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Control of a *Salmonella* Virulence Locus by an ATP-Sensing Leader mRNA

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Abstract

The facultative intracellular pathogen *Salmonella enterica* resides within a membrane-bound compartment inside macrophages¹. This compartment must be acidified for *Salmonella* to survive within macrophages², possibly because acid pH promotes expression of *Salmonella* virulence proteins^{3,4}. We reasoned that *Salmonella* may sense its surroundings have turned acidic not only upon protonation of the extracytoplasmic domain of a protein sensor⁵ but also by an increase in cytosolic ATP levels because conditions that enhance the proton gradient across the bacterial inner membrane stimulate ATP synthesis^{6,7}. We now report that an increase in cytosolic ATP promotes transcription of the coding region for the virulence gene *mgtC*, which is the most highly-induced horizontally-acquired gene when *Salmonella* is inside macrophages⁸. This transcript was induced both upon media acidification as well as by physiological conditions that increase ATP levels independently of acidification. ATP is sensed via the coupling/uncoupling of transcription of the unusually long *mgtC* leader mRNA and translation of a short open reading frame located in this region. A mutation in the *mgtC* leader mRNA that eliminated the response to ATP hindered *mgtC* expression inside macrophages and attenuated *Salmonella* virulence in mice. Our results define a singular example of an ATP-sensing leader mRNA. Moreover, they indicate that pathogens can interpret extracellular cues by the impact they have on cellular metabolites.

The *mgtC* gene is required for survival inside macrophages and for growth in low Mg²⁺ media in several phylogenetically unrelated intracellular pathogens including *S. enterica*⁹, *Yersinia pestis*¹⁰, *Brucella suis*¹¹, *Burkholderia cenocepacia*¹² and *Mycobacterium tuberculosis*¹³. In *Salmonella*, *mgtC* heads the *mgtCBR* operon, which specifies the inner membrane protein MgtC, the Mg²⁺ transporter MgtB and the MgtR peptide promoting MgtC degradation¹⁴. Transcription from the *mgtCBR* operon is controlled by the virulence regulatory system PhoP/PhoQ¹⁵. *mgtC* expression must be tightly regulated for a normal course of infection because inactivation of the *mgtC* gene attenuates *Salmonella* virulence in mice⁹ whereas preventing transcription of AmgR, a PhoP-dependent anti-sense RNA that promotes the preferential degradation of the *mgtC* portion of the polycistronic *mgtCBR* message, renders *Salmonella* hypervirulent¹⁶.

Mild acid pH induces expression of the *mgtC*¹⁷ (Supplementary Fig. 1) and *mgtB*¹⁸ genes in a *phoP*-dependent manner in wild-type *Salmonella*. This could be ascribed to acid pH sensing by PhoP's cognate sensor PhoQ⁵. However, *Salmonella* appears to utilize a different mechanism to promote *mgtC* expression in response to mild acid pH because when bacteria

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Author Contribution

E.L. conducted the experiments. E.L. and E.A.G. designed the study and wrote the paper. All authors read the paper and contributed to its final form.

were switched from media at pH 7.7 to media at pH 5.1, the mRNA levels of the *mgtC* (Fig. 1a) and *mgtB* (Fig. 1b) coding regions increased even in a *phoP* phoQ* strain, which lacks the pH sensor PhoQ and harbors a PhoP allele that can function in its absence¹⁹. This stimulation appears to be specific to *mgtC* because the mRNA levels corresponding to the *mgtA* coding region did not increase (Fig. 1c) despite the *mgtA* promoter also being under direct transcriptional control of PhoP¹⁵.

The stimulation of *mgtC* expression promoted by mild acid pH could be mediated by an increase in the proton gradient across the inner membrane, hence creating a change in cytosolic ATP. Indeed, the intracellular pH dropped by ~0.9 units and the ATP concentration rose ~2.5 fold within one hour of switching the *phoP* phoQ* strain from media at pH 7.7 to media at pH 5.1 (Fig. 1d). Dissipation of the proton gradient with a protonophore decreased ATP levels (Fig. 1d) and prevented induction of *mgtC* and *mgtB* at pH 5.1 (Fig. 1a and 1b). (A similar drop in intracellular pH was displayed by wild-type *Salmonella* displayed [i.e., from pH 8.15±0.112 in media with pH 7.7 to pH 7.25±0.079 in media with pH 5.1 and to pH 5.04±0.031 in media with pH 5.1 in the presence of the protonophore].)

That cellular ATP is the signal upregulating *mgtC* transcription when *Salmonella* experiences mild acidification is supported by two additional experiments performed with strains carrying plasmid pGFP303, which harbors the nucleotide sequence corresponding to the natural *mgtCBR* promoter and leader region fused to a promoterless *gfp* gene at the *mgtC* start codon. Fluorescence was six fold higher when a purine auxotroph was grown in defined media with high adenine than with low adenine (Fig. 2a). The higher fluorescence is not due to differences in growth rates (data not shown) or a decrease in cellular pH (Fig. 2b), but reflects the higher ATP levels present in bacteria grown at the higher adenine concentration (Fig. 2b). Moreover, it is specific to ATP because a change in uridine levels in the growth media did not alter the fluorescence of a uridine auxotroph harboring pGFP303 (data not shown). Furthermore, it requires *mgtCBR* regulatory sequences because changes in the adenine concentration in the media did not affect the fluorescence produced by the adenine auxotroph harboring the plasmid vector (Fig. 2a). And when wild-type *Salmonella* harboring pGFP303 was grown in glucose, fluorescence was two times higher than when it was grown in glycerol (Fig. 2c), reflecting the larger ATP amounts generated when glucose is used as carbon source (Fig. 2d).

We analyzed the *mgtCBR* leader mRNA seeking sequence and structural features that may suggest how it might sense ATP. We identified two short ORFs – designated *mgtM* and *mgtP* – preceded by putative ribosome binding sites (RBS) (Fig. 4a). Here, we focus on *mgtM* because, as described below, it mediates the response to ATP. We determined that *mgtM* is translated *in vivo* (Supplementary Fig. 2) and that similarly sized ORFs preceded by potential RBSs are conserved in the *mgtCBR* leader regions from other enteric bacteria (Supplementary Fig. 3). The sequences adjacent to and including *mgtM* have the potential to adopt two alternative structures – stem-loops A and B – in *Salmonella* (Fig. 4a) and other examined species (Supplementary Fig. 3). Inline probing experiments verified the formation of stem-loop B in the wild-type *mgtCBR* leader RNA (Supplementary Fig. 4) and of stem-loop A in a mutant leader RNA with the G₉₅→C substitution, which is predicted to hinder formation of stem-loop B (Supplementary Fig. 4).

The deduced amino acid sequence of *mgtM* is not conserved in other species (Supplementary Fig. 5), but its length and location relative to stem-loop A, as well as the presence of A nucleotides near the base and within stem-loop A are (i.e., positions 44-46 and 56-57; Fig. 4b). Because the left arm of stem-loop A includes the last four *mgtM* codons (Fig. 4a), translation of the complete *mgtM* is anticipated to hinder formation of stem-loop

A and favor formation of stem-loop B. Therefore, changes in intracellular ATP levels may affect the coupling/uncoupling of transcription of the *mgtCBR* leader with translation of *mgtM*. This would determine whether stem-loop A versus B forms thereby dictating whether transcription continues into the *mgtCBR* coding regions. This is analogous to the mechanism by which cytoplasmic UTP levels control expression of the pyrimidine biosynthetic gene *pyrBI* in *Escherichia coli*, except that low UTP promotes *pyrBI* transcription when RNAP pauses at a U-rich segment of the *pyrBI* leader mRNA²⁰ whereas we propose that high ATP furthers expression of the *mgtCBR* coding regions.

In agreement with the notion that a transcription attenuation-like mechanism^{20,21} regulates transcription elongation into the *mgtCBR* coding region, a strain with a chromosomal mutation in the *mgtM* start codon and harboring a *lac* fusion in the *mgtC* coding region produced over four times more β -galactosidase than the isogenic strain with the wild-type *mgtC* leader in response to low Mg^{2+} (Fig. 4c and 4d). Likewise, strains harboring stop codons at the 4th, 6th or 7th positions of *mgtM* (Fig. 4a) also derepressed *mgtC-lac* expression (Fig. 4c). This derepression is due to a defect in *mgtM* translation (as opposed to resulting from an effect on the structure of the *mgtC* leader mRNA) because a plasmid expressing the amber suppressor *supF* restored normal *mgtC-lac* transcription to an *mgtM* mutant with an amber stop codon at the 6th position but not to an isogenic mutant with an ochre stop codon at the same position (Fig. 4e). As expected, the *supF*-expressing plasmid had no effect on *mgtC-lac* transcription in a strain harboring the wild-type *mgtC* leader (Fig. 4e).

An *mgtM* derivative with a stop codon at the 9th position failed to express *mgtC-lac* (Fig. 4c), possibly because translation of *mgtM* beyond the 8th codon would result in formation of stem-loop B (Fig. 4a). *mgtM* translation exerts its regulatory effect on the associated *mgtCBR* coding regions in *cis* (as opposed to *mgtM* encoding a *trans*-acting peptide) because a plasmid expressing the *mgtM* ORF failed to restore normal *mgtC-lac* expression to an *mgtM* stop codon mutant, behaving like the vector control (Fig. 4f); and it had no effect on a strain with a wild-type *mgtC* leader (Fig. 4f). Our data suggest that whenever translation stops before the ribosome reaches the 9th *mgtM* codon, stem-loop A forms, which promotes expression of the *mgtCBR* coding region; and when *mgtM* is translated beyond a certain position, stem-loop B is favored, which reduces transcription of the *mgtCBR* coding region. As predicted, the dramatic *mgtC-lac* derepression exhibited by the *mgtM* start codon mutant (Fig. 4c and 4d) was eliminated by the simultaneous introduction of C₈₇→G and G₉₅→C substitutions (Fig. 4d), which further formation of stem-loop B (Fig. 4a).

The phenotypes described above are not an artifact resulting from the absence of a functional *mgtC* gene in the *mgtC-lac* strains. This is because mutation of the *mgtM* start codon derepressed *mgtC* levels in an otherwise wild-type *Salmonella* that experienced mild acidification; and also because introduction of a stop codon at the 9th position of *mgtM* resulted silenced *mgtC* expression in an isogenic strain subjected to the same conditions (Supplementary Fig. 6)).

The conserved A nucleotides at positions 44-46 or 56-57 of the *mgtCBR* leader (Fig. 4a) are critical for the response to ATP because: on the one hand, the mild acid induction of the *mgtC* and *mgtB* genes disappeared in a strain with a chromosomal mutation at position 44-46 in the *mgtCBR* leader (Fig. 1a and 1b). Likewise, growth in glucose no longer promoted higher fluorescence than growth in glycerol in wild-type *Salmonella* carrying a pGFP303 derivative with the As at position 44-46 substituted by Ts (Fig. 2c). And the increased fluorescence displayed by the *mgtC* mutant also required the A nucleotides at position 44-46 (Fig. 2e). Yet, this mutant leader retained a wild-type structure

(Supplementary Fig. 7) and ability to respond to Mg^{2+} (Supplementary Fig. 8)²². Moreover, changes in the adenine concentration in the media failed to affect the fluorescence produced by the purine auxotroph with derivatives of pGFP303 where the A nucleotides at position 44-46 were replaced by either Ts, Gs or Cs, or with a plasmid with the A nucleotides at position 56-57 replaced by Gs (to avoid destabilizing stem-loop A, the latter two nucleotides were not replaced by Ts or Cs; Fig. 4a) (Fig. 2a). On the other hand, an engineered *mgtCBR* leader with a scrambled *mgtM* sequence that retained the As at positions 44-46 and 56-57 displayed a wild-type response to ATP (Fig. 2a).

The RNAs from the wild-type and ATP-blind mutant *mgtC* leader displayed indistinguishable in vitro profiles when investigated at two pHs (Supplementary Fig. 7b). This provides further support to the notion that the increase in *mgtCBR* expression resulting from mild acidification is mediated by cytosolic ATP levels as opposed to protons being sensed directly by the *mgtC* leader mRNA. Cumulatively, these data provide a singular example of a bacterial mRNA leader that senses ATP, utilizing a mechanism that is different from those previously described in eukaryotic organisms²³⁻²⁵.

We wondered whether ATP sensing by the *mgtCBR* mRNA leader is required for *Salmonella* virulence given that *mgtC* is expressed in a variety of tissues during infection in several different animal hosts^{8,26-28} and that inactivation of the *mgtC* gene hinders survival inside macrophages and virulence in mice⁹. We established that when *Salmonella* was inside the macrophage-like cell line J774.A1, the mRNA levels of the *mgtA* and *mgtC* leaders region rose in parallel (Supplementary Fig. 9), which likely reflects activation of their respective promoters during infection²⁹. By contrast, the mRNA levels corresponding to the *mgtC* and *mgtB* coding regions increased dramatically (i.e., 10 fold by 9 h post internalization relative to *Salmonella* grown in tissue culture media; Fig. 3d), but no induction was observed for the coding region of the *mgtA* gene (Fig. 3d), which is not required for *Salmonella* virulence⁹. This may reflect differences between the *mgtCBR* (Fig. 4a) and *mgtA*³⁰ leaders. Indeed, the mRNA levels of the *mgtA* coding region did not increase under conditions promoting higher ATP levels (Fig. 2a) or mild acidification (Fig. 1a). Induction of the *mgtC* and *mgtB* coding regions inside macrophages requires the ability of the *mgtCBR* leader mRNA to sense ATP because it was defective in a chromosomal mutant with the A nucleotides at position 44-46 replaced by Ts (Fig. 3e). This mutant was attenuated for virulence following intraperitoneal inoculation of mice (Fig. 3f), albeit not as much as a strain deleted for the *mgtC* and *mgtB* coding regions (Fig. 3f), implying that other signaling inputs remain functional in this mutant. Cumulatively, our findings demonstrate that the *mgtCBR* leader and the MgtC protein regulate ATP homeostasis by sensing and controlling cytosolic ATP levels, respectively. These properties are critical for *Salmonella* virulence, possibly because *Salmonella* resides within a phagosome that is mildly acidic¹, which is a condition that can generate high ATP levels in the bacterium^{6,7}. Finally, our data highlight how pathogens can interpret host-derived signals, such as acid pH, by the changes they experience in their cellular constituents.

Methods

Bacterial strains, plasmids, oligodeoxynucleotides and growth conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table 1. All *S. enterica* serovar Typhimurium strains are derived from the wild-type strain 14028s³¹ and were constructed by phage P22-mediated transductions as described³². All DNA oligonucleotides are listed in Supplementary Table 2. Bacteria were grown at 37°C in Luria-Bertani broth (LB), N-minimal media (pH 7.7)³³ supplemented with 0.1% casamino acids, 38 mM glycerol and the indicated concentrations of $MgCl_2$. To examine the effect of pH or carbon source on gene expression, we used a modified N-minimal medium containing 0.2%

glucose instead of 38 mM glycerol. *Escherichia coli* DH5 α was used as the host for preparation of plasmid DNA. Ampicillin was used at 50 $\mu\text{g ml}^{-1}$, chloramphenicol was used at 20 $\mu\text{g ml}^{-1}$, kanamycin at 20 $\mu\text{g ml}^{-1}$, tetracycline at 10 $\mu\text{g ml}^{-1}$ and fusaric acid at 12 $\mu\text{g ml}^{-1}$.

Effect of pH on gene expression

Cells were grown overnight in N-minimal medium containing 10 mM Mg^{2+} . 1/100 dilution of the overnight culture was used to inoculate 20 ml of the same medium and grown for 3 h. Cells were then washed and transferred to 20 ml of N-minimal medium containing 500 μM Mg^{2+} and grown for 1 h. The cells were harvested and washed with N-minimal medium containing 500 μM Mg^{2+} and an aliquot corresponding to 1/10 volume of cells for pH 7.7 before switching to pH 5.1. Then, cells were resuspended in 20 ml of N-minimal medium containing 500 μM Mg^{2+} at pH 5.1 with or without 0.5 μM of CCCP and growth continued for 1 h. Bacteria were stabilized using RNAlprotect Bacteria Reagent (Qiagen) and RNA was isolated for further analysis.

Effect of exogenous adenine on gene expression

The adenine auxotrophic strain EG9652 harboring plasmid pGFP303, its derivatives, pfpv25*mgtA* or the plasmid vector were grown overnight in N-minimal medium containing 10 mM Mg^{2+} , 500 μM adenine and ampicillin. 1 ml of the overnight culture was washed in the N-minimal medium without Mg^{2+} and adenine and resuspended in 1 ml of the same media. The suspended bacteria were inoculated 1/50 volume in N-minimal medium with 10 μM Mg^{2+} and ampicillin in the presence of either 25 μM or 250 μM adenine. Fluorescence and the OD₆₀₀ of the cultures were monitored for 6.5 h with shaking 37°C in a Victor³ plate reader (Perkin Elmer). To prevent evaporation, the 96-well plate was covered with mineral oil. GFP expression of a given strain was determined by plotting fluorescence over OD₆₀₀. Note that the *purB* strains were freshly transduced before each experiment to prevent the isolation of suppressor mutations.

Measurement of intracellular ATP in *Salmonella*

We measured intracellular ATP levels using a luminometer (BioTek Synergy H1) as described with modification³⁴. Briefly, bacteria were grown overnight in N-minimal media containing 10 mM Mg^{2+} and 0.2% glucose as a carbon source. 1 ml of the overnight culture was washed in the N-minimal medium without Mg^{2+} and resuspended in 1 ml of the same media. 1/100 dilution of bacteria were inoculated in 2 ml of N-minimal media containing 10 μM Mg^{2+} (with proper antibiotics if necessary) and grown for 4 h. Cells were normalized by OD₆₀₀ and resuspended in 500 μl of phosphate-buffered saline (PBS). Nucleic acids were extracted by adding 100 μl of ice cold 3 M perchloric acid. After incubating for 5 min, extracts were neutralized with 225 μl of neutralization buffer (1 M KOH, 0.5 M Tris, 0.5 M KCl) and centrifuged for 10 min. 50 μl of the supernatant was diluted with 50 μl of L buffer (25 mM KCl, 50 mM MgSO_4 , and 100 mM HEPES pH 7.4) and intracellular ATP was measured using an ATP determination kit (Invitrogen) according to manufacturer's instruction. Intracellular ATP levels [pmoles/ml cells at given OD₆₀₀] were converted using reference to standards of known concentration.

To measure intracellular ATP levels in response to exogenous adenine levels (Fig. 2b), *purB* *Salmonella* were grown overnight in N-minimal medium containing 10 mM Mg^{2+} and antibiotic. 1 ml of the overnight culture was washed in the N-minimal medium without Mg^{2+} and adenine and resuspended in 1 ml of the same media. 1/50 dilution of suspended bacteria were inoculated in modified N-minimal media containing 10 μM Mg^{2+} and 0.3 mM inorganic phosphate and grown for 6.5 h. Then, 120 μCi of P^{32} -phosphorus was added and labeled for 15 min. The labeled NTPs were extracted by formic acid extraction as described

previously and analyzed by PEI-cellulose TLC plate³⁵. The levels of intracellular ATP were quantified by Typhoon FLA-9000 phosphorimager (GE healthcare).

Examining gene expression inside macrophages

Macrophage infection was performed as described⁸ with the following modifications: J774 A.1 macrophages were seeded in 6-well plates in RPMI medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 10 mM HEPES at a density of 10⁶ per well one day prior to infection with *Salmonella*. Bacteria were grown overnight in LB media at 37°C, washed with PBS and used to infect macrophages at a multiplicity of infection (MOI) of 100:1. Plates were centrifuged at 1,000 *g* for 5 min (defined as time 0) and incubated for 1 h for phagocytosis. Extracellular bacteria were killed with RPMI media containing 50 µg/ml gentamicin. The media was replaced after 1 h with same media containing 10 µg/ml gentamicin and continued incubation as indicated in the legend to Figures 3d and 3e. At each time point, infected macrophages were lysed and stabilized with Tri reagent (Applied Biosystems) and RNA was extracted according to manufacturer's instruction. Control RNA from *Salmonella* cultured in tissue culture media was obtained as described previously⁸. *Salmonella* mRNA levels at each time point were normalized by the mRNA levels following growth in tissue culture media. To estimate the intracellular bacteria at each time point, cells were lysed with PBS containing 0.1% triton X-100 and plated on LB plate with proper dilutions or DNA was extracted from the same RNA sample according to manufacturer's instruction (Applied Biosystems) and measured by quantitative RT-PCR.

Mouse virulence assays

Six to eight week old female C3H/HeN mice were inoculated intraperitoneally with ~10⁴ colony forming units. Mouse survival was followed for 21 days. Virulence assays were conducted three times with similar outcomes and data correspond to groups of five mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Mild acid pH promotes transcription of the *mgtC* coding region in a *Salmonella* strain lacking the extracytoplasmic pH sensor PhoQ

Relative mRNA levels of the coding regions of the *mgtC* (a), *mgtB* (b) and *mgtA* (c) genes produced by a *Salmonella* *phoP*^{*} *phoQ* strain (EG10232) or derivatives with conserved A 44-46 nucleotides in the *mgtCBR* leader substituted by Ts (EL486). Bacteria were grown in N-minimal media with 500 μ M Mg²⁺ at pH 7.7 for 1 h and then for an additional 1 h in the same media at pH 5.1 in the absence or presence of 0.5 μ M of the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP). mRNA levels of target genes were normalized to that of 16S ribosomal RNA *rrs* gene. Shown are the mean and SD from two independent experiments. **d**, Intracellular ATP levels and pH of *Salmonella* (EG10232) grown in media with pH 7.7 and one hour after being switched to media with pH 5.1 to an OD₆₀₀: 0.453.

Values are in pmol of ATP per ml of cells per OD₆₀₀. Shown are the mean and SD from two independent measurements.

Figure 2. ATP promotes gene transcription in a manner dependent on conserved A nucleotides in the *mgtCBR* leader region

a, Fold change in fluorescence produced by an adenine auxotroph (EG9652) harboring the plasmid vector pfpv25, pGFP303 or derivatives with substitutions of conserved A nucleotides at position 44-46 by Ts (pGFP303 A₄₄₋₄₆→T), Gs (pGFP303 A₄₄₋₄₆→G), or Cs (pGFP303 A₄₄₋₄₆→C), with substitutions of conserved 55-56 A nucleotides by Gs (pGFP303 A₅₅₋₅₆→G), or with substitution of most of the *mgtM* sequence except for A nucleotides at 44-46 and 55-56 (pGFP303 *mgtM*_{scrambled}). Plasmid pfpv25*mgtA* harbors a transcriptional fusion between the PhoP-dependent *mgtA* promoter and *mgtA* leader sequence and a promoterless *gfp* gene. Bacteria were grown in N-minimal media with 10 μM Mg²⁺ in the presence of either 25 μM (low) or 250 μM (high) adenine. Fluorescence was monitored as described in Methods. Fold change was calculated by dividing the fluorescence of cells grown in the high adenine by that of cells grown in low adenine. Note that the ratio of fluorescence displayed at high and low adenine is the same for bacteria harboring pfpv25*mgtA* or the vector pfpv25. Data correspond to a representative of four independent experiments conducted in duplicate. **b**, Intracellular ATP levels and intracellular pH of *purB* *Salmonella* (EG9652) grown in the presence of the indicated

concentrations of adenine were determined by labeling with ^{32}P -phosphorus and then separating by TLC. **c**, Fluorescence produced by wild-type *Salmonella* (14028s) harboring pGFP303 or pGFP303 A₄₄₋₄₆→T following growth for 4 h in N-minimal media with 10 μM Mg^{2+} in the presence of either 38 mM glycerol (~0.35%) or 0.2% glucose as a carbon source. **d**, Intracellular ATP levels of cells grown in the indicated carbon sources to an OD₆₀₀: 0.419 were determined by luminescence as described in Methods. Shown are the mean and SD from two independent measurements. **e**, Fluorescence produced by wild-type (14028s), *mgtC* (EL4), *mgtB* (EL5) and *mgtA* (EG9521) *Salmonella* harboring the vector (pfpv25), pGFP303, or its derivative pGFP303 A₄₄₋₄₆→T following growth for 4 h in N-minimal media with 10 μM Mg^{2+} .

Figure 3. Expression of the *mgtC* coding region inside macrophages is dependent on its leader region's ability to sense ATP, a property required for *Salmonella* virulence
d, Relative mRNA levels of the *mgtC*, *mgtB* and *mgtA* coding regions of wild-type *Salmonella* harboring the wild-type *mgtCBR* leader (14028s) inside J774 A.1 macrophages at the indicated times after infection. **e**, Same as in (**d**) except using a mutant *Salmonella* with the chromosomal A nucleotides at position 44-46 of the *mgtCBR* leader substituted by Ts (EL341). **f**, Survival of C3H/HeN mice inoculated intraperitoneally with ~10⁴ colony forming units of wild-type *Salmonella* (14028s) or ATP-sensing defective mutant (EL341) or deleted for both the *mgtC* and *mgtB* coding regions (EL6).

Figure 4. Regulation of the *Salmonella mgtCBR* virulence operon by the PhoP/PhoQ system and *mgtCBR* leader region

a, The sensor PhoQ responds to extracytoplasmic low Mg²⁺, acid pH and antimicrobial peptides by promoting phosphorylation of the PhoP protein, which binds to the *mgtCBR* promoter resulting in transcription initiation. Acidic pH also produces a proton gradient across the inner membrane resulting higher intracellular ATP levels. Intracellular ATP levels control transcription elongation into the *mgtCBR* coding regions by affecting the coupling/uncoupling of transcription of the *mgtCBR* leader and translation of the short ORF *mgtM*, which affects the formation of alternative stem-loops A versus B. The *mgtMRBS* is underlined. Positions and sequences of stop codon mutations or nucleotide substitutions in the strains used in the experiments presented in (c) and (d) are indicated and denoted below the *mgtM* sequence. **b**, Conservation of A nucleotides in *mgtM* relative to stem-loop structures in the *mgtC* leader. Alignment of the nucleotide sequences corresponding to *mgtM* from the indicated species. Sequences in bold correspond to *mgtM*. The *mgtMRBS*s are underlined. Asterisks correspond to nucleotides conserved in all listed species. Conserved A nucleotides are colored in orange and sequences with a potential to adopt stem-loop structures are underlined. **c**, β-galactosidase activity (Miller units) produced by *Salmonella* with a chromosomal *mgtC-lac* fusion (EG9527) and isogenic derivatives with mutation of the start codon (EG19269) or with stop codons at different positions (EG19285, EG19289, EG19293 and EG19298) in *mgtM*. Bacteria were grown in N-minimal media containing low (L; 10 μM) or high (H; 10 mM) Mg²⁺ for 4 h. **d**, β-galactosidase activity (Miller units) produced by *Salmonella* with a chromosomal *mgtC-lac* fusion (EL92) and isogenic derivatives with C₈₇→G and G₉₅→C substitution (EL96) or mutation of the start codon in *mgtM* (EL97) or both (EL98). Bacteria were grown as described in (c). Shown in (c) and (d) are the mean and SD from three independent experiments. **e**, β-galactosidase activity produced by *Salmonella* with a chromosomal *mgtC-lac* fusion harboring either plasmid *psupF* or the empty vector pUH21-2*lacA* (EL86, EL87) and isogenic derivatives with an amber stop codon (EL90, EL91) or ochre stop codon (EL88, EL89) at position 39-41 of *mgtM*. Bacteria were grown as described above except in the presence of ampicillin and 0.2 mM IPTG. **f**, β-galactosidase activity produced by *Salmonella* with a chromosomal *mgtC-lac* fusion (EG9527) or a isogenic strain with a stop codon at position

39-41 in *mgtM* (EG19289) harboring either the vector or plasmid *p_{mgtM}*. Bacteria were grown as described except in the presence of ampicillin and 0.1 mM IPTG. Shown in **(e)** and **(f)** are the mean and SD from two independent experiments.