

SUPPLEMENTARY INFORMATION

Nitric oxide is a host cue for *Salmonella* Typhimurium systemic infection in mice

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–Supplementary Figures:

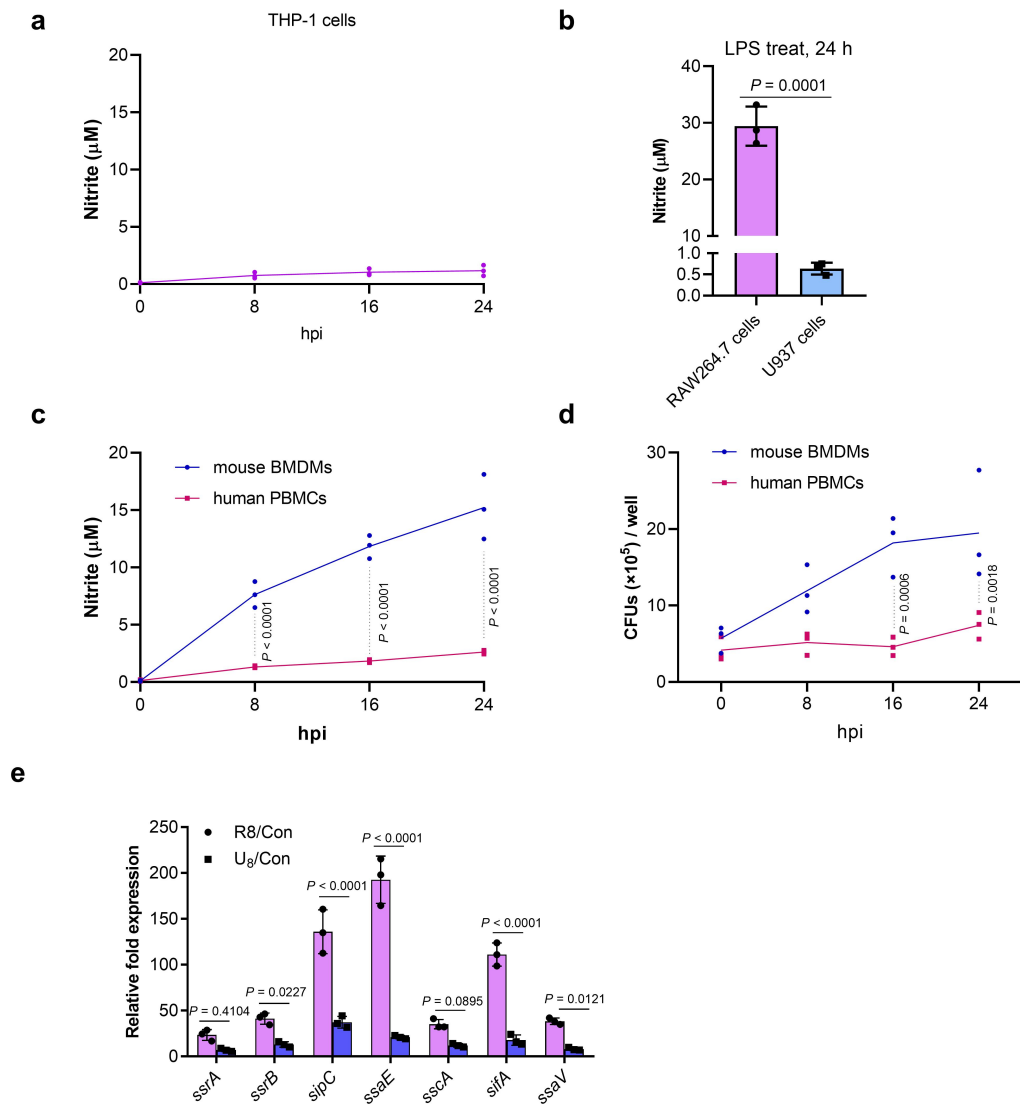
Supplementary Fig. 1; Supplementary Fig. 2; Supplementary Fig. 3; Supplementary Fig. 4; Supplementary Fig. 5; Supplementary Fig. 6

–Supplementary Tables:

Supplementary Table 1; Supplementary Table 2; Supplementary Table 3; Supplementary Table 4; Supplementary Table 5

–Supplementary Figures

Supplementary Fig. 1

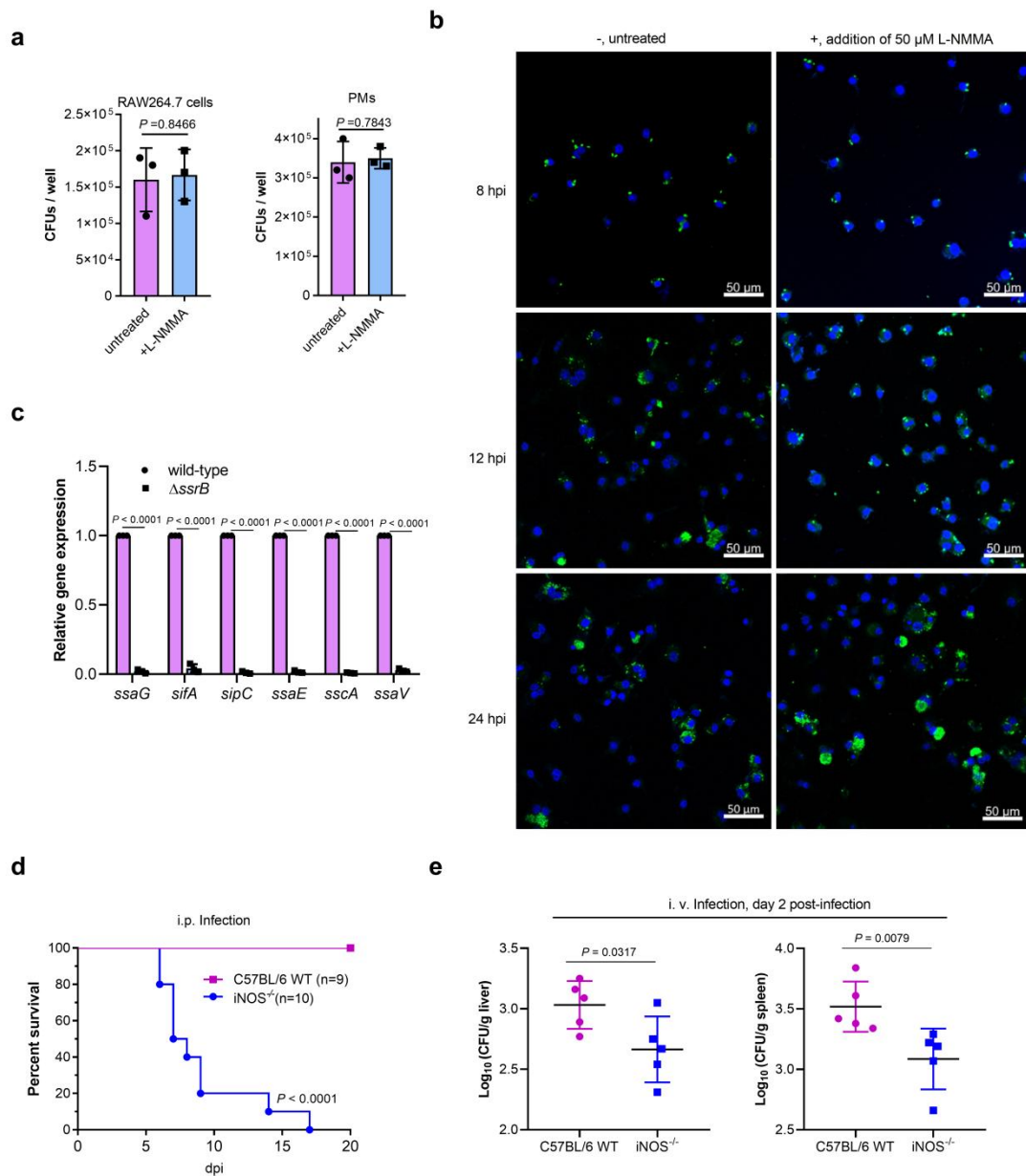


Supplementary Fig. 1 (related to Fig. 1) Host NO levels correlate positively with *S. Typhimurium* replication and SPI-2 expression levels in mouse macrophages.

a, Nitrite production by human THP-1 cell lines left uninfected (0 hpi) or infected with *S. Typhimurium* wild-type strain ATCC 14028s (n = 3 independent experiments). Cells were infected at a MOI of 10, and nitrite levels in the supernatant were measured using Griess assays at the indicated time points. **b**, Nitrite production by

RAW264.7 cells and U937 cells stimulated with LPS (10 ng/mL) for 24 h (n = 3 independent experiments). **c**, Nitrite production by mouse primary bone-marrow-derived macrophages (BMDMs) and human peripheral blood mononuclear cells (PBMCs) left uninfected (0 hpi) or infected with *S. Typhimurium* wild-type (n = 3 independent experiments). **d**, Bacteria burden of *S. Typhimurium* wild-type in BMDMs and PBMCs (n = 3 independent experiments). Bacterial CFU ($\times 10^5$) /well (y axis) and time after addition of gentamicin (x axis) are indicated. **e**, Confirmation of RNA-seq results by qRT-PCR for seven SPI-2 genes (n = 3 independent experiments). RAW264.7 cells or U937 cells were infected with *S. Typhimurium* wild-type for 8 h, cells were then lysed and the intracellular bacteria were collected for RNA extraction and qRT-PCR (R₈: RAW264.7, 8 hpi; U₈: U937, 8 hpi). RNA extracted from bacteria in the RPMI-1640 medium was used as a control (Con). All data are presented as mean $\pm$ SD. *P* values were determined using two-tailed unpaired Student's *t*-test (**b**) or two-way ANOVA (**c–e**). hpi, hours post-infection. Source data are included in Supplementary Data 1.

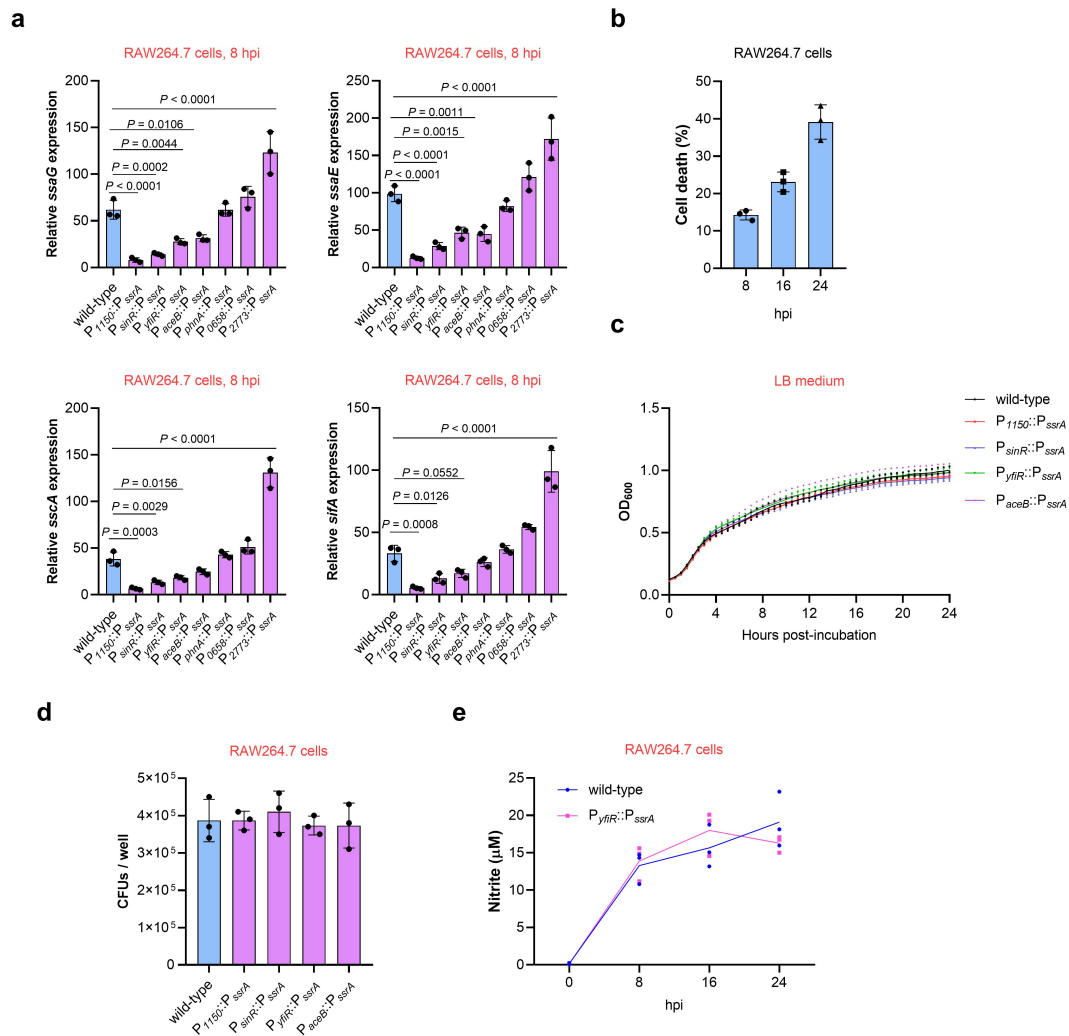
Supplementary Fig. 2



Supplementary Fig. 2 (related to Fig. 2) Lack of host NO reduced *S. Typhimurium* SPI-2 gene expression and bacterial replication in mouse macrophages, and reduced bacterial burden in mouse systemic organs at early infection stages. a, Uptake of *S. Typhimurium* by RAW264.7 cells and PMs derived from C57BL/6 mice in the presence or absence of 50 μ M L-NMMA ($n = 3$ independent experiments). RAW264.7 cells and PMs were infected with wild-type *S. Typhimurium* at an MOI of 10. L-NMMA was added 2 h prior to infection. At 15 min

post-infection, the infected cells were washed three times with PBS, and intracellular bacteria were released for CFU enumeration. **b**, Representative immunofluorescence images of RAW264.7 cells infected with wild-type *S. Typhimurium* at 8, 12, and 24 hpi. RAW264.7 cells were seeded on coverslips and infected with wild-type *S. Typhimurium* at an MOI of 10. Infected cells were fixed at indicated time points and stained with anti-*S. Typhimurium* LPS antibody (green) and DAPI (blue) and then analyzed by confocal microscopy. Scale bars, 50 μ m. **c**, qRT-PCR analysis of *ssaG*, *sifA*, *sipC*, *ssaE*, *sscA*, and *ssaV* mRNA levels in *S. Typhimurium* wild-type and *ssrB* mutant (n = 3 independent experiments). RNA was extracted from bacteria grown in N-minimal medium. **d**, Survival plots of C57BL/6 wild-type (WT) and iNOS^{-/-} mice after intraperitoneal (i.p.) inoculation with ~5000 CFUs *S. Typhimurium* wild-type. n = 9 mice for WT, n = 10 mice for iNOS^{-/-}. **e**, Bacterial counts recovered from the liver and spleen of C57BL/6 WT and iNOS^{-/-} mice intravenously (i.v.) infected with ~500 CFUs *S. Typhimurium* wild-type at day 2 post-infection, n = 5 mice per group. All data are presented as mean $\pm$ SD. *P* values were determined using two-tailed unpaired Student's *t*-test (**a**), two-way ANOVA (**c**), log-rank Mantel-Cox test (**d**), or Mann-Whitney U test (**e**). Source data are included in Supplementary Data 1.

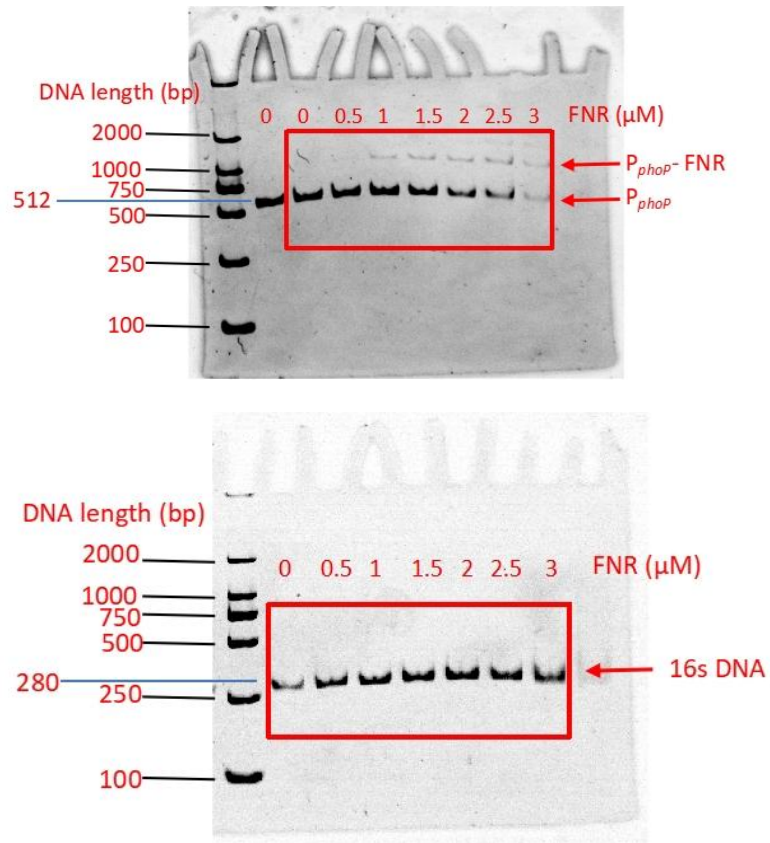
Supplementary Fig. 3



Supplementary Fig. 3 (related to Fig. 3) *S. Typhimurium* SPI-2 expression levels correlate positively with bacterial replication in mouse macrophages and *in vivo* virulence. **a**, qRT-PCR analysis of *S. Typhimurium* *ssaG*, *ssaE*, *sscA* and *sifA* mRNA levels in wild-type and different promoter-replaced strains (n = 3 independent experiments). RNA was extracted from bacteria collected from RAW264.7 cells at 8 hpi. RNA extracted from bacteria in the RPMI-1640 medium was used as a control. **b**, Death of RAW264.7 cells (%) after *S. Typhimurium* infection (n = 3 independent experiments). RAW264.7 cells were infected with wild-type at an MOI of 10, and release of lactate dehydrogenase (LDH) was measured in supernatants at 8, 16, and 24 hpi. **c**, Growth curves of *S. Typhimurium* wild-type and different promoter-replaced

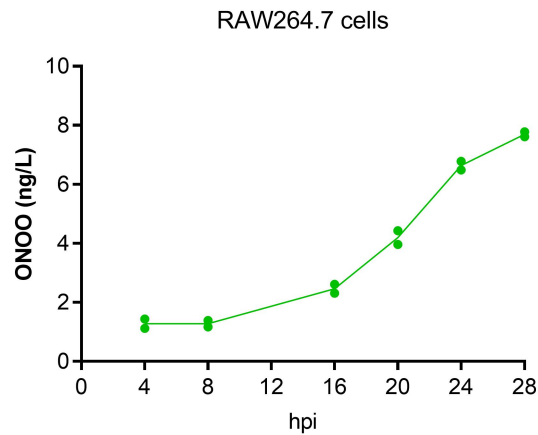
strains in LB medium (n = 3 independent experiments). **d**, Uptake of *S. Typhimurium* wild-type and different promoter-replaced strains by RAW264.7 cells (n = 3 independent experiments). RAW264.7 cells and PMs were infected with different *S. Typhimurium* strains at an MOI of 10. At 15 min post-infection, the infected cells were washed three times with PBS, and intracellular bacteria were released for CFU enumeration. **e**, Nitrite production by RAW264.7 cells left untreated (0 h) or infected with *S. Typhimurium* wild-type or promoter-replaced strain $P_{yfiR}::P_{ssrA}$ (n = 3 independent experiments). Cells were infected at a MOI of 10, and nitrite levels in the supernatant were measured using Griess assays at 0, 8, 16 and 24 hpi. All data are presented as mean $\pm$ SD. *P* values were determined using one-way ANOVA (**a,d**), or two-way ANOVA (**e**). hpi, hours post-infection. Source data are included in Supplementary Data 1.

Supplementary Fig. 4



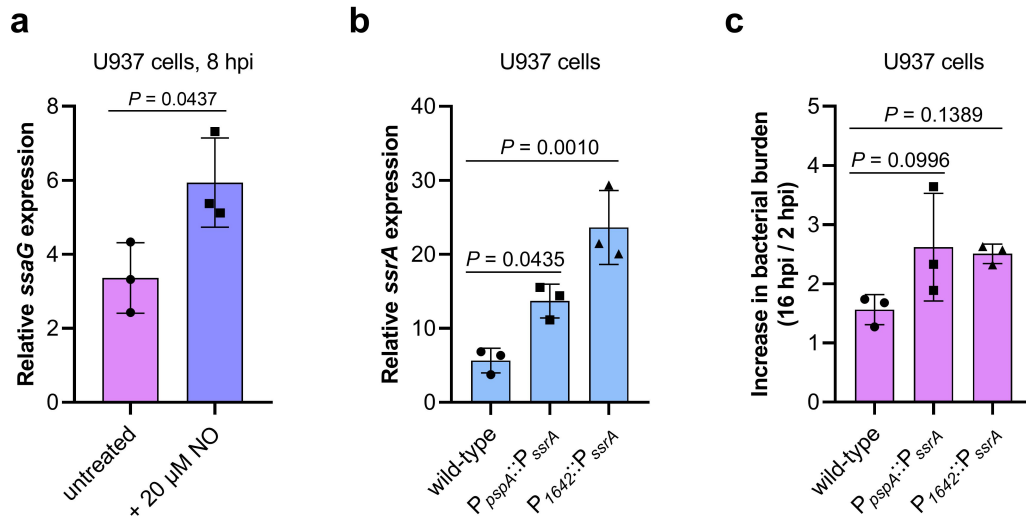
Supplementary Fig. 4 (related to Fig. 5e) Full gels of the EMSA. Boxes highlight lanes used in the Fig. 5e.

Supplementary Fig. 5



Supplementary Fig. 5 ONOO⁻ concentrations in RAW264.7 cells infection with *S. Typhimurium* at 4, 8, 16, 20, 24, and 28 hpi. RAW264.7 cells infected with *S. Typhimurium* wild-type at MOI of 10 were lysed at the indicated time points and the supernatant was collected for the measurement of ONOO⁻ (n = 2 independent experiments). hpi, hours post-infection. Source data are included in Supplementary Data 1.

Supplementary Fig. 6



Supplementary Fig. 6 Increasing SPI-2 expression in human U937 cells leads to limited increase in intracellular replication of *S. Typhimurium*. **a**, qRT-PCR analysis of *ssaG* expression in U937 cells at 8 hpi in the presence or absence of 20 μ M NO (n = 3 independent experiments). **b**, qRT-PCR analysis of *ssrA* mRNA levels in wild-type and two promoter-replaced strains after infection of U937 cells (n = 3 independent experiments). U937 cells were infected with bacteria at an MOI of 10. RNA was harvested from intracellular bacteria at 8 hpi. Fold changes in *ssrA* gene intracellular expression relative to its expression in RPMI medium are presented. **c**, Increase in bacterial burden of wild-type and two promoter-replaced strains in U937 cells at 16 hpi relative to 2 hpi (n = 3 independent experiments). All data are presented as mean $\pm$ SD. *P* values were determined using two-tailed unpaired Student's *t*-test (**a**) or one-way ANOVA (**b,c**). hpi, hours post infection. Source data are included in Supplementary Data 1.

–Supplementary Tables

Supplementary Table 1 Expression of SPI-2 genes in different macrophages

Gene	Fold change (RAW264.7–8 h/ control)	Fold change (U937–8 h/ control)	Fold change (RAW264.7–8 h vs U937–8 h)	qRT-PCR verification (RAW264.7–8 h vs U937–8 h)
<i>ssrB</i>	77.42	13.98	5.54	40.9 vs 13.2
<i>ssrA</i>	64.45	14.46	4.46	26.6 vs 7.4
<i>ssaB</i>	>1240	>195	6.36	
<i>ssaC</i>	360.24	98.78	3.65	
<i>ssaD</i>	191.56	39.79	4.81	
<i>ssaE</i>	>3298	>228	14.46	206.4 vs 20.8
<i>sseA</i>	2782.34	830.96	3.35	
<i>sseB</i>	746.49	107.42	6.95	
<i>sscA</i>	95.33	40.84	2.33	36.3 vs 12.0
<i>sseC</i>	426.72	43.20	9.88	
<i>sseD</i>	87.9394	18.99	4.63	
<i>sseE</i>	227.11	41.48	5.47	
<i>sscB</i>	19.45	8.26	2.36	
<i>sseF</i>	7.82	4.79	1.63	
<i>sseG</i>	2.13	4.18	0.51	
<i>ssaG</i>	671.10	14.62	45.92	
<i>ssaH</i>	80.15	11.90	6.74	
<i>ssaI</i>	723.75	55.71	12.99	
<i>ssaJ</i>	276.06	68.88	4.01	
<i>ssaK</i>	184.49	17.98	10.26	
<i>ssaL</i>	25.49	11.08	2.30	
<i>ssaM</i>	562.24	55.17	10.19	
<i>ssaV</i>	50.12	11.26	4.45	35.3 vs 8.7
<i>ssaN</i>	17.73	6.90	2.57	
<i>ssaO</i>	28.81	2.45	11.77	
<i>ssaP</i>	82.00	3.52	23.30	
<i>ssaQ</i>	20.13	2.66	7.58	
<i>ssaR</i>	98.51	47.53	2.07	
<i>ssaS</i>	26.12	30.04	0.87	
<i>ssaT</i>	264.20	43.31	6.10	
<i>ssaU</i>	94.06	11.62	8.10	
<i>sifA</i>	86.81	21.19	4.10	110.5 vs 20.1
<i>sifB</i>	784.05	54.18	14.47	
<i>sseJ</i>	175.43	27.98	6.27	

Supplementary Table 2 Gene promoters selected for replacement of P_{ssrA}

Gene	Fold change (RAW264.7–8 h/control)
<i>STM14_1150</i>	3
<i>yfiR</i>	6
<i>aceB</i>	12
<i>sinR</i>	14
<i>phnA</i>	18
<i>STM14_0658</i>	81
<i>STM14_2773</i>	455

Supplementary Table 3 Expression levels of the SPI-2 regulators in mouse and human macrophages

	RAW264.7–8 h/ control	U937–8 h/ control
repressor		
<i>hns</i>	3.60	0.43
<i>hha</i>	1.68	0.97
<i>ydgT</i>	44.54	1.51
<i>pmrB</i>	1.19	1.49
<i>pmrA</i>	0.30	0.60
Activator		
<i>fis</i>	4.76	2.70
<i>IHF_a</i>	2.88	1.02
<i>IHF_b</i>	0.43	1.02
<i>slyA</i>	4.68	2.59
<i>phoQ</i>	4.25	6.62
<i>phoP</i>	14.22	6.85
<i>envZ</i>	0.53	0.69
<i>ompR</i>	0.32	0.36
<i>hilD</i>	4.35	3.69

Supplementary Table 4 Expression levels of the NO sensors in mouse and human macrophages

Gene	RAW264.7–8 h/ control	U937–8 h/ control
<i>fnr</i>	14.61	3.71
<i>ybaG</i>	1.21	1.23
<i>soxR</i>	3.71	7.51
<i>oxyR</i>	0.31	0.83
<i>nrdR</i>	1.53	2.83
<i>ygaA</i>	0.10	2.29
<i>fur</i>	1.30	0.69
<i>yjeB</i>	4.80	2.64

Supplementary Table 5 Bacterial strains and plasmids used in this study

Plasmid or strain	Genotype or description	Source
Plasmids		
pKD46	Red recombinase system under an arabinose-inducible promoter; Ap ^R	Lab collection
pKD3	Template plasmid containing the Cm cassette for λ Red recombination; Cm ^R	Lab collection
pET28a	T ₇ expression vector; Km ^R	Lab collection
pWSK129	low-copy-number expression vector; Km ^R	Lab collection
pWSK-3×FLAG	pWSK129 carrying 3×FLAG sequence and Cm cassette; Cm ^R , Km ^R	Lab construction
pFnr	pWSK129 carrying the 14028 <i>fnr</i> gene; Km ^R	This study
pET-Fnr	pET28a carrying the 14028 <i>fnr</i> gene; Km ^R	This study
Strains		
wild-type	Wild-type <i>S. Typhimurium</i> strain ATCC 14028	ATCC
P _{1150::P_{ssrA}}	14028 strain P _{ssrA} replaced by P ₁₁₅₀ ; Cm ^R	This study
P _{sinR::P_{ssrA}}	14028 strain P _{ssrA} replaced by P _{sinR} ; Cm ^R	This study
P _{yfiR::P_{ssrA}}	14028 strain P _{ssrA} replaced by P _{yfiR} ; Cm ^R	This study
P _{aceB::P_{ssrA}}	14028 strain P _{ssrA} replaced by P _{aceB} ; Cm ^R	This study
P _{phnA::P_{ssrA}}	14028 strain P _{ssrA} replaced by P _{phnA} ; Cm ^R	This study
P _{0658::P_{ssrA}}	14028 strain P _{ssrA} replaced by P ₀₆₅₈ ; Cm ^R	This study
P _{2773::P_{ssrA}}	14028 strain P _{ssrA} replaced by P ₂₇₇₃ ; Cm ^R	This study
P _{pspA::P_{ssrA}}	14028 strain P _{ssrA} replaced by P _{pspA} ; Cm ^R	This study
P _{1642::P_{ssrA}}	14028 strain P _{ssrA} replaced by P ₁₆₄₂ ; Cm ^R	This study
Δ <i>fnr</i>	14028 strain <i>fnr</i> gene deleted	This study
Δ <i>fnr</i> +pFnr	Δ <i>fnr</i> containing plasmid pFnr; Km ^R	This study
WT <i>fnr</i> -FLAG	14028 strain <i>fnr</i> gene tagged with 3×FLAG tag; Cm ^R	This study