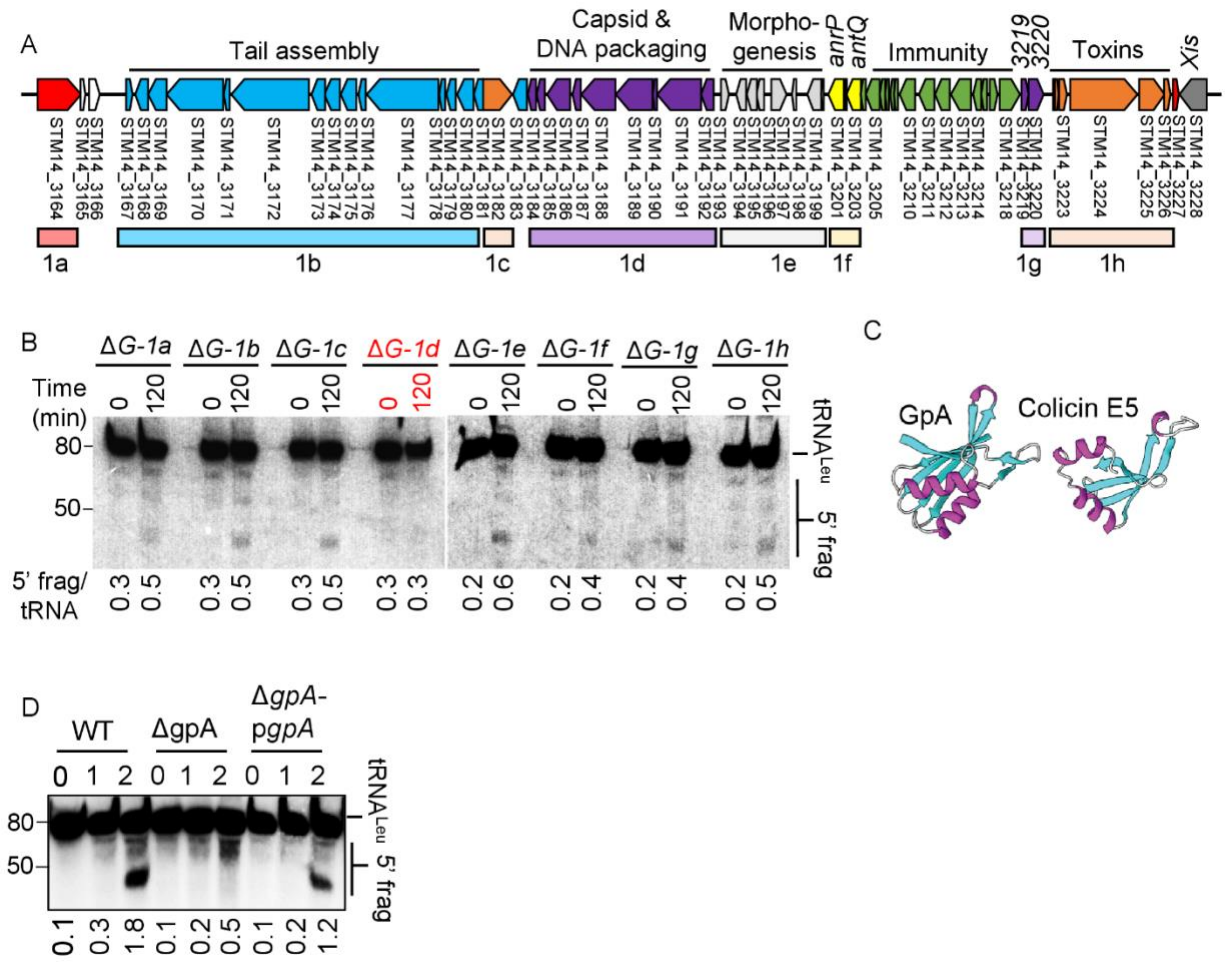

Fig S6. Capsid and DNA packaging region in Gifsy-1 mediates tRNA fragmentation in *S. enterica* during oxidative stress. (A) *S. enterica* 14028s genomic organization of Gifsy-1 region showing deletions spanning the viral genome. (B, D) $tRNA^{LeuPQTV}$ fragmentation in indicated *S. enterica* strains assessed by Northern blotting. Cultures were grown to mid-log phase in LB broth and treated with 5 mM H_2O_2. Densitometric ratios of 5' fragment / intact tRNA are shown below each lane (B, D). (C) Similarity of Gifsy-1 GpA nuclease domain with colicin E5 nuclease region as determined by Alphafold. The α helices and β sheets are represented in magenta and cyan colors, respectively.

Supplementary Figure 7

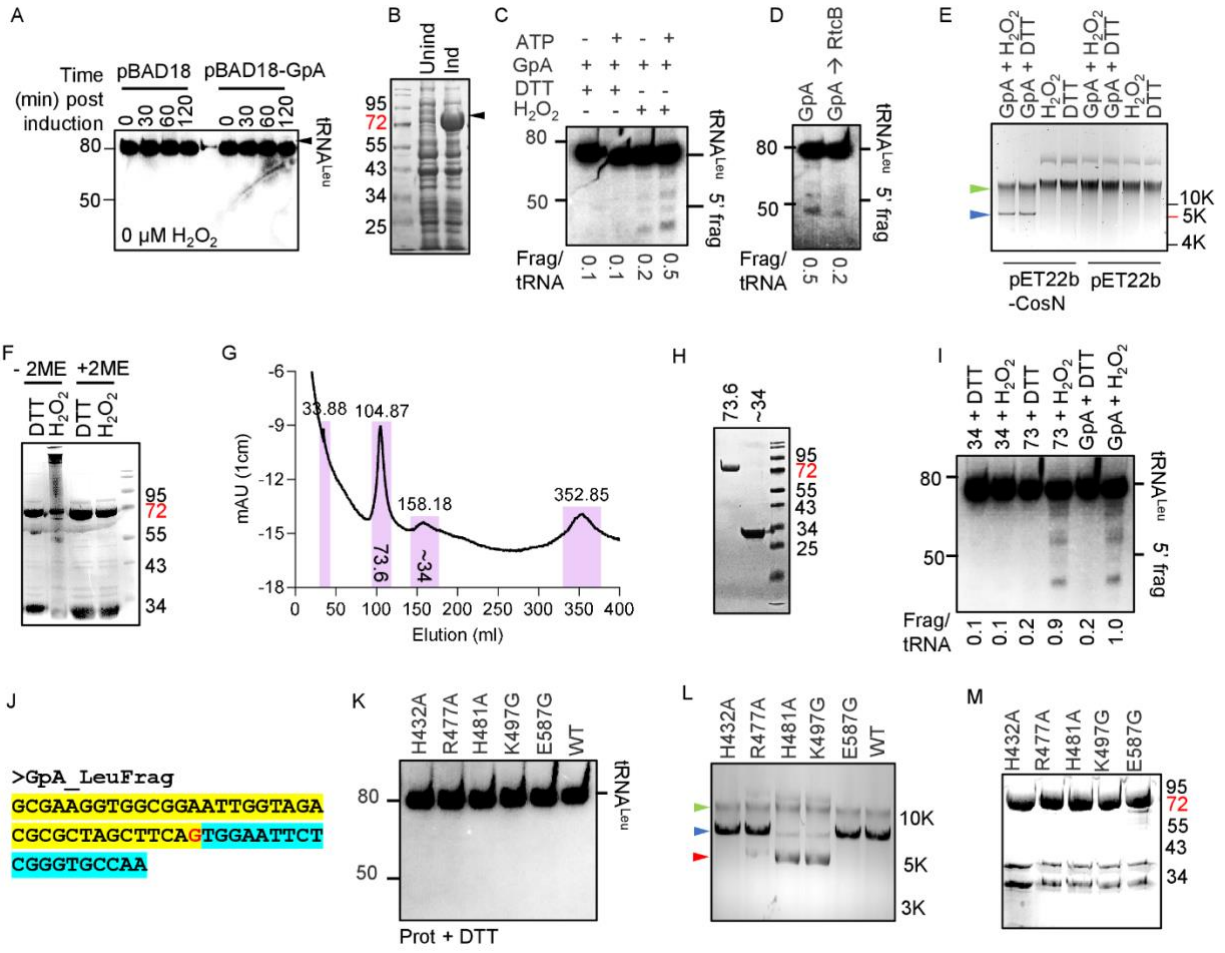

Fig S7: *S. enterica* Gifsy-1 terminase cleaves tRNA^{Leu} during oxidative stress. (A) The *gpA* gene was cloned under arabinose control in the pBAD vector and transformed into *Salmonella* strain 14028s. The GpA protein was induced in log phase cultures grown in LB broth upon the addition of 0.5% arabinose. Total RNA was extracted and assessed for tRNA^{LeuPQTV} fragmentation by Northern blotting. (B) Expression of recombinant GpA protein. A C-terminal 6X-His fusion of the *gpA* gene was cloned into pBAD-18 vector and transformed into *E. coli* Top10. Early log phase cultures were induced with 0.5% arabinose and the overexpressed GpA protein (arrow) was visualized in 10% SDS-PAGE. (C) ATP-dependent tRNA fragmentation by oxidized GpA protein. tRNA^{LeuPQTV} fragmentation was assessed by Northern blotting in total RNA incubated with H_2O_2- or DTT-treated recombinant GpA protein. Where indicated, the reactions contained 0.5 mM ATP. Densitometric ratios of 5' fragment / intact tRNA are shown below each lane (C, D, I). (D) tRNA fragment repair by recombinant RtcB ligase as assessed in Northern blots. Total RNA treated with oxidized GpA for 1 h was cleaned up and treated with RtcB ligase for 1 h at 37°C. Reactions were stopped with 25 mM EDTA and run on 8M Urea PAGE. (E) In vitro *cos* DNA cleavage by recombinant GpA protein. pET22b plasmid carrying the

Gifsy-1 *cos* site was treated with GpA protein and assessed for *cosN* cleavage in agarose gel electrophoresis. Cleavage products including linearized (blue arrow) and nicked (green arrow) fragments and undigested supercoiled (red arrow) products are shown. pET22b plasmid without the *cos* site insert served as a negative control. (F) Purified GpA proteins were buffer exchanged with 20 mM Tris, pH 7.8, and treated with either 1 mM DTT or 500 mM H_2O_2 for 30 min. Where indicated, the specimens were treated with 2-mercaptoethanol before separation on SDS-PAGE gels. Mass spectrometry of the 73.6 kDa in the DTT-treated and the high molecular weight band stuck at the top of the resolving gel observed in H_2O_2-treated proteins mapped to the GpA encoded in STM14-3191 locus of the *S*. Typhimurium strain 14028s (see **Table S5**). (G) Size exclusion chromatographic curve showing protein peak fractions detected at OD_{280} obtained after anaerobic AKTA purification of specimens containing affinity-purified recombinant GpA protein. (H) Fractions containing 34 and 73.6 kDa proteins were analyzed on SDS-PAGE gels. (I) $tRNA^{LeuPTV}$ fragmentation of total RNA from *S. enterica* in Northern blots after treatment with fractions obtained by size exclusion chromatography in H. Where indicated, the proteins were treated with DTT or H_2O_2 before being added to the reactions. (J)The site of $tRNA^{LeuPQTV}$ cleavage by recombinant GpA protein treated with H_2O_2 after purification by size exclusion chromatography was identified by sequencing of 3' RACE products as described in Fig S3E. The adapter sequence is highlighted in blue. (K) $tRNA^{LeuPQTV}$ fragmentation after total RNA was treated with reduced recombinant WT and mutant GpA proteins for 1 h. (L) In vitro *cos* cleavage by recombinant wild-type and mutant GpA proteins. pET22b plasmid carrying the Gifsy-1 *cos* site was treated with GpA protein and assessed for *cosN* cleavage by agarose gel electrophoresis. Linearized (blue arrow) and nicked (green arrow) fragments as well as undigested supercoiled (red arrow) products are shown. (M) Purified GpA mutant proteins are visualized in SDS-PAGE.

Supplementary Fig 8

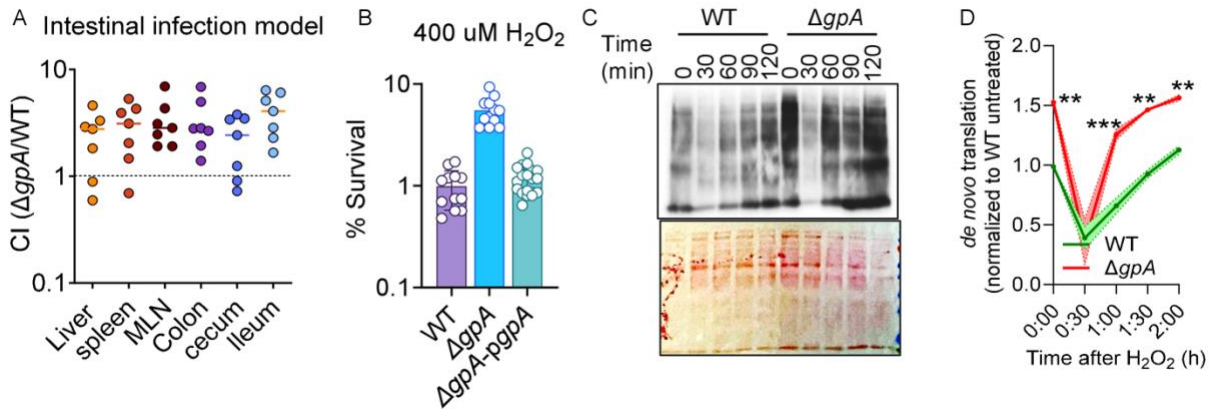

Fig S8: Gifsy-1 terminase sensitizes *S. enterica* to the oxidative stress engendered by phagocyte NADPH oxidase. (A) Competitive index of *S. enterica* in tissues of streptomycin-treated C57BL/6 mice 4 days after oral inoculation with 2 x 10^8 CFU of equal numbers of wild-type (WT) and Δ*gpA* strains. (n=7). ***p* < 0.01 was assessed by Mann-Whitney test. (B) Killing of WT and Δ*gpA S. enterica* grown overnight in LB broth and treated for 2 h with 400 μM H_2O_2 in PBS. Where indicated, the Δ*gpA* mutant was complemented with the pWSK29 *gpA* plasmid. Data are mean ± SD (N = 16-26). *****p* ≤ 0.0001 was determined by one way ANOVA with Dunnet's multiple comparison test. Immunoblot and densitometry (C, D) of the puromycin$^+$ proteome in the indicated *S. enterica* strains grown to log phase in MOPS-GLC minimal medium. The samples were treated with 400 μM H_2O_2. Each lane in the immunoblot is normalized against its Ponceau-S-stained lanes and plotted relative to WT untreated sample. The data are mean ± SD (n=2), ***P* ≤ 0.01, ****P* ≤ 0.001 as determined by two-way ANOVA with Sidak's multiple comparison test.

Supplementary Fig 9

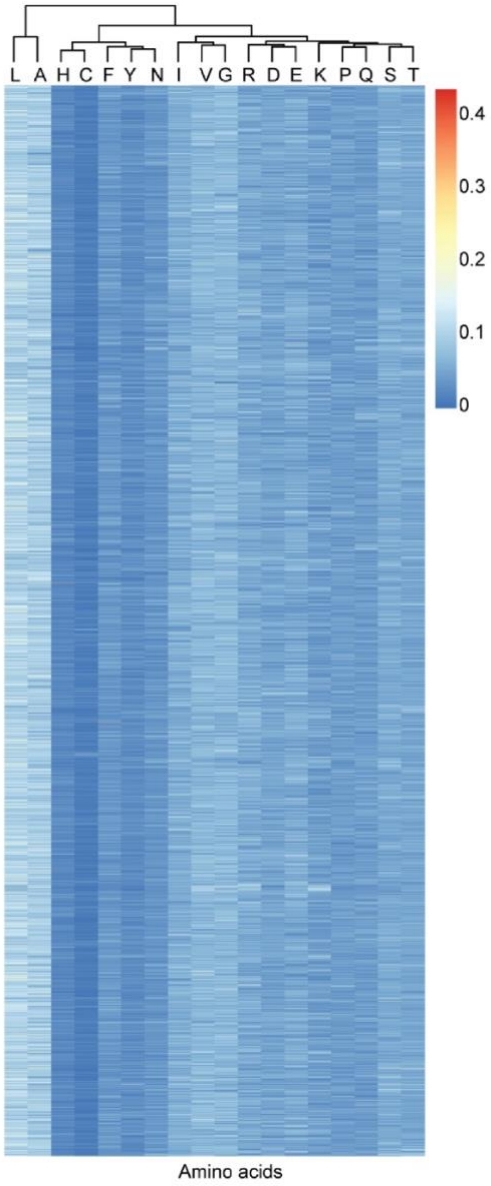

Fig S9: Relative amino acid and leucine codon distribution in *S. enterica* 14028s genome. Heatmap of amino acid distribution calculated from CAIcal server. The x-axis depicts the amino acids identified by conventional lettering. The y-axis represents the genes in the order in the *S*. Typhimurium strain 14028s genome. The scale represents relative frequency of amino acids in a scale of 0-1, 1 being 100%.

Table S6: Bacterial strains used in the study

Strain	Relevant characteristics	Reference
Salmonella		
14028s	Wild type of *S. enterica* serovar *Typhimurium*	ATCC
SL1344	Wild type of *S. enterica* serovar *Typhimurium*	Lab stock
LT2	Wild type of S. enterica serovar Typhimurium	Lab stock
AV22192	*rtcBA*::Chl^R	This study
AV22190	*rtcR*::Chl^R	This study
AV23120	Δ*rtcBA*	This study
AV23121	Δ*rtcR*	This study
AV16021	Δ*recA*	
AV23108	Gifsy-1::Kan^R	This study
AV23109	Gifsy-1A::Kan^R	This study
AV23110	Gifsy1B::Kan^R	This study
AV23111	Gifsy1C::Kan^R	This study
AV23112	Gifsy1D::Kan^R	This study
AV23113	Gifsy1E:Kan^R	This study
AV23114	Gifsy1F::Kan^R	This study
AV23115	Gifsy1H::Kan^R	This study
AV23116	STM14_3219-20::Kan^R (same as Gifsy1G::Kan^R)	This study
AV23117	STM14_3220::Kan^R	This study
AV23118	*dinJ-yafQ*::Kan^R (*ΔdinJ ΔyafQ*)	This study
MZ2915	ΔSTM14_3218-3220	MMCC
MZ1644	Gifsy-1(-)/ΔGifsy-1	(*38*)
MZ1647	Gifsy-1,2(-)/ΔGifsy-1,2	(*38*)
MZ1648	Gifsy-1,2,3(-)	(*39*)
MZ1645	Gifsy-2(-)/ΔGifsy-2	(*39*)
MZ1646	Gifsy-3(-)/ΔGifsy-	(*39*)
AV24013	*rtcBA*::Chl^R carrying pWSK29-*rtcBA*	This study
AV24014	*rtcR*::Chl^R carrying pWSK29-*rtcR*	This study
AV24015	*gpA*::Chl^R	This study
AV24016	*gpA*::Chl^R carrying pWSK29-*gpA*	This study
E.coli		
Top10	Cloning host	Invitrogen
AV23119	*E. coli* Top10 expressing Gifsy-1 GpA under arabinose promoter	This study
AV24007	*E. coli* BL21 DE3 expressing Gifsy-1 GpA	This study
AV24008	*E. coli* BL21 DE3 expressing Gifsy-1 GpA-H432A	This study
AV24009	*E. coli* BL21 DE3 expressing Gifsy-1 GpA-R477A	This study
AV24010	*E. coli* BL21 DE3 expressing Gifsy-1 GpA-H481A	This study
AV24011	*E. coli* BL21 DE3 expressing Gifsy-1 GpA-K497G	This study
AV24012	*E. coli* BL21 DE3 expressing Gifsy-1 GpA-E587G	This study
AV24026	*E. coli* Top10 carrying Gifsy-1-cosN in pET22b	This study

Table S7: Primers and probes used in the study

Name	Sequence	Purpose
	Probes for Northern blots	
5MetZW	GGTTATGAGCCCGACGAGCTACCAGGCTG CTCCACCCCGCG	tRNA-Met probe
5LeuPQTV	CCTGAAGCTAGCGCGTCTACCAATTCCGC CACCTTCGC	tRNA-LeuPQTV probe
5LeuZ	TTTAAATCCCTTGTGTCTACCGATTCCACC ATCCGGGC	tRNA-LeuZ probe
5LeuX	TTTGAATCAACTGCGTCTACCGATTTCGCC ACTTCGGC	tRNA-LeuX probe
5Tyr	GATTTACAGTCTGCTCCCTTTGGCCGCTCG GGAACCCCACC	tRNA-Tyr probe
5His	GAATCACAATCCAGGGCTCTACCAACTGA GCTATAGCCAC	tRNA-His probe
5Gln	CCGGAATCAGAATCCGGTGCCTTACCGCT TGGCGATAC	tRNA-Gln probe
	qPCR primers	
RTrtcAF	TACCCGGACAAGATAGCCAC	*rtcA*
RTrtcAR	CTCCGCTCACTTGCTTACCA	
RTrtcBF	GCATCTTTACGGCATTCGAC	*rtcB*
RTrtcBR	GTGATGAGCCGAACGAAAGC	
RTrtcRF	AACTGGAAAACGTCGTTGCG	*rtcR*
RTrtcRR	CGTCATTCACCGTGGCTTTC	
RT-rpoDF	ATGACGCCGCCGATGACGAC	*rpoD*
RT-rpoDR	AACACTTCAGACAGCTTCAG	
RTSufA F	GGATTTGGCTATGTTCTGGA	*sufA*
RTSufA R	CCTGTAACGGTGCGAAAAGC	
RTSufB F	GGTTCGGCGATTACCTGGAA	*sufB*
RTSufB R	CATTTTGGTGCCGGTGTCAG	
RTSufC F	GTGACCCACTATCAGCGCAT	*sufC*
RTSufC R	TCGCCGGAACGGACAATTCG	
RTKatG F	GTGTCAGCATCAGCGTACCT	*katG*
RTKatG R	CAACGTTAACTGCTGCGCTT	
RT3219F	ATGGTGTTATGGCTCGTTCA	STM14_3219
RT3219R	AGCATCCTAGATACCCACAG	
RT3220F	TGCAGTTGCTTGTAAACGAC	STM14_3220
RT3220R	ATACTTCAGATCTGCCATGG	
RT3218F	CTGATAACCTCGACGTCATC	STM14_3218
RT3218R	GGAGGTTGTTATCATCGATG	
RTnuoAF	CTGTGCTGCCTGATGCTGGT	*nuoA*
RTnuoAR	CAGCCGCTTTCGCGGATAGA	
RTcyoAF	CGCTGCTGGATCCCAAAGGA	*cyoA*

RTcyoAR	GTCCAGACCACGGCTTCCAC	
RTcydAF	GTTTGTGCCGCTAACGCTCG	*cydA*
RTcydAR	CGCACCGAAGATGTCCCCAA	
RTatpIF	TCTGGGGCATCTCCGCAGTA	*atpI*
RTatpIR	ATCAGCGGCAAAAACACCGC	
RtattLF	CTTTTTCAATCTATATGTCAA	*Gifsy-1 attl*^{R}
RTattLR	GATAACTATCTGAAATAGCCA	
	Gene KO primers	
rtcBAkm5	AACTTGTCGCCACCCTAATGATTAAAACGAGAAAAAAATGGTGTAGGCTGGAGCTGCTTC	*rtcBA* deletion
rtcBAkm3	TTAGTCGCTTACCCGGACAAGATAGCCACTTTCGGTTGCATTCCGGGGATCCGTCGACC	
rtcRcm5	GGTGTTGACGGAGAACCATATTCAACTGGAGCTGTGTTACATATGAATATCCTCCTTA	
rtcRcm3	CGATAAAATTATCTCGTCTTTTAGAAGGAAATCTAAGATGGTGTAGGCTGGAGCTGCTTC	*rtcR* deletion
3218 KOF	TTATCAAAATGACTCATTCTCAACATAGCAAGGAATAATTTGCGAGAGTAGGGAACTGCC	3218-3220; 3219-3220; 3220 gene deletions
3219 KOF	CCATGGCGGAGTAACAAAAGAATCCGCGATAGGTCTTTTTTGCGAGAGTAGGGAACTGCC	
3220 KOF	TCTGTGGGTATCTAGGATGCTTAAGAAAGGCGATAAATAATGCGAGAGTAGGGAACTGCC	
3220 KOR	GGATAAAAAACGTCTTTGTTGCCCGGTTAAATTAGAGGTGCATATGAATATCCTCCTTAG	
DrelBEF	AATTTGAACTGTATAGAGACACAGTACAGGAGACTAATCGTGCGAGAGTAGGGAACTGCC	*dinJ-yafQ* gene deletion
DrelBER	CCTGAAAGCACCGGTTAACATATGGGCAAAAACGGCGCTTCATATGAATATCCTCCTTAG	
Gifsy1AF	TTGCCTGAATGAAAATAAATGTAATAATGATAGCTTGGTAGCGATTGTGTAGGCTGGAGC	Gifsy-1A fragment deletion
Gifsy1AR	GCTCTATATATAAATATATTAATTGCATATTTTTTTAAAGGATAAGCTGTCAAACATGAG	
Gifsy1BF	TCTTTAAAAACTACTGCATGTAAAAGGGTCTCCTCTTGTTGCGATTGTGTAGGCTGGAGC	Gifsy-1B fragment deletion
Gifsy1BR	GAAATTACTTACCGGATCACCTATACCAACTGAGGAGGATGATAAGCTGTCAAACATGAG	
Gifsy1CF	CGGATTCCAGCTTTTCCAGAACGGCCCGGCGGATTAATGTGCGATTGTGTAGGCTGGAGC	Gifsy-1C fragment deletion

Gifsy1CR	CGCCCTGAAGGACGGGGATTCCCACTTCAACGAGCCGAATGATAAGCTGTCAAACATGAG	
Gifsy1DF	CAAGCCCTTTCATCAGAATGCCTCCGGGAAACCCCGGCCTGCGATTGTGTAGGCTGGAGC	Gifsy-1D fragment deletion (STM14_3219-3220 deletion)
Gifsy1DR	GAAGATCGCAGATCCTTTCCTGTTTCCGGGAGACTTTTCCGATAAGCTGTCAAACATGAG	
Gifsy1EF	TAACAATTAATCATTTTAACGCTGACTGAGGGTCTTACATGCGATTGTGTAGGCTGGAGC	Gifsy-1E fragment deletion
Gifsy1ER	GCATGACCCATGACCAGTGGACTTCACTGGTCAATTTTTTGATAAGCTGTCAAACATGAG	
Gifsy1FF	GATTTACATAAAATATAGCCGTTTTAATCCAGTTTTGCAAGCGATTGTGTAGGCTGGAGC	Gifsy-1F fragment deletion
Gifsy1FR	GCAATTTTGTTCGTATTTATCAGTTCACAGGATTTACTCAGATAAGCTGTCAAACATGAG	
Gifsy1HF	GTGATAAACGTTCCGCTGGCCGGCGATAAGGCAAACGAGGGCGATTGTGTAGGCTGGAGC	Gifsy-1H fragment deletion
Gifsy1HR	CGTTTTCTCCAGTGGCCCCGCAGCGGGCCATCGCTAATATGATAAGCTGTCAAACATGAG	
TerKF	CGTACCGCTTACCACCGTGCAGTGGGCTGATGAGTATTACTTAGAAGAACTCGTCAAGAA	Gifsy-1 *gpA* deletion
TerKR	CCTGCTCTTCGCTTTTCCTTGATGTCGCCAGCGCCTCCAGCTCAGGGCGCAAGGGCTGCT	
Cloning primers		
pBEcoF	GATAGAATTCCCACGATAGCGGATGTGGAG	GpA cloning into pBAD
pBCHinR	CTTAAGCTTTCAATGATGATGATGATGGTGATTGTTGCCATTAAC	
G1AtpF	cgcCATATGATTTCCGGAGAGCGCAGG	GpA cloning into pET22b
G1NucR	ccgCTCGAGGTGATTGTTGCCATTAACTC	
CosNF	GTTATCCAGCAGATCCTTTC	*cosN* cloning
CosNR	TGTAAGACCCTCAGTCAGCG	
R477A_F	GAAATCGTCTATAAAGCCTCAAAAAAACACGGC	GpA-R477A cloning
R477A_R	GCCGTGTTTTTTTGAGGCTTTATAGACGATTTC	
H481A_F	AAACGCTCAAAAAAAGCCGGCATTTTCCGCG	GpA-H481A cloning

H481A_R	CGCGGAAAATGCCGGCTTTTTTTGAGCGTTT	
E587G_F	AAGGACGTCGTAACGGGGCGCTGGATTGTCTTG	GpA-E587G cloning
E587G_R	CAAGACAATCCAGCGCCCCGTTACGACGTCCTT	
K497G_F	GCCTCCGTTTACGGAGGGCCCGTTATTACCATG	GpA-K497G cloning
K497G_R	CATGGTAATAACGGGCCCTCCGTAAACGGAGGC	
H432A_F	CAAATTATCATGGGACGGGCCGATGATGAAGATACCCTG	GpA-H432A cloning
H432A_R	CAGGGTATCTTCATCATCGGCCCGTCCCATGATAATTTG	
3’ RACE oligos		
3RACE-Ada	5Adenylation-UGGAAUUCUCGGGUGCCAAGG-3Ddc	
LeuP-For	GCGAAGGTGGCGGAATTGGTAG	
RT-Pri	CCTTGGCACCCGAGAATTC	
LeuW-For	GCGGGAGTGGCGAAATTGG	

Captions for Tables S1 to S5

Table S1: Differentially expressed gene fold changes and P values in H2O2 treated Salmonella when compared with untreated controls (n=4)

Table S2: Upregulated pathways identified by ClueGO analysis in Cytoscape

Table S3: Downregulated pathways identified by ClueGO analysis in Cytoscape

Table S4: Gene ontology and enrichment analysis of leucine poor proteins (<7.5%) in Salmonella proteome

Table S5: M/z values and peptides derived from protein bands excised from SDS-PAGE of purified and oxidized GpA

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