# Supporting Information

## Choi and Groisman 10.1073/pnas.1303420110

### SI Materials and Methods

**Construction of Chromosomal Mutants.** To create the *ssrB* promoter mutant strain, we introduced a *tetRA* cassette in the *ssrB* promoter region (the *spiR-ssrB* intergenic region) as follows: The *tetRA* fragment was amplified from strain MS7953s by using primers 13094/13095, which are complementary to the *tetRA* and the *ssrB* promoter regions. The PCR product was purified by using a QIAquick PCR purification kit (QIAGEN) and used to electroporate *Salmonella* strain 14028s harboring plasmid pKD46 (1). The resulting strain containing the *tetRA* cassette was kept at 30 °C. Then, this *tetRA* cassette in the *ssrB* promoter region was replaced by annealed oligonucleotides (13104/13105) to create the *ssrB* promoter mutation. These annealed oligonucleotides were used to electroporate the $p_{ssrB}$::*tetRA* strain harboring pKD46, and bacterial suspension was plated on media containing fusaric acid to select against the *tetRA* genes (2), and incubated at 37 °C. The transformants were further purified on fusaric acid-containing plates and screened for their ability to grow in the presence of tetracycline and ampicillin. Those tetracycline- and ampicillin-sensitive colonies were further investigated for the presence of nucleotide substitutions in the *ssrB* promoter region by sequencing a PCR product generated with primers 13088/13089. The nucleotide changes in the *ssrB* promoter mutant are indicated in Fig. 4*A*.

To create a strain containing a $p_{ssaG}$-*lacZ* chromosomal fusion, a *cat* cassette was introduced in the *ssaG* gene as follows: The *cat* fragment was amplified from pKD3 by using primers 12340/12341, then introduced into *Salmonella* 14028s harboring plasmid pKD46. The resulting strain was kept at 30 °C and transformed with pCE36 to create a $p_{ssaG}$-*lacZ* chromosomal fusion.

**Construction of Plasmids.** Plasmid pFPV25AAV expressing an unstable variant of the green fluorescent protein (i.e., GFPaav) (3) was constructed as follows: A promoterless *gfp* (AAV) gene encoding GFPaav was amplified by using pFPV25 (4) and primers 1550 and 3072, and then introduced between the EcoRI and HindIII sites of pFPV25 (4) to replace the original *gfp* gene with *gfp* (AAV). A 300-bp promoter region of *ssaG* was amplified by using genomic DNA from strain 14028s and primers 8577/8578, and then ligated into plasmid pFPV25AAV that had been digested with EcoRI and BamHI to generate pFPV25AAV-$p_{ssaG}$. DNA sequencing verified the presence of the *ssaG* promoter and *gfp* (AAV) sequences in the pFPV25AAV-$p_{ssaG}$ plasmid.

Plasmids pJC107 and pJC108 harbor fusions of the $p_{lac1–6}$ promoter (5) to the regions located between the transcriptional start sites and putative initiation codons of the *spiR* and *ssrB* genes, respectively, and a promoterless *lacZ* gene. The leader regions were amplified by using genomic DNA from strain 14028s and primers 13124/13154 and 13133/13155, respectively. The resulting PCR products were then ligated into plasmid pYS1000 (5) that had been digested with SalI and XhoI to generate pJC107 and pJC108. DNA sequencing verified the presence of the expected leader sequences from the *spiR* and *ssrB* genes and $p_{lac1–6}$ sequences in the pJC107 and pJC108 plasmids.

Plasmid p*pmrA* expressing PmrA was constructed as follows: The *pmrA* gene was amplified using primers 13525/13526 and genomic DNA from strain 14028s, and then introduced between the BamHI and HindIII sites of pUHE21-2*lacI*$^q$ (6). DNA sequencing verified the presence of the *pmrA* gene in the p*pmrA* plasmid.

**β-Galactosidase Assays.** Kinetic β-galactosidase assays were carried out in triplicate, and the activity was determined as described (7); the rate of 2-nitrophenyl β-D-galactopyranoside conversion (i.e., velocity, with unit of $mOD_{415}$ per minute) divided by the $OD_{595}$ of the bacterial culture; those values were measured by using a multidetector (SpectraMax 340PC$^{384}$; Molecular Devices). Activities were normalized to those produced by the wild-type strain. Data correspond to mean values of three independent experiments performed in duplicate.

**Chromatin Immunoprecipitation.** Bacterial cells were grown overnight in *N*-minimal medium containing 1 mM $MgCl_2$, were washed with $Mg^{2+}$-free medium, and diluted with 25 mL of denoted media at 1:100. Cells were grown as described and cross-linked with 1% formaldehyde. Chromatin immunoprecipitation assays were performed, and the data was analyzed as described (8). Quantification of PmrA-bound DNA was carried out by quantitative RT-PCR (qRT-PCR) by using SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7500 Sequence Detection System (Applied Biosystems). The relative amount of cDNA was determined by using a standard curve obtained from PCR with serially diluted genomic DNA, and results were normalized to the levels of *rpoD*. Data are an average from at least three independent experiments. Primers used for qRT-PCR are 12342/13087 for *ssaG*, 12344/12345 for *ssrB*, 3007/3008 for *pmrC*, and 4149/4150 for *rpoD*.

**Electrophoretic Mobility Shift Assay.** DNA fragments corresponding to the *ssrB* promoter region were generated by PCR using primers 13088/13089 and genomic DNA of 14028s and the *ssrB* promoter mutant strains as template. The DNA fragments were gel purified with QIAquick column (Qiagen) and 100 ng of DNA labeled with T4 polynucleotide kinase and [γ-$^{32}$P]-ATP. Unincorporated [γ-$^{32}$P]-ATP was removed by using G-50 microcolumns (GE Healthcare). A total of $2 \times 10^4$ cpm of labeled probe (~10 fmol), 200 ng of poly (dI-dC) (Sigma), and purified His-tagged PmrA were mixed with binding buffer [20 mM Hepes at pH 8.0, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 50 $\mu g \cdot mL^{-1}$ BSA, and 10% (vol/vol) glycerol] in a total volume of 20 μL and incubated for 20 min at room temperature. Samples were then electrophoresed on 4–20% Tris-borate-EDTA gels (Life Technologies), and the gels were dried and autoradiographed.

**DNase I Footprinting Assay.** DNA fragments corresponding to the *ssrB* region were generated by PCR using primer 2459 and $^{32}$P-labeled primer 2072 with genomic DNA from strain 14028s as template DNA. PmrA proteins were incubated with a probe in the same condition used in the electrophoretic mobility shift assay for 20 min at room temperature. DNase I (GIBCO) (0.01 units), 10 mM $CaCl_2$, and 10 mM $MgCl_2$ were added and incubated for 3 min at room temperature. The reaction was stopped by the addition of 100 μL of phenol chloroform, and the aqueous phase was precipitated with ethanol. The precipitate was dissolved in sequence-loading buffer and electrophoresed on a 6% acrylamide/7 M urea gel together with a sequence ladder initiated with the labeled primer by using the T7 Sequenase 2.0 DNA-sequencing kit (Amersham Biosciences), and the gels were dried and autoradiographed.

**Macrophage Infections.** The murine-derived macrophage cell line J774A.1 was cultured in Dulbecco modified Eagle medium (DMEM; Life Technologies) supplemented with 10% FBS (Life Technologies) at 37 °C under 5% $CO_2$. Bone marrow-derived macrophages (BMDM) were isolated from C3H/HeN mice (Charles River Laboratories) and cultured for 6 d (37 °C, 5% $CO_2$) in DMEM supplemented with 10% FBS and 30% L929 supernatant. Confluent monolayers for infection with bacteria

were prepared in 24-well or 6-well tissue culture plates. Each well was seeded with $5 \times 10^5$ (24-well plate) or $2 \times 10^6$ (6-well plate) cells suspended in DMEM/10% FBS and incubated for 1 h at 37 °C under 5% $CO_2$. Bacterial cells were washed with PBS, suspended in prewarmed DMEM, and then added to the cell monolayer with a multiplicity of infection of 10. Following 30 min of incubation, the wells were washed three times with prewarmed PBS to remove extracellular bacteria and then incubated with prewarmed medium supplemented with 100 μg/mL gentamicin for 1 h to kill extracellular bacteria. Next, the wells were washed three times with PBS incubated with prewarmed medium supplemented with 10 μg/mL gentamicin.

**RNA Isolation from Intracellular *Salmonella* and Real-Time PCR To Determine Transcript Levels.** Macrophages were infected with *Salmonella* strains, and samples were harvested at the indicated times by using TRIzol solution. Total RNA was purified by using RNeasy Kit (Qiagen) with on-column DNase treatment, and cDNA was synthesized by using TaqMan (Applied Biosystems) and random hexamers. Quantification of transcripts was carried out by qRT-PCR using SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7500 Sequence Detection System (Applied Biosystems). The relative amount of cDNA was determined by using a standard curve obtained from PCR with serially diluted genomic DNA, and results were normalized to the levels of *rpoD*. Data shown are an average from at least three independent experiments.

**Cytotoxicity Assay.** The percentage of macrophage death was determined by measuring the release of host cytoplasmic lactate dehydrogenase (LDH). At denoted time points, the supernatants were collected, and the release of LDH was quantified by using the Cytotoxicity detection kit (Roche). The absorbance at 490 nm was measured by using multidetector (SpectraMax 340PC$^{384}$; Molecular Devices), and the percentage of host cell death was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. The spontaneous release is the amount of LDH released from the cytoplasm of uninfected macrophages, whereas the maximum release is the amount released by total lysis of uninfected macrophages.

**Mouse Virulence Assays.** Six-week-old female C3H/HeN or BALB/c mice were purchased from Charles River Laboratories. Five mice in each group were infected i.p. with ~$10^3$ *Salmonella* cells that had been grown overnight in LB broth and resuspended in 0.1 mL of PBS. Mouse survival was monitored for 21 d. All procedures were performed according to approved protocols by the Yale School of Medicine Committee on the Use and Care of Animals.

For analysis of bacterial colonization, mice were euthanized by using carbon dioxide at 5 d after infection, and the spleen and liver were removed aseptically. The organs were homogenized in 1 mL (spleen) or 3 mL (liver) of ice-cold PBS and serially diluted. Bacterial loads were determined by plating the diluents on LB agar media.


1. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.
2. Maloy SR, Nunn WD (1981) Selection for loss of tetracycline resistance by Escherichia coli. *J Bacteriol* 145(2):1110–1111.
3. Andersen JB, et al. (1998) New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* 64(6):2240–2246.
4. Valdivia RH, Falkow S (1996) Bacterial genetics by flow cytometry: Rapid isolation of Salmonella typhimurium acid-inducible promoters by differential fluorescence induction. *Mol Microbiol* 22(2):367–378.
5. Cromie MJ, Shi Y, Latifi T, Groisman EA (2006) An RNA sensor for intracellular Mg(2+). *Cell* 125(1):71–84.
6. Soncini FC, García Véscovi E, Solomon F, Groisman EA (1996) Molecular basis of the magnesium deprivation response in Salmonella typhimurium: Identification of PhoP-regulated genes. *J Bacteriol* 178(17):5092–5099.
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8. Shin D, Lee EJ, Huang H, Groisman EA (2006) A positive feedback loop promotes transcription surge that jump-starts Salmonella virulence circuit. *Science* 314(5805):1607–1609.


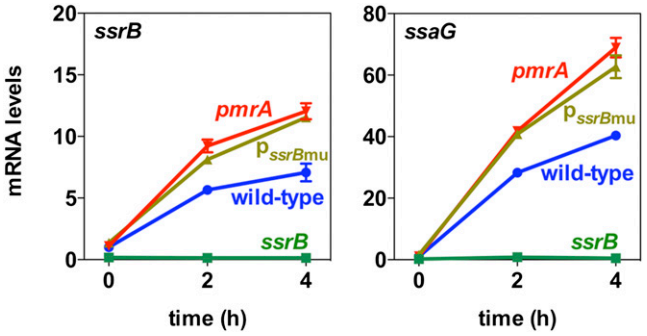


**Fig. S1.** PmrA controls expression of SPI-2 genes in BMDM from C3H/HeN mice. BMDM were infected with wild-type, *pmrA*, *ssrB*, and *ssrB* promoter mutant ($p_{ssrBmu}$) *Salmonella*. mRNA levels of the *ssrB* and *ssaG* genes were determined at the indicated times after infection.

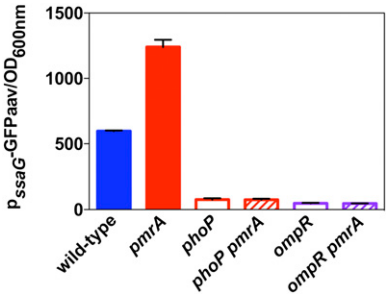


**Fig. S2.** Repression of *Salmonella* pathogenicity island (SPI)-2 genes by PmrA is not observed in organisms lacking the SPI-2 activators PhoP and OmpR. Fluorescence from a plasmid-linked *ssaG-gfp* transcriptional fusion was determined in wild-type, *pmrA, phoP, phoP pmrA, ompR,* and *ompR pmrA Salmonella* grown in *N*-minimal medium at pH 4.6 with 1 mM $Mg^{2+}$ (LH) for 8 h. Values are normalized by that of the wild-type strain.

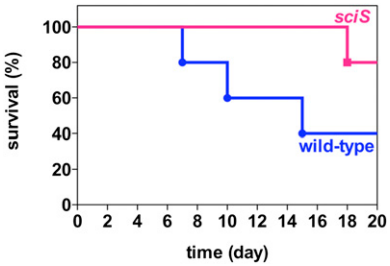


**Fig. S3.** SciS is required for *Salmonella* virulence in C3H/HeN mice. C3H/HeN mice were inoculated i.p. with ~$10^3$ cfu of wild-type and *sciS Salmonella*, and mouse survival was monitored daily.

**Table S1. Bacterial strains and plasmids used in this study**

| Strain or plasmid | Description | Source |
|---|---|---|
| *S. enterica* strains | | |
| 14028s | Wild-type | 1 |
| EG7139 | *pmrA*::*cat* | 2 |
| EG14010 | *ompR*::Tn*10* | This work |
| EG14411 | *ssrB*::$Cm^R$ | 3 |
| EG18502 | *pmrA*-HA | 4 |
| MS7953s | *phoP7953*::Tn*10* | 1 |
| JC001 | *ssrB pmrA*:: *cat* | This work |
| JC008 | $p_{ssrB}$::*tetRA* | This work |
| JC019 | 4 nt substitution in the PmrA box of the *ssrB* promoter | This work |
| JC049 | *phoP7953*::Tn*10 pmrA*::*cat* | This work |
| JC213 | *ompR*::Tn*10 pmrA*::*cat* | This work |
| JC216 | $p_{ssaG}$::*lacZY* | This work |
| JC217 | $p_{ssaG}$::*lacZY pmrA*::*cat* | This work |
| *E. coli* strains | | |
| DH5α | F–*sup*E44 Δ*lac*U169 (*f* 80 *lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 | 5 |
| Plasmids | | |
| pKD3 | $rep_{R6Kg}$ $Ap^R$ FRT $Cm^R$ FRT | 6 |
| pKD46 | $rep_{pSC101}$ts $Ap^R$ $p_{araBAD}$ γ β exo | 6 |
| pCP20 | $rep_{pSC101}$ts $Ap^R$ $Cm^R$ *cI*857 $lP_R$ *flp* | 6 |
| pCE36 | $rep_{R6Kg}$ $Km^R$ FRT *lacZY* $t_{his}$ | 6 |
| pFPV25 | $rep_{pMB1}$ $Ap^R$ promoterless *gfp* | 7 |
| pFPV25AAV | $rep_{pMB1}$ $Ap^R$ promoterless *gfp* (AAV) | This work |
| pFPV25AAV-$p_{ssaG}$ | $rep_{pMB1}$ $Ap^R$ promoterless *gfp* (AAV) $p_{ssaG}$ | This work |
| pYS1000 | $rep_{p15A}$ $Cm^R$ $p_{lac1–6}$ $t_{his}$ | 8 |
| pJC107 | $rep_{p15A}$ $Cm^R$ $p_{lac1–6}$ $t_{his}$ 5′$UTR_{spiR}$ | This work |
| pJC108 | $rep_{p15A}$ $Cm^R$ $p_{lac1–6}$ $t_{his}$ 5′$UTR_{ssrB}$ | This work |
| pUHE21-2 *lacI*$^q$ | $rep_{pMB1}$ $Ap^R$ *lacI*$^q$ | 2 |
| p*pmrA* | $rep_{pMB1}$ $Ap^R$ *lacI*$^q$ *pmrA* | This work |


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2. Soncini FC, García Véscovi E, Solomon F, Groisman EA (1996) Molecular basis of the magnesium deprivation response in Salmonella typhimurium: Identification of PhoP-regulated genes. *J Bacteriol* 178(17):5092–5099.
3. Bijlsma JJ, Groisman EA (2005) The PhoP/PhoQ system controls the intramacrophage type three secretion system of Salmonella enterica. *Mol Microbiol* 57(1):85–96.
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8. Cromie MJ, Shi Y, Latifi T, Groisman EA (2006) An RNA sensor for intracellular Mg(2+). *Cell* 125(1):71–84.

**Table S2. Primers used in this study**

| Primers | Sequence (from 5′ to 3′) |
|---|---|
| 1550 | CAC CTG ACG TCT AAG AAA CC |
| 2072 | CGG AAT TCC CAG GGT AAT AAG GCA TTC |
| 2459 | CCG GAA TTC TCT TGC TGG CTG ATA TTG |
| 3007 | GCG CAA TGT GCT GGT GTT TAT |
| 3008 | CAC ACT ATT GAC CAC GCT AAA CG |
| 3072 | CAT TAA AGC TTG CAT GCC TGC AGG AGA TTT AAA CTG CTG CAG CGT AGT TTT CGT CGT TTG CTG CAG GCC TTT TGT ATA GTT CAT CCA TGC |
| 4149 | ACC GTG GCA CAA ATG ATG CT |
| 4150 | TCG GCA ATC GCC TTA TCT G |
| 8577 | TCC GGG ATC CAA TGC TTT TCC TTA AAA TAA ATA CAT CG |
| 8578 | TCC GGA ATT CAC CGC GAC GGT AAT GAC TCA TTC ATA CTG |
| 12340 | TAC GAT GTA TTT ATT TTA AGG AAA AGC ATT ATG GAT ATT GCA CAA TTT GTA GGC TGG AGC TGC TTC G |
| 12341 | TTC ATT TTG TCA TTA ATG GCC TGG CCT GCC TGG TGC GCC ATG TGG GAA TAT GAA TAT CCT CCT TAG TTC |
| 12342 | GGA TGT TCA TTG CTT TCT AAA TTT TG |
| 12344 | TAC GTA TGA TCT TCA AAA ACT ACA C |
| 12345 | CTT ATA TTC TTT CAT TTT GCT GCC C |
| 13087 | AGA GCA TAT CCA CTA ATT GTG CAA |
| 13088 | TGA GAA TCG CGT AAA AAT GGG GAA A |
| 13089 | GCC AGG GTA ATA AGG CAT TCA TAA T |
| 13094 | CGC CAG AAA ATA CGT ATG ATC TTC AAA AAC TAC ACC ATT ACT TAA TTA AGA CCC ACT TTC ACA TT |
| 13095 | AAG ATC TTA TAT TCT TTC ATT TTG CTG CCC TCG CGA AAA TTA AGA CCC TAA GCA CTT GTC TCC TG |
| 13104 | CGC CAG AAA ATA CGT ATG ATC TTC AAA AAC TAC ACC ATT ACT TAA TAT TAT CGG ACC TTT CGC GAG GGC AGC AAA ATG AAA GAA TAT AAG ATC TT |
| 13105 | AAG ATC TTA TAT TCT TTC ATT TTG CTG CCC TCG CGA AAG GTC CGA TAA TAT TAA GTA ATG GTG TAG TTT TTG AAG ATC ATA CGT ATT TTC TGG CG |
| 13124 | CAT GCG TCG ACC TTT ACA CTT TAA GCT TTT TAT GTT TAT GTT GTG TGG AAC ATC GCC ATC TTA TTA AAA AGT AAT TGT AG |
| 13133 | CAT GCG TCG ACC TTT ACA CTT TAA GCT TTT TAT GTT TAT GTT GTG TGG AAG TAT GCT ATG TCA TAG ACA TTG AGA ATC GC |
| 13154 | TGA GCA ACT CGA GAA TGC TTC CCT CCA GTT GCC TGT TGC A |
| 13155 | TAT ATT CCT CGA GTT TGC TGC CCT CGC GAA AAT TAA GAT A |
| 13525 | GAT ATT CTG CAA GGA TCC AGG AGA CTA AGC GAA TGA A |
| 13526 | CAT CAG GCA AGC TTA GCT TTC CTC AGT GGC AAC |