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A protein that controls the onset of a *Salmonella* virulence program

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Abstract

The mechanism of action and contribution to pathogenesis of many virulence genes are understood. By contrast, little is known about anti-virulence genes, which contribute to the start, progression, and outcome of an infection. We now report how an anti-virulence factor in *Salmonella enterica* serovar Typhimurium dictates the onset of a genetic program that governs metabolic adaptations and pathogen survival in host tissues. Specifically, we establish that the anti-virulence protein CigR directly restrains the virulence protein MgtC, thereby hindering intramacrophage survival, inhibition of ATP synthesis, stabilization of cytoplasmic pH, and gene transcription by the master virulence regulator PhoP. We determine that, like MgtC, CigR localizes to the bacterial inner membrane and that its C-terminal domain is critical for inhibition of MgtC. As in many toxin/anti-toxin genes implicated in antibiotic tolerance, the *mgtC* and *cigR* genes are part of the same mRNA. However, *cigR* is also transcribed from a constitutive promoter, thereby creating a threshold of CigR protein that the inducible MgtC protein must overcome to initiate a virulence program critical for pathogen persistence in host tissues.

Keywords anti-virulence protein; CigR; pathogenesis; *Salmonella*; virulence protein MgtC

Subject Categories Microbiology, Virology & Host Pathogen Interaction; Transcription

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Introduction

In the context of pathogenesis, the genes of a pathogen can be divided into three distinct groups: virulence genes, which promote virulence; anti-virulence genes, which decrease virulence; and genes that neither promote nor decrease virulence. Investigations over the last several decades have revealed the mechanism of action and contribution to pathogenesis of an increasing number of virulence genes. By contrast, little is known about how anti-virulence genes function. Anti-virulence genes have been identified in a wide variety of pathogens (Cunningham *et al*, 2001; Shimono *et al*, 2003; Ionescu *et al*, 2013). The interplay between virulence genes and anti-virulence genes is believed to regulate the pathogenicity of pathogens (Brown *et al*, 2016). Here, we report how an anti-virulence protein governs the onset of a *Salmonella* virulence program.

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen that causes gastroenteritis in humans and a typhoid fever-like disease in mice (Coburn *et al*, 2007; Fabrega & Vila, 2013). *Salmonella* can survive and replicate in acidic vacuoles within host phagocytic cells (Buchmeier & Heffron, 1991; Lee *et al*, 2013), where it senses low pH (Prost *et al*, 2007; Choi & Groisman, 2016) and expresses virulence genes via the PhoP/PhoQ two-component regulatory system (Alpuche Aranda *et al*, 1992; Guo *et al*, 1997; Bijlsma & Groisman, 2005; Retamal *et al*, 2009). *Salmonella* survival in host phagocytic cells is made possible by the precise timing at which virulence gene products are produced (Jones & Falkow, 1996; Marcus *et al*, 2000). Indeed, growth within acidic vacuoles requires the coordinated expression of several virulence factors (Eriksson *et al*, 2003).

The *mgtC* gene mediates intramacrophage survival and proliferation within host tissues in several intracellular pathogens (Blanc-Potard & Groisman, 1997; Grabenstein *et al*, 2006; Rang *et al*, 2007; Alix & Blanc-Potard, 2008; Lee & Groisman, 2012; Belon *et al*, 2014; Pontes *et al*, 2015). In *S. enterica* serovar Typhimurium, the MgtC protein confers virulence in two distinct ways. On the one hand, MgtC inhibits *Salmonella*'s own F_1F_o ATP synthase (Lee *et al*, 2013), the machine responsible for the synthesis of the majority of adenosine triphosphate (ATP) in the cell (Senior, 1990). Thus, MgtC enables *Salmonella* to maintain its cytoplasmic pH near 7 when experiencing a mildly acidic pH inside macrophages (Lee *et al*, 2013) and to reduce transcription of ribosomal rRNA when cytosolic conditions prevent the assembly of functional ribosomes (Pontes *et al*, 2016). On the other hand, MgtC prevents degradation of PhoP (Yeom *et al*, 2017), the master regulator of *Salmonella* pathogenesis (Groisman *et al*, 1989; Miller *et al*, 1989), thereby advancing virulence gene expression. Thus, MgtC's actions commit *Salmonella* to low cytosolic ATP, slow growth, and expression of genes requiring large amounts of the regulator PhoP.

We now report that *Salmonella*'s commitment to the MgtC-dependent program requires not only the signals promoting expression of the *mgtC* gene, but also that MgtC protein amounts supersede those of CigR, an anti-virulence protein (Kidwai *et al*, 2013; Yin *et al*, 2016) that binds MgtC, thereby preventing MgtC

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from inhibiting the F_1F_o ATP synthase. Surprisingly, the *cigR* and *mgtC* genes are located on the *Salmonella* pathogenicity island 3 (SPI-3; Blanc-Potard *et al*, 1999) and are part of the same transcription unit under MgtC-inducing conditions. However, the *cigR* gene is also transcribed constitutively and independently of MgtC, setting a threshold of CigR protein that MgtC must overcome to exert its virulence and metabolic functions.

Results

CigR is an inner membrane protein that binds to the inner membrane protein MgtC

It was previously suggested that CigR is an effector protein secreted into host cells (Niemann *et al*, 2011, 2013). However, this does not appear to be the case because (i) CigR lacks the characteristics of effector proteins (Sato *et al*, 2011); (ii) CigR secretion was detected only in a *Salmonella* mutant lacking control of secretion and overexpressing a regulatory protein (Niemann *et al*, 2011); and (iii) CigR localizes to the inner membrane in wild-type *Salmonella* (Fig EV1A), in agreement with transmembrane domain predictions (TMHMM v. 2.0). Given that MgtC is also an inner membrane protein (Lee *et al*, 2013) and that inactivation of *cigR* renders *Salmonella* hypervirulent in mice and inside macrophages (Kidwai *et al*, 2013; Yin *et al*, 2016), we wondered whether CigR interacts with MgtC, which would implicate these two proteins in the same virulence pathway.

We determined that the CigR and MgtC proteins interact in a specific manner. First, bacterial two-hybrid analysis demonstrated CigR binding to MgtC but not to PmrB (Fig EV1B–E), an inner membrane protein used as a negative control (Wösten *et al*, 2000). Second, anti-HA antibodies pulled down MgtC-FLAG (see low-intensity band in center bottom panel of Fig 1A), and anti-FLAG antibodies pulled down CigR-HA (Fig 1A), in a strain expressing C-terminally FLAG-tagged MgtC and C-terminally HA-tagged CigR from their normal chromosomal locations. And third, CigR did not bind to YqjA-FLAG, an inner membrane protein used as a negative control (Fig 1A). In addition, CigR exhibited decreased binding to an MgtC variant with the N92T substitution (Fig 1B), which is defective in the ability to inhibit the F_1F_o ATP synthase (Lee *et al*, 2013) but retains normal localization to the inner membrane (Fig EV1F). By contrast, CigR interacted with the MgtC variants with the E84A, C99A, or W226A amino acid substitutions (Lee *et al*, 2013), as it did with the wild-type MgtC protein (Fig 1B). Furthermore, the anti-HA antibody did not precipitate MgtC-FLAG from a strain expressing untagged CigR (Fig 1A), and the anti-FLAG antibody did not precipitate CigR in a strain expressing untagged MgtC (Fig 1A). In sum, the CigR protein specifically interacts with the MgtC protein *in vivo*.

CigR and AtpB compete for binding to MgtC

We hypothesized that CigR competes with the F_1F_o ATP synthase subunit AtpB for binding to MgtC because the MgtC N92T variant was defective for interaction with AtpB (Lee *et al*, 2013) and CigR (Figs 1B and EV1G). To test this hypothesis, we performed co-immunoprecipitation experiments using different amounts of *in vitro* synthesized MgtC-FLAG, AtpB-HA, and CigR-HA proteins reconstituted into proteoliposomes. As AtpB-HA amounts increased, binding between CigR and MgtC decreased (Fig EV2A, lower box). Likewise, an increase in CigR-HA amounts decreased the interaction between MgtC and AtpB (Fig EV2A, upper box). These results argue that CigR and AtpB compete with each other for binding to MgtC.

To examine the binding specificity of CigR and AtpB for MgtC, we calculated the half inhibitory concentration (IC_{50}) values of CigR-HA and AtpB-HA for MgtC-FLAG using increasing amounts of competitor AtpB-HA and CigR-HA, respectively (Figs 1C and EV3). The IC_{50} values of CigR-HA and AtpB-HA for MgtC-FLAG are 8.9 and 22.3 μM, respectively (Fig 1C). Thus, MgtC binding to AtpB is stronger than to CigR (Figs 1C and EV3). Additionally, CigR does not appear to bind to AtpB because the intensity of the CigR-HA band was similar in the presence and absence of AtpB-FLAG following a pull-down with anti-FLAG antibodies (Fig EV2B). Competition between CigR and AtpB was also detected *in vivo* as expression of the *cigR* gene from a heterologous promoter reduced the interaction between the AtpB and MgtC proteins (Fig 1D). These experiments demonstrate that CigR binds MgtC directly and that this binding hinders MgtC binding to AtpB. The CigR protein determines whether *Salmonella* embarks on a pathway that alters normal bacterial physiology.

The *cigR* gene decreases survival inside macrophages and increases both ATP levels and intracellular pH, in an *mgtC*-dependent manner

If CigR exerts its anti-virulence function by targeting MgtC, a *cigR* mutant should exhibit the opposite behavior of an *mgtC* mutant. As predicted, a strain deleted for the *cigR* gene achieved four times the number of wild-type *Salmonella* inside the macrophage-like cell line J774A.1 by 20 h post-infection (Fig 2A). This is in contrast to the *mgtC* mutant, which survived five times less than the wild-type strain (Fig 2A; Blanc-Potard & Groisman, 1997; Rang *et al*, 2007), albeit not to the low levels as the *phoP* mutant (Fig 2A). The enhanced intramacrophage survival of the *cigR* mutant is due to inactivation of *cigR* (as opposed to the *cigR* mutation being polar on a downstream gene) because a plasmid harboring the *cigR* gene restored wild-type survival, whereas the plasmid vector did not (Fig 2A).

The *cigR* mutant exhibited lower ATP levels (Fig 2B) and ATPase activity (Fig 2C) than wild-type *Salmonella,* the opposite phenotypes of those displayed by the *mgtC* mutant (Fig 2B and C; Lee *et al*, 2013). The plasmid harboring the *cigR* gene restored wild-type ATP levels to the *cigR* mutant, but the vector control did not (Fig 2B). [As previously reported (Lee *et al*, 2013), a mutant deleted for the *mgtB* gene, which is co-transcribed with *mgtC* (Snavely *et al*, 1991), retained wild-type ATP levels (Fig 2B).] Furthermore, the cytoplasmic pH of the *cigR* mutant was higher than that of wild-type *Salmonella* both during growth in defined laboratory media (Fig 2D) and inside macrophages (Fig 2E), unlike the lower pH displayed by the *mgtC* mutant in both conditions (Fig 2D and E; Lee *et al*, 2013).

The *cigR* mutant produced more PhoP-activated mRNAs than wild-type *Salmonella* (Fig 2F). This result reflects that MgtC protects PhoP from degradation (Yeom *et al*, 2017). Thus, when CigR is absent, the higher amounts of free MgtC further PhoP-dependent gene transcription (Yeom *et al*, 2017).

That CigR works primarily (if not exclusively) by inhibiting MgtC is supported by three additional lines of evidence. First, an *mgtC cigR* double mutant retained the behavior of the *mgtC* single mutant:

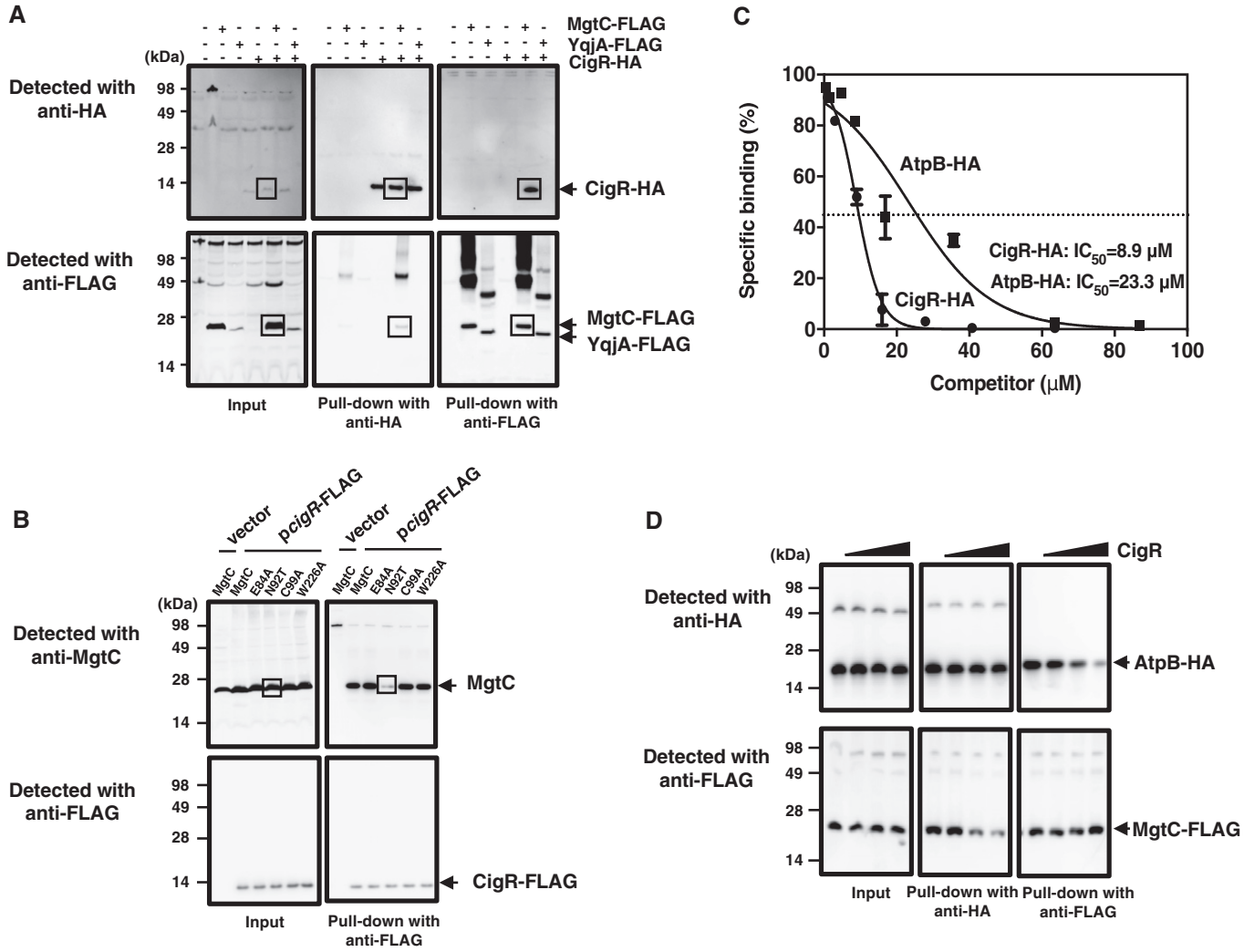

Figure 1. The AtpB and CigR proteins compete for binding to MgtC.

A Western blot analysis of crude extracts prepared from JY6, EG16539, YS251, JY92, and JY95 *Salmonella* strains grown in N-minimal media pH 7.7 containing 10 μM $MgCl_2$ for 6 h, followed by immunoprecipitation and detection with anti-HA and anti-FLAG antibodies, as indicated. Relevant bands are boxed.

B Western blot analysis of crude extracts prepared from wild-type (14028s), EL549, EL551, EL552, and EL553 *Salmonella* strains harboring a plasmid expressing CigR-FLAG grown in N-minimal media pH 7.7 containing 10 μM $MgCl_2$ and 0.5 mM IPTG for 6 h. These strains express the wild-type MgtC protein or derivatives with the indicated amino acid substitutions. Immunoprecipitation and detection was carried out with anti-MgtC and anti-FLAG antibodies, as indicated. Relevant bands are boxed.

C Dissociation curves for CigR-HA to MgtC-FLAG, and AtpB-HA to MgtC-FLAG (from Fig EV3G and H; see Materials and Methods). The IC_{50} corresponds to the concentration at which half of the protein is dissociated from the MgtC-FLAG protein. Shown are the mean and SD from three independent experiments.

D Western blot analysis of crude extracts from strain EL481 harboring a plasmid expressing the *cigR* gene following growth in N-minimal media pH 7.7 containing 10 μM $MgCl_2$ and IPTG (0.01, 0.1, and 1 mM) for 6 h. Immunoprecipitation and detection were carried out with anti-HA and anti-FLAG antibodies. Data are representative of three independent experiments, which gave similar results.

Source data are available online for this figure.

It displayed lower intramacrophage survival (Fig 2A) and higher ATP levels (Fig 2B) than wild-type *Salmonella*. Second, a plasmid expressing the *cigR* gene from a heterologous promoter increased ATP levels in wild-type *Salmonella* but not in the *mgtC* mutant (Fig 2G). Taken together with the data presented above (Fig EV1A), these results indicate that the anti-virulence protein CigR exerts its effects by targeting the virulence protein MgtC. Moreover, they reinforce the notion that the inner membrane protein CigR operates inside *Salmonella*.

The C-terminal domain of CigR is required for inhibition of MgtC

A tBLASTN analysis using the deduced amino acid sequence of the *S. enterica* serovar Typhimurium *cigR* gene revealed the presence of *cigR* homologs in several enteric bacterial species (Appendix Fig S1). The C-terminal region of CigR, which harbors the single predicted transmembrane domain, is much more conserved (82~98% amino acid identity) than the rest of the protein (53~85% amino acid identity for the full-length CigR; Appendix Fig S1).

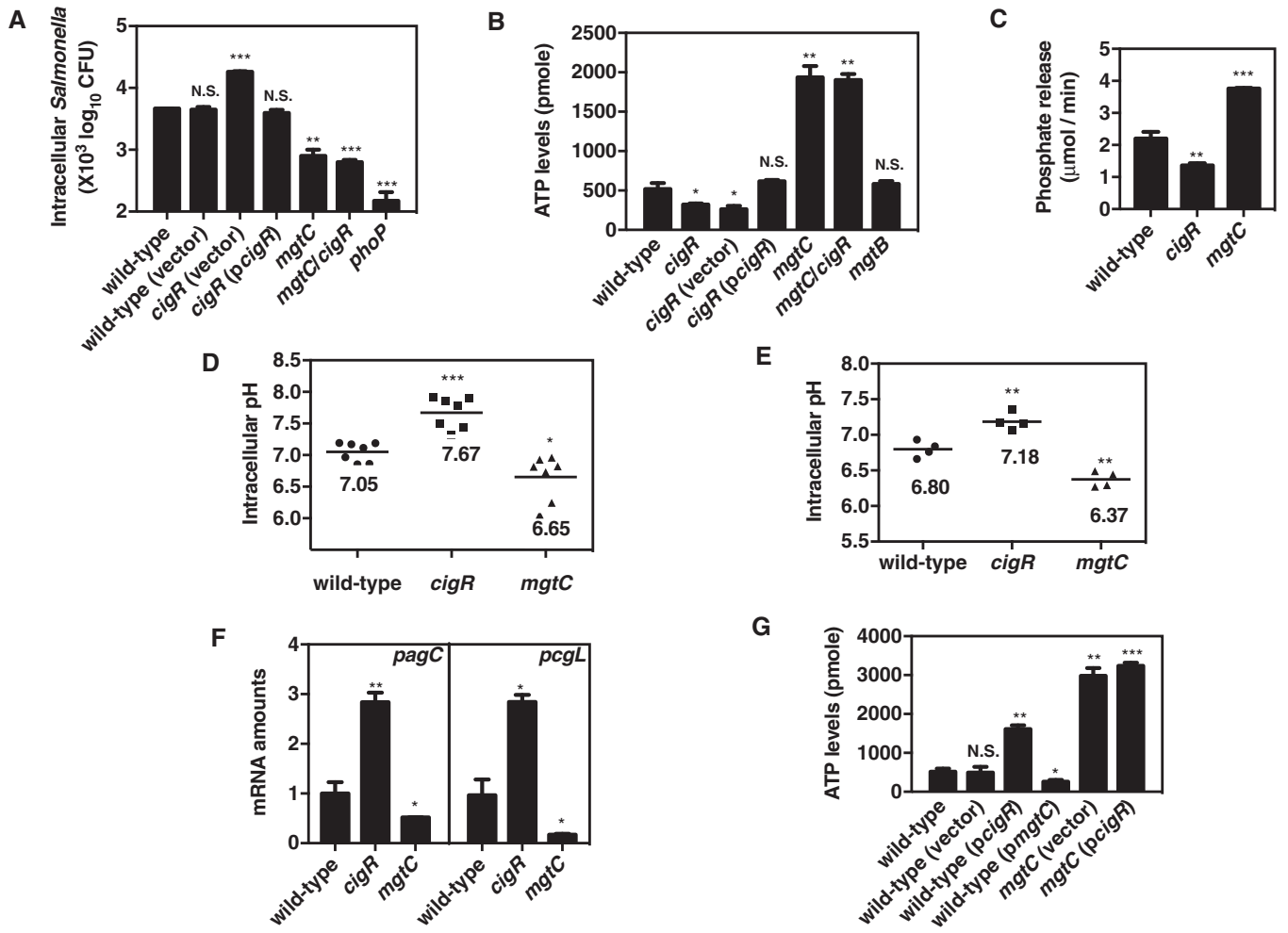

Figure 2. The *cigR* and *mgtC* mutants display opposite behaviors.

A Survival inside J774A.1 macrophages of wild-type (14028s), *mgtC* (EL4), *cigR* (JY12), *mgtC cigR* (JY6), and *phoP* (MS7953s) *Salmonella* without/with the indicated plasmids 20 h after infection. The mean and SD from two independent experiments are shown. Please note $\log_{10}$ scale of *y*-axis.

B ATP levels in wild-type (14028s), *mgtC* (EL4), *cigR* (JY12), *mgtC cigR* (JY6), and *mgtB Salmonella,* and in the *cigR* mutant harboring the plasmid vector or p*cigR*. Intracellular ATP levels correspond to picomoles of ATP per ml of cells at a given OD_{600}. The mean and SD from three independent experiments are shown. Bacteria were grown in N-minimal media pH 7.7 containing 10 µM $MgCl_2$ for 5 h.

C ATP hydrolysis rates in inverted vesicles prepared from wild-type (14028s), *mgtC* (EL4), and *cigR* (JY12) *Salmonella*. The reaction was initiated by adding ATP and monitored for 5 min. The mean and SD from two independent experiments are shown.

D Intracellular pH of wild type (14028s), *mgtC* (EL4), and *cigR* (JY12) *Salmonella* following bacterial growth in N-minimal medium pH 7.7 for 1 h and then switched to the same media at pH 4.6 for 1 h. Lines represent the average pH of seven independent replicates.

E Intracellular pH of wild type (14028s), *mgtC* (EL4), and *cigR* (JY12) *Salmonella* when inside macrophages. Bacteria grown in LB medium overnight were used to infect J774A.1 macrophages. pH measurements were carried out 6 h post-internalization. Numbers represent the average pH of four independent replicates.

F mRNA amounts of the PhoP-activated *pagC* and *pcgL* genes in wild type (14028s), *mgtC* (EL4), and *cigR* (JY12) *Salmonella*. The mean and SD from two independent experiments are shown.

G ATP levels present in wild type (14028s) and *mgtC* (EL4) *Salmonella* harboring no added plasmid, the plasmid vector, or plasmids expressing the *cigR* or *mgtC* genes. Bacteria were grown in N-minimal media pH 7.7 containing 10 µM $MgCl_2$ with 200 µM IPTG for 4 h. ATP levels correspond to picomoles of ATP per ml of cells at given OD_{600}. Shown are the mean and SD from three independent experiments.

Data information: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-tailed *t*-test with each sample vs. wild type, N.S., not significant.

Therefore, we hypothesized that the C-terminal region is crucial for CigR function.

To test this hypothesis, we compared the behaviors of wild-type *Salmonella* and a strain deleted for the whole *cigR* open reading frame to those of strains with deletions or nucleotide substitutions in the part of the chromosomal copy of the *cigR* gene specifying the C-terminal region of CigR (Appendix Fig S1). We deleted the nucleotide sequence corresponding to three conserved domains (D1, D2, and D3), each four amino acids long (Appendix Fig S1). Because tryptophan residues are generally critical for protein function (Bogan & Thorn, 1998; Rasmussen *et al,* 2007), we also substituted the nucleotides specifying the single tryptophan at position 133 so that *cigR* specified an alanine residue at this position.

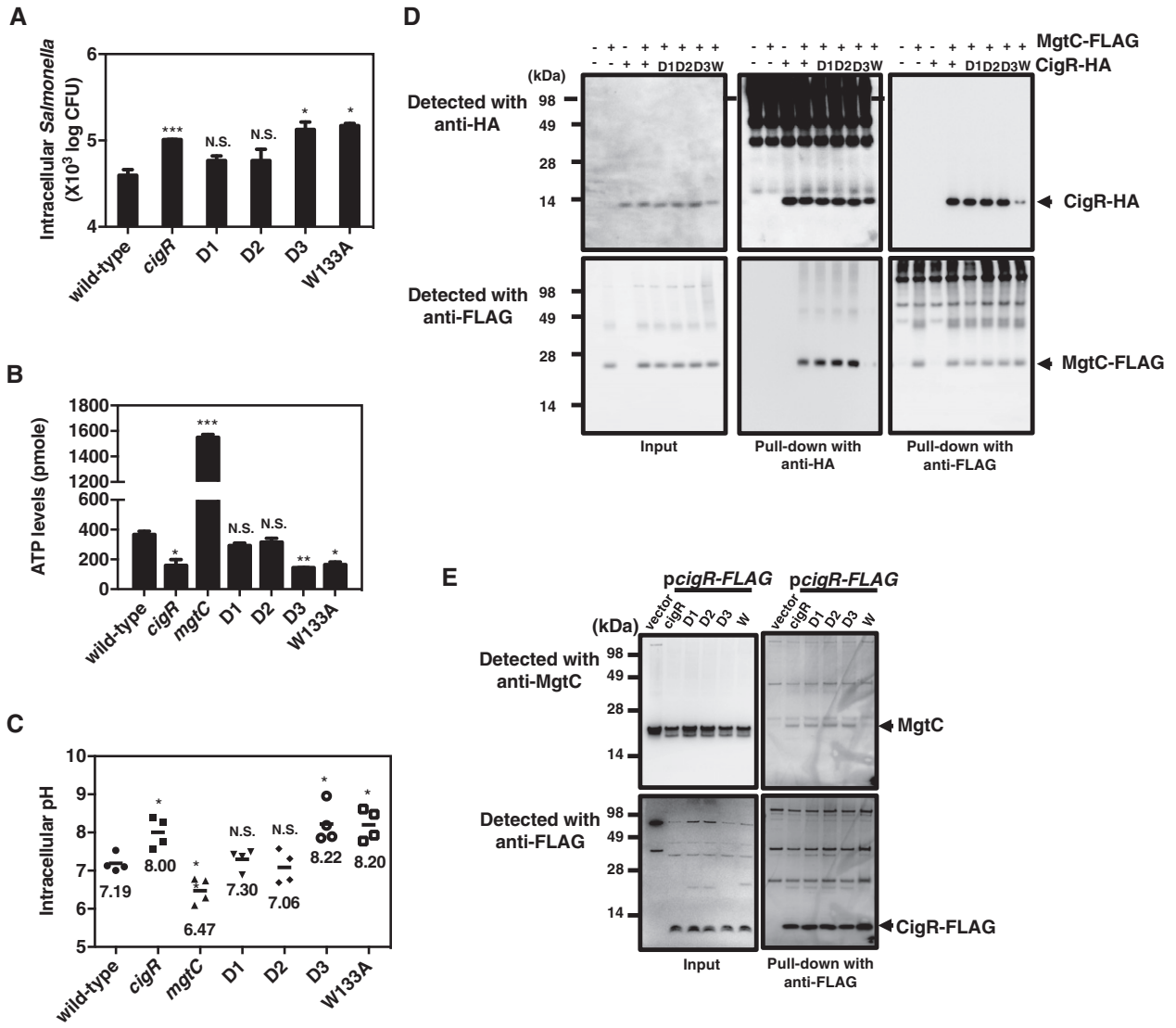

Figure 3. The C-terminal domain of CigR is required for inhibition of MgtC.

A–C Bacterial survival inside macrophages (A), ATP levels (B), and intracellular pH (C) of wild-type *Salmonella* (14028s) and mutants with deletions or nucleotide substitution in the *cigR* open reading frame (JY139, JY150, JY151, and JY152). (A) *Salmonella* survival inside J774A.1 macrophages 20 h after infection. The mean and SD from two independent experiments are shown. Please note $\log_{10}$ scale of *y*-axis. (B) ATP levels, corresponding to picomoles of ATP per ml of cells at a given OD_{600}, of the strains listed in (A). Bacteria were grown for 4 h in N-minimal media pH 7.7 containing 10 μM $MgCl_2$. The mean and SD from three independent experiments are shown. (C) Intracellular pH of the *Salmonella* strains listed in (A) was measured inside the macrophage-like cell line J774A.1. Numbers represent the average pH of four independent replicates, which gave similar bacterial colony counts when plated on LB agar plates. Data information: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-tailed *t*-test with each sample vs. wild type, N.S., not significant.

D Western blot analysis of proteoliposomes reconstituted from *in vitro* synthesized MgtC-FLAG and CigR-HA proteins, followed by immunoprecipitation and detection with antibodies directed to the HA or FLAG epitopes, as indicated. At the end of the reconstitution reaction, an aliquot (input) and fractions immunoprecipitated with antibodies directed to the HA or FLAG epitopes were analyzed. Proteoliposomes were prepared as described in Materials and Methods. The data are representative of two independent experiments, which gave similar results.

E Western blot analysis of crude extracts prepared from wild-type *Salmonella* (14028s) harboring a plasmid expressing the CigR-FLAG protein grown in N-minimal media pH 7.7 containing 10 μM $MgCl_2$ and IPTG (0.12 mM for wild type, 0.2 mM for D1 and D2, 0.3 mM for D3, and 0.4 mM for W) for 6 h. These strains express the wild-type CigR-FLAG protein or derivatives with the indicated amino acid substitution or domain mutations. Immunoprecipitation and detection were carried out with antibodies directed to the MgtC protein and FLAG epitope, as indicated. The data are representative of two independent experiments, which gave similar results.

Source data are available online for this figure.

Bacteria expressing the D3 and W133A CigR variants displayed the enhanced intramacrophage survival (Fig 3A), lower ATP levels during growth in laboratory media (Fig 3B), and higher pH inside macrophages (Fig 3C) that are characteristic of the *cigR*-deleted strain. The D3 and W133A CigR proteins localized to the inner membrane like wild-type CigR (Fig EV1H). By contrast, the D1 and

D2 mutants retained a wild-type behavior (Fig 3A–C). Although bacteria expressing the D3 and W133A CigR variants exhibited the same phenotypes, the corresponding proteins differed in their ability to interact with the MgtC protein *in vivo* and *in vitro*. Specifically, the D3 variant interacted with MgtC like wild-type CigR or the D1 and D2 variants (Fig 3D and E). By contrast, the W133A CigR variant was defective in binding to MgtC (Figs 3D and E, and EV1I). Cumulatively, these data establish that W133 is required for CigR binding to MgtC and that one or more residues substituted in the D3 region are necessary for inhibition of the MgtC protein.

The *cigR* gene is transcribed together with and independently of the *mgtC* gene

Toxins and anti-toxins are typically encoded by adjacent genes and produced from the same transcript (Gerdes *et al*, 2005). Although the *mgtC* and *cigR* genes are separated by two genes (Fig 4A–C), we hypothesized that they are part of the same polycistronic mRNA because they exhibit comparable expression behavior in a different *Salmonella* strain (http://bioinf.gen.tcd.ie/cgi-bin/salcom.pl; Kröger *et al*, 2013). As hypothesized, similar RNA polymerase (RNAP) occupancy of the *mgtC* and *cigR* coding regions as well as of the intervening *mgtB* and *mgtR* genes was observed during growth in 10 μM Mg^{2+} (Fig EV4A), a condition promoting *mgtCBR* expression (Soncini *et al*, 1996). Moreover, reverse transcriptase PCR (RT–PCR) assays produced *mgtC-cigR*, *mgtC-mgtB*, *mgtB-cigR*, and *cigR-cigR* amplicons of the predicted sizes (Fig EV4B–F). These results establish that the *mgtCBR* operon is longer than previously reported (Alix & Blanc-Potard, 2008).

Surprisingly, the *cigR* gene is also transcribed independently of the *mgtC* gene. That is to say, transcription from the *mgtC* promoter is strictly dependent on the PhoP protein (Soncini *et al*, 1996), whose amounts and activity increase during growth in 10 μM Mg^{2+} and decrease during growth in 10 mM Mg^{2+} (Soncini *et al*, 1996). Thus,

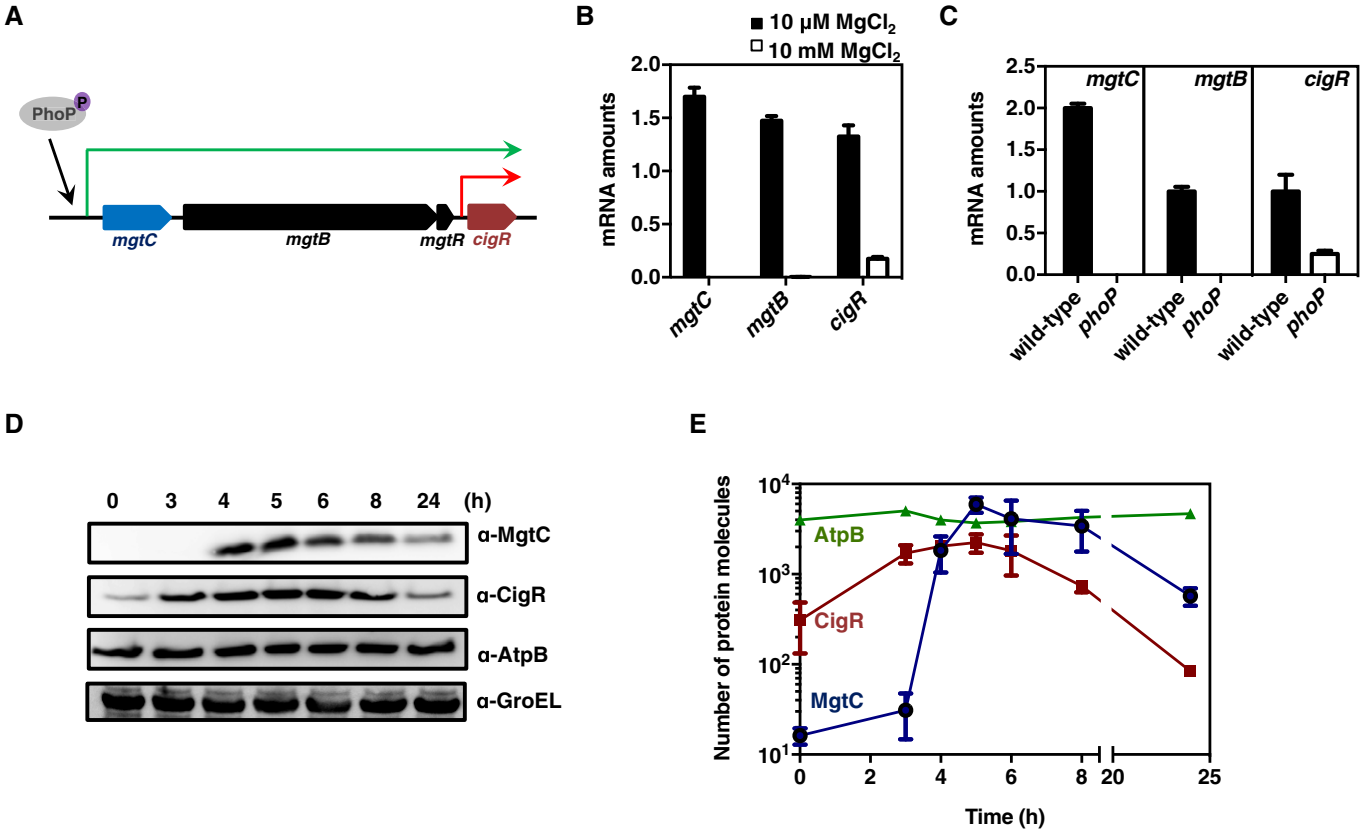

Figure 4. Transcription of the *cigR* gene is partially PhoP-dependent.

A Diagram of the *mgtC-cigR* chromosomal region showing the PhoP-dependent (green color) and PhoP-independent (red color) promoters.

B mRNA amounts of the *mgtC*, *mgtB*, and *cigR* genes produced by wild-type *Salmonella* (14028s) grown in N-minimal media pH 7.7 with 10 mM or 10 μM $MgCl_2$ for 4 h. Shown are the mean and SD from three independent experiments.

C mRNA amounts of the *mgtC*, *mgtB*, and *cigR* genes produced by wild-type (14028s) and *phoP* mutant (MS7953s) *Salmonella* following growth in N-minimal media pH 7.7 with 10 μM $MgCl_2$ for 4 h. mRNA amounts of target genes were normalized to those of the *rpoD* gene. The mean and SD from two independent experiments are shown.

D Western blot analysis of crude extracts from wild-type *Salmonella* (14028s) following growth in N-minimal media with 10 μM $MgCl_2$ for the indicated times using antibodies directed to the MgtC, CigR, AtpB, or GroEL proteins.

E Number of MgtC, CigR, and AtpB protein molecules present in cell extracts from wild-type *Salmonella* (14028s) following growth under non-inducing conditions (10 mM $MgCl_2$, time zero) and inducing conditions (10 μM $MgCl_2$) at the indicated times. Shown are the mean and SD from three independent experiments.

Source data are available online for this figure.

the *mgtC* and *mgtB* mRNA amounts are very low in wild-type *Salmonella* grown in 10 mM Mg^{2+} (Fig 4B) and in a *phoP* mutant grown in 10 μM Mg^{2+} (Fig 4C). By contrast, significant amounts of *cigR* mRNA were produced in these strains and growth conditions (Fig 4B and C).

We identified a PhoP-independent *cigR* promoter (Figs 4A and EV5A) which, when mutated, resulted in a strain in which *cigR* transcription was like *mgtC*'s (i.e., strictly dependent on growth in 10 μM Mg^{2+}; Fig EV5B). These results imply that the CigR protein is present in wild-type *Salmonella before* the onset of *mgtC*-inducing conditions. Taken together with the genetic (Figs 2 and 3A–C) and biochemical (Figs 1, and 3D and E) data presented above, these results suggest that inhibition of the F_1F_o ATP synthase and stabilization of the PhoP protein take place only when MgtC protein amounts exceed those of CigR.

CigR controls MgtC's virulence function at early times inside macrophages

To test the model presented above, we examined CigR and MgtC protein amounts and MgtC-dependent phenotypes in a set of isogenic strains at different times following a switch from non-inducing to *mgtC*-inducing conditions. CigR protein amounts were > 20 times higher than MgtC's at early times after wild-type *Salmonella* experienced *mgtC*-inducing conditions (Figs 4D and E, and EV5C and D). MgtC protein amounts increased dramatically starting at 3 h and exceeded CigR's by 4 h. By contrast, the amounts of the MgtC target AtpB (Lee *et al*, 2013) did not change over the 24-h course of the experiment (Fig 4D and E).

The *cigR* promoter mutant and the mutant lacking the *cigR* coding region exhibited similar ATP levels at 3 h post-switch to *mgtC*-inducing conditions (Figs 5A and Appendix Fig S2). This was also the case for wild-type and *mgtC Salmonella* (Figs 5A and Appendix Fig S2). These results reflect that the *mgtC* gene is barely expressed at this time (Fig 4E) and that *cigR* transcription largely originates from the PhoP-independent promoter (Fig 4A). By 5 h, the ATP levels of the *mgtC* mutant were much higher than those of the *cigR* mutant, which, in turn, were lower than those of the wild-type strain (Fig 5B). The ATP levels of the *cigR* promoter mutant resembled those of the wild-type strain (Fig 5B), likely due to the stronger *mgtC* promoter providing the bulk of *cigR* transcription at 5 h. Thus, MgtC-dependent phenotypes are manifested only when MgtC protein amounts exceed CigR's. As expected, the CigR protein was absent from the *cigR* promoter mutant before the onset of *mgtC*-inducing conditions, mimicking the expression of the MgtC protein in this strain (Fig EV5E).

To test whether initiation of MgtC's virulence program requires that MgtC supersedes the CigR threshold, we investigated the behavior of an *mgtC* mutant harboring a plasmid that transcribed the *mgtC* gene from an inducible promoter. In contrast to wild-type *Salmonella,* in which the MgtC protein is hardly detectable at early times (i.e., 3 h; Fig 4E), MgtC expression in the engineered strain at early times lowered ATP levels and growth under low Mg^{2+} conditions (Appendix Fig S3A and B). Furthermore, the mRNA amounts of the PhoP-activated genes *pagC* and *pgtE* increased at early times in the engineered strain (Appendix Fig S3C and D). These results argue that MgtC can overcome the CigR threshold even at early times when MgtC is expressed from a heterologous (as opposed to its normal) promoter. Investigation of this strain at 6 h revealed that the MgtC-expressing plasmid rescued all four investigated MgtC-dependent phenotypes (Appendix Fig S3E–G).

We determined that the CigR protein also controls MgtC when *Salmonella* is inside macrophages. First, the *cigR* promoter mutant survived slightly better than the wild-type strain both at 5 and 20 h post-internalization (Fig 5C). Second, survival of the *cigR* deletion mutant was significantly higher than that of the *cigR* promoter mutant at 20 h (Fig 5D). And third, the *mgtC* mutant was slightly defective at 5 h (Fig 5C) and very defective at 20 h (Fig 5D). These results reflect that at 5 h post-internalization, the CigR protein is detected in wild-type and *mgtC* strains, but not in the *cigR* promoter mutant (Fig 5E), and that by 20 h, the *cigR* promoter mutant produces CigR at amounts similar to those of the wild-type and *mgtC* strains (Fig 5E). As *cigR* inactivation did not impact macrophage lysis (Fig EV5F), CigR regulates *Salmonella*'s ability to survive and replicate inside macrophages.

Discussion

Pathogens respond to specific signals by regulating precisely when and where virulence genes are expressed. In addition, the manifestation of a pathogenic behavior may be subject to the action of anti-virulence genes (Brown *et al*, 2016). We have now determined that the anti-virulence protein CigR controls the timing of a virulence program by imposing a threshold for the key virulence factor MgtC (Fig 6). CigR achieves this task by outcompeting MgtC for binding to two virulence factors: the F_1F_o ATP synthase (Fig 1D), preventing the MgtC-dependent decrease in ATP levels (Fig 2B), and the master virulence regulator PhoP, resulting in higher mRNA abundance of two PhoP-activated MgtC-dependent genes in the *cigR* null mutant than in wild-type *Salmonella* (Fig 2E). The CigR protein is produced continuously (Fig 4D). Therefore, when an organism first experiences *mgtC*-inducing conditions, the high basal levels of CigR are sufficient to bind MgtC and prevent it from executing its virulence program (Fig 5C–E). In other words, CigR sets a threshold for *Salmonella*'s commitment to a pathway that promotes profound physiological changes.

The MgtC protein is part of a genetic program that, by reducing ATP levels (Lee *et al*, 2013), lowers ribosome production, protein synthesis, and bacterial growth rate (Pontes *et al*, 2016), and is anticipated to increase antibiotic tolerance. We have now determined that execution of this program requires not only the signals that promote expression of the *mgtC* gene, but also that the amounts of the resulting MgtC protein supersede those of the anti-virulence protein CigR (Fig 6). In this way, CigR functions as a gatekeeper that prevents the accidental entry into a low ATP state when *Salmonella* experiences *mgtC*-inducing signals only transiently (Fig 5A and Appendix Fig S2).

Three negative regulators have been implicated in the control of MgtC amounts and activity: the CigR protein, the peptide MgtR, and the anti-sense RNA AmgR. MgtR promotes MgtC degradation and is encoded in the same operon as *mgtC* (Alix & Blanc-Potard, 2008). AmgR is complementary to the *mgtC* portion of the *mgtCBRcigR* polycistronic mRNA (Lee & Groisman, 2010). Whereas *mgtR* and *amgR* are transcribed exclusively in a PhoP-dependent manner, *cigR* is also transcribed independently of PhoP (Fig 4). Because CigR is present *before* MgtC, it controls the onset of the MgtC-dependent

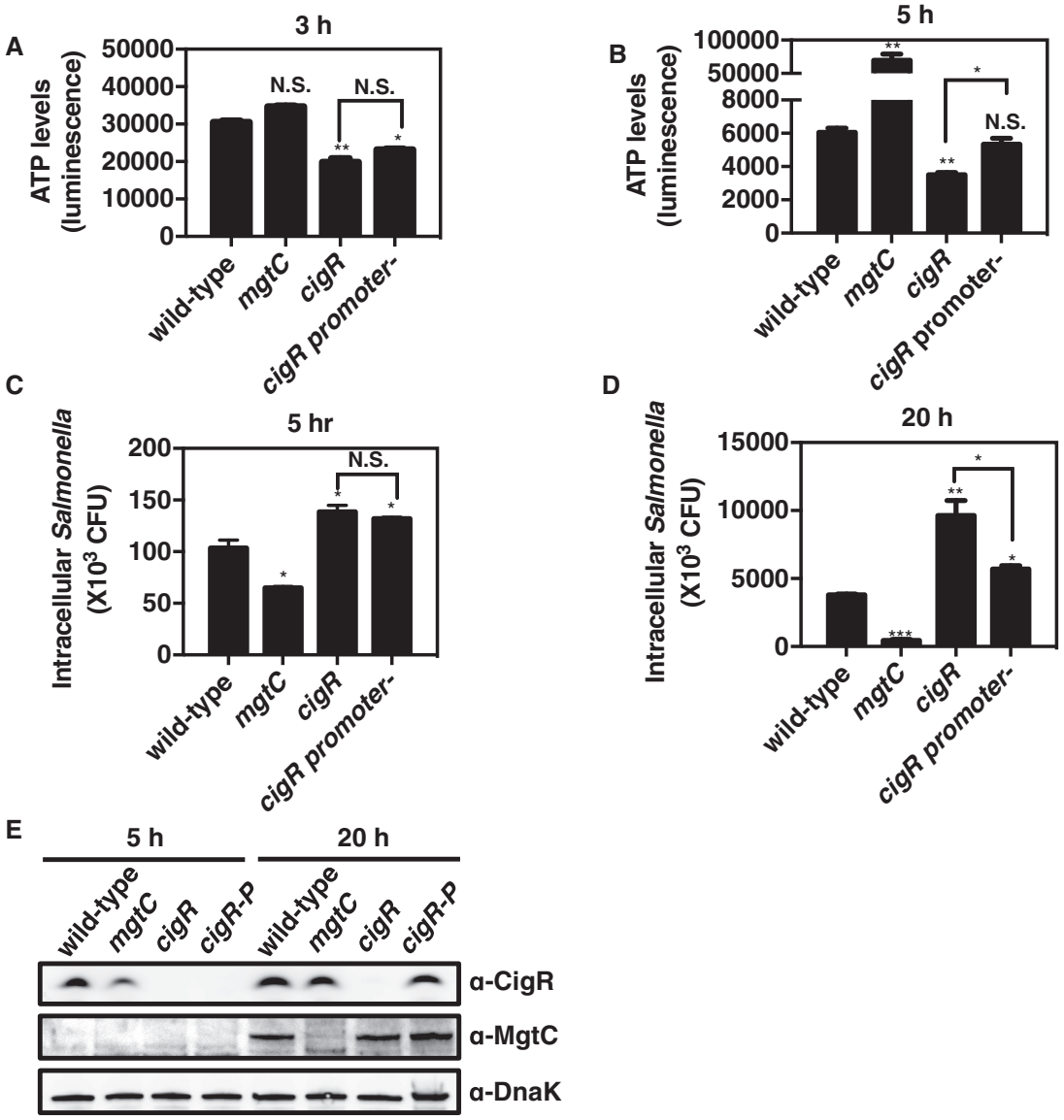

Figure 5. PhoP-independent CigR expression sets threshold for control of MgtC protein.

A, B ATP levels in wild type (14028s), *mgtC* (EL4), *cigR* (JY12), and *cigR* promoter mutant (JY372) *Salmonella* strains following growth in N-minimal media pH 7.7 containing 10 μM $MgCl_2$ for 3 (A) or 5 h (B). ATP levels correspond to luminescence amounts. The mean and SD from three independent experiments are shown.

C, D Survival inside J774A.1 macrophages of the strains listed in (A, B) at 5 (C) or 20 (D) h after infection. The mean and SD from two independent experiments are shown.

E Western blot analysis of crude extracts from wild type (14028s), *cigR* (JY12), *mgtC* (EL4), and *cigR* promoter mutant (JY372) *Salmonella* strains inside J774 A.1 macrophages determined at the indicated times after infection.

Data information: N.S., not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-tailed *t*-test with each sample vs. wild-type or *cigR* mutant vs. *cigR* promoter mutant. N.S., not significant.
Source data are available online for this figure.

activities. This is in contrast to MgtR and AmgR, which, being made after MgtC, control the extent of time MgtC is present.

Our findings illustrate how specific, ordered interactions between virulence and anti-virulence determinants govern the progression of an infection process. That is to say, once MgtC amounts surpass CigR's, MgtC protects the master virulence regulator PhoP from degradation (Yeom *et al*, 2017; Fig 6). This protection enables normal transcription of PhoP-activated genes, including *pcgL* (Fig 2G), which specifies another anti-virulence protein, one that destroys a metabolite promoting pathogen growth in host tissues (Mouslim *et al*, 2002).

The genes specifying the MgtC and CigR proteins are often found in enteric pathogens. By contrast, *Yersinia* and *Mycobacterium* have MgtC but not CigR, whereas *Citrobacter* and *Rahnella* have CigR but not MgtC. In addition, the *mgtC-cigR* operon structure is conserved within *Salmonella* species whereas enteric bacteria specify *mgtC* and

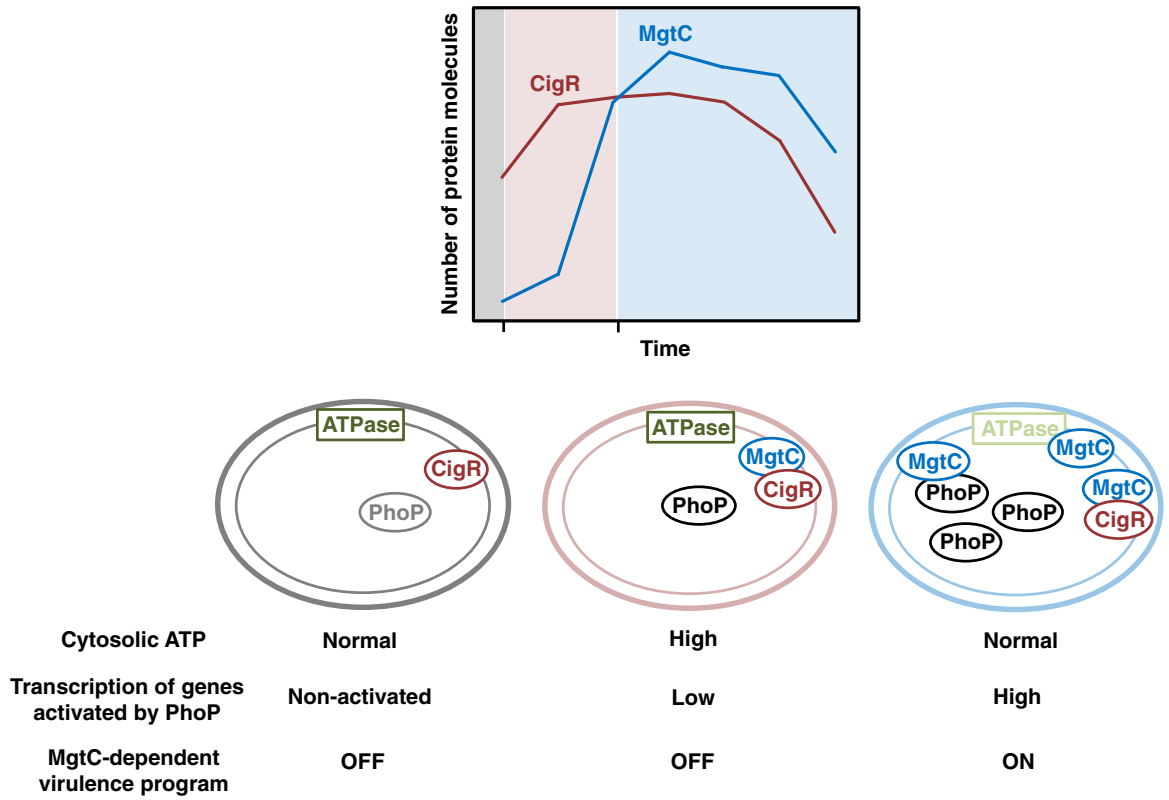

Figure 6. An anti-virulence protein controls the onset of a *Salmonella* virulence program.

Under non-inducing conditions for the master virulence regulator PhoP (gray color), the anti-virulence protein CigR is expressed independently of PhoP, and the virulence protein MgtC is not expressed (left). At early times under inducing conditions (pink color), PhoP promotes expression of MgtC, which, sequestered by CigR, does not inhibit the F_1F_o ATP synthase (ATPase) or protect PhoP from degradation (middle). At late times under inducing conditions (blue color), MgtC amounts supersede CigR amounts, which result in MgtC binding to and inhibition of the ATPase (right). In addition, MgtC protects PhoP from degradation, thereby increasing PhoP amounts and enabling transcription of a subset of PhoP-activated genes (right).

cigR at different parts of the genome. *cigR* is a pseudogene in *S. enterica* serovar Typhi (Parkhill *et al*, 2001), which may advance the MgtC-dependent virulence program in this human-restricted pathogen.

CigR localizes to the *Salmonella* inner membrane (Fig EV1A), binds to the MgtC protein (Fig 1A), which also localizes to the *Salmonella* inner membrane, and mediates all investigated behaviors including replication and control of bacterial pH inside macrophages in an *mgtC*-dependent manner (Fig 2). Therefore, CigR operates within *Salmonella*. This is in contrast to CigR's previously proposed site of action inside host mammalian cells (Niemann *et al*, 2011, 2013), which was based on detection of a reporter based on a fusion protein between adenylate cyclase (CyaA; Sory *et al*, 1995) and CigR in a *Salmonella* mutant lacking control of secretion and over-expressing a regulatory protein (Niemann *et al*, 2011). That the anti-virulence protein CigR operates within *Salmonella* is reminiscent of other anti-virulence gene products, such as PmrA (Choi & Groisman, 2013) and AmgR (Lee & Groisman, 2010), that also function within this pathogen.

Finally, the CigR-MgtC interaction is reminiscent of that established between anti-toxins and toxins implicated in antibiotic tolerance (Gerdes *et al*, 2005). For example, the toxin HipA promotes slow growth and tolerance to antibiotics (Schumacher *et al*, 2015), and the MgtC protein also promotes slow growth by reducing ATP levels (Pontes *et al*, 2016), a condition that results in antibiotic tolerance (Conlon *et al*, 2016; Shan *et al*, 2017). Moreover, HipB binds to HipA, preventing it from exerting its function (Schumacher *et al*, 2015), whereas CigR sequesters MgtC with like effects (Figs 1 and 2).

Materials and Methods

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are presented in Table 1. All *S. enterica* serovar Typhimurium strains are derived from strain 14028s (Fields *et al*, 1986). Phage P22-mediated transductions were carried out as described (Davis *et al*, 1980). DNA oligonucleotides used in this study are presented in Table 2. Bacteria were grown at 37°C in Luria-Bertani broth (LB), N-minimal media (pH 7.7 or pH 4.8; Snavely *et al*, 1991) supplemented with 0.1% casamino acids, 38 mM glycerol, and the indicated concentrations of $MgCl_2$. *Escherichia coli* DH5α was used as the host for preparation of plasmid DNA. Ampicillin was used at 50 μg/ml, chloramphenicol at 25 μg/ml, kanamycin at 50 μg/ml, and tetracycline at 12.5 μg/ml.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source
Escherichia coli		
BTH101	Host strain used for bacterial two-hybrid system	Karimova *et al* (1998)
DH5α	Host strain used for generation and propagation of plasmid constructs	Hanahan (1983)
Salmonella enterica serovar Typhimurium		
14028s	Wild type	Fields *et al* (1986)
EG16539	*mgtC*-FLAG	Lee *et al* (2013)
EL4	Δ*mgtC*	Lee *et al* (2013)
EL5	Δ*mgtB*	Lee *et al* (2013)
EL481	*mgtC*-FLAG/*atpB*-HA	Lee *et al* (2013)
EL549	E84A*mgtC*	This study
EL551	N92T*mgtC*	Lee *et al* (2013)
EL552	C99A*mgtC*	This study
EL553	W226A*mgtC*	This study
JY2	*cigR*-HA	This study
JY6	Δ*mgtC/cigR*	This study
JY12	Δ*cigR*	This study
JY92	*mgtC*-FLAG/*cigR*-HA	This study
JY95	*yqjA*-FLAG/*cigR*-HA	This study
JY139	W133A*cigR*	This study
JY150	D1*cigR*	This study
JY151	D2*cigR*	This study
JY152	D3*cigR*	This study
JY372	*cigR* promoterless	This study
MS7953s	*phoP*::Tn10	Fields *et al* (1989)
YS251	*yqjA*-FLAG	Shi *et al* (2004)
Plasmids		
pCP20	$Rep_{pSC101}{}^{ts}$ I cI857 FLP Amp^R Cm^R	Datsenko and Wanner (2000)
pFPV25.1	Rep_{ColE1} Amp^R *rpsM*::*gfp*mut3	Valdivia and Falkow (1996)
pKD46	$Rep_{pSC101}{}^{ts}$ Amp^R P_{araBAD}-gbexo	Datsenko and Wanner (2000)
pKD3	Rep_{R6Kg} Amp^R FRT Cm^R FRT	Datsenko and Wanner (2000)
pKD4	Rep_{R6Kg} Amp^R FRT Km^R FRT	Datsenko and Wanner (2000)
pKT25	KmR rep_{p15A} (pACYC184 derivative)	Battesti and Bouveret (2012)
pKT25-*zip*	$rep_{pSC101}Km^R$ T25-*zip*	Battesti and Bouveret (2012)
pKT25-*cigR*	$rep_{pSC101}Km^R$ T25-*cigR*	This study
pKT25-*mgtC*	$rep_{pSC101}Km^R$ T25-*mgtC*	This study
pKT25-*pmrB*	$rep_{pSC101}Km^R$ T25-*pmrB*	This study

Table 1 (continued)

Strain or plasmid	Relevant characteristics	Source
pUHE21-2*lacI*^{q}	Rep_{pMB1} *lacI*^{q} Amp^R	Soncini *et al* (1995)
pUHE-MgtC	Rep_{pMB1} *lacI*^{q} Amp^R P_{lac}-*mgtC*	Chamnongpol and Groisman (2002)
pUHE-CigR	Rep_{pMB1} *lacI*^{q} Amp^R P_{lac}-*cigR*	This study
pUHE-CigR-FLAG	Rep_{pMB1} *lacI*^{q} Amp^R P_{lac}-*cigR*-FLAG	This study
pUHE-D1*cigR*-FLAG	Rep_{pMB1} *lacI*^{q} Amp^R P_{lac}-D1*cigR*-FLAG	This study
pUHE-D2*cigR*-FLAG	Rep_{pMB1} *lacI*^{q} Amp^R P_{lac}-D2*cigR*-FLAG	This study
pUHE-D3*cigR*-FLAG	Rep_{pMB1} *lacI*^{q} Amp^R P_{lac}-D3*cigR*-FLAG	This study
pUHE-W133A*cigR*-FLAG	Rep_{pMB1} *lacI*^{q} Amp^R P_{lac}-W133A*cigR*-FLAG	This study
pUT18	Amp^R rep_{pMB1} (pBlueScriptII derivative)	Battesti and Bouveret (2012)
pUT18-*zip*	rep_{pMB1} Amp^R *zip*-UT18	Battesti and Bouveret (2012)
pUT18-*mgtC*	rep_{pMB1} Amp^R *mgtC*-UT18	Yeom *et al* (2017)
pUT18-*pmrB*	rep_{pMB1} Amp^R *pmrB*-UT18	Yeom *et al* (2017)
pUT18-*cigR*	rep_{pMB1} Amp^R *cigR*- UT18	Yeom *et al* (2017)
pUT18-*mgtC-E84A*	rep_{pMB1} Amp^R *mgtC-E84A*-UT18	This study
pUT18-*mgtC-N92T*	rep_{pMB1} Amp^R *mgtC-N92T*-UT18	This study
pUT18-*cigR-D1*	rep_{pMB1} Amp^R *cigR-D1*-UT18	This study
pUT18-*cigR-D3*	rep_{pMB1} Amp^R *cigR-D3*-UT18	This study
pUT18-*cigR-W133A*	rep_{pMB1} Amp^R *cigR-W133A*- UT18	This study

Construction of chromosomal mutants and plasmids

To construct *cigR* mutant strains, a *kan* cassette was introduced in the *cigR* gene as follows: The *kan* fragment was amplified from plasmid pKD4 using primers 13852/13853, then introduced into wild-type 14028s and *mgtC* mutant strains harboring plasmid pKD46 (Datsenko & Wanner, 2000). The resulting strain was kept at 30°C and transformed with plasmid pCP20 to remove the *kan* cassette (Datsenko & Wanner, 2000).

To construct a strain specifying CigR-HA, a *cat* cassette was introduced in the *cigR* gene as follows: A *cat* fragment was amplified from plasmid pKD3 using primers 13861/13862, then introduced into wild-type 14028s, *mgtC*-FLAG, and *yqjA*-FLAG strains harboring plasmid pKD46. The resulting strains were kept at 30°C and transformed with pCP20 to remove the *cat* cassette.

To create the *cigR* promoter mutant strain, we introduced a *tetRA* cassette in the *cigR* promoter region as follows: The *tetRA* fragment

Table 2. Primers used in this study.

No.	Sequence (from 5′ to 3′)
13852	TAATAATCGCCGTGACCACCGCGGTACTGAGCGCGATCAGTGTAGGCTGGAGCTGCTTC
13853	AATATCATGAATAATCGTCGTGGTTTAACCGCCGTCCTGTATGAATATCCTCCTTAGT
13861	GTCACGGCGATTATTAATGGCGTATTTGATTACCCATACGATGTTCCAGATTACGCTTAAGTGTAGGCTGGAGCTGCTTC
13862	TCCAAACTGGCTGCGCCAATAACGCCTGGTTATGAATATCCTCCTTAGT
12595	GTCAGGATCCCATGGAGGAACGTATGTTAAT
12596	GTCATGGTACCCGTTGACTATCAATGCTCCAGT
1388	AAGGTACCCTGATGCGTTTTCAGC
1389	CAGGATCCACGGCGTATTACCCGT
16108	CGCTCTAGAGATGAATAATCGTCGTGGTTTAACCGCCGTCCTGGCGACG
16109	CGCGGTACCCGATCAAATACGCCATTAATAATCGCCGTGACCAC
14721	GGGATACCATGAAAATTTAAGACCCACTTTCACA
14722	AAAATAAGAATCGATGCTAAGCACTTGTCTCCTG
14723	ACGACGATTATTCATGATATTGCCCTTCTGATGCTTATTTTAAGCGGGGCTCGCCAACAGCAGCTTA
14724	TAAGCTGCTGTTGGCGAGCCCCGCTTAAAATAAGCATCAGAAGGGCAATATCATGAATAATCGTCGT
14718	CGCAAGCTTTTACTTGTCATCGTCGTCCTTGTAGTCATCAAATACGCCATTAATAATCGCCGTGACCACCGCG
15088	CGCAGGATCCATGAATAATCGTCGTGGTTTAACCGCCGTCCTGGCGA
15089	CGCAAGCTTTTAATCAAATACGCCATTAATAATCGCCGTGACCACCGCG
12718	CATCATGCGCGCAGGGATGAATGTG
12719	CACATTCATCCCTGCGCGCATGATG
12722	GCGGCAACGCTATGGGCTTCGGCGGGCATCGGC
12723	GCCGATGCCCGCCGAAGCCCATAGCGTTGCCGC
12724	ATATCACCGCAATTCACGCGAGCATTGATAGTCA
12725	TGACTATCAATGCTCGCGTGAATTGCGGTGATAT
14057	GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGGAGGAACGTATGTTAATGTTTCCTTAT
14058	AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTACTTGTCATCGTCGTCCTTGTAGTCTTGACTATCAATGCTCCAGTGAATTGCGGT
14065	GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGAATAATCGTCGTGGTTTAACCGCCGTC
14066	AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTAAGCGTAATCTGGAACATCGTATGGGTAATCAAATACGCCATTAATAATCGCCGTGACCA
14300	GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGGCTTCAGAAAATATGACGCCGCAGGAATACATAGG
14301	AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTAAGCGTAATCTGGAACATCGTATGGGTAATGCTCTTCGGACGCCATCGACAGATAG
15106	CGGCTTAGGGCAGTTCAAAAATGCGCTGGCG
15107	AGTTGCCTGGCGACAGCATAGCTAATGTCC
15108	TTTTGCCCGGCATTTCCTTT
15109	ATTTCGCAGTTGTCGTTGCC
15110	ATGAATAATCGTCGTGGTTTAACCGCCGTCCTGGCGA
15111	TTAATCAAATACGCCATTAATAATCGCCGTGACCACCGCG
15112	ACGCAACACCGTGGCGGTGGTGCGAACC
7530	CAGCCCGCGCACATTC
7531	TTGTCTCTGGGATTGGCTTTCT
7763	TCAGAAAATGATAAGCAGCATAAAAAA
7764	CCCTGACGATGGCTGTTCA
15171	ACGTCGAGTCGGACATTAGC
15172	ATTGTCCCAGCATAGACGCC
4149	ACCGTGGCACAAATGATGCT
4150	TCGGCAATCGCCTTATCTG
15208	GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATATGGCTTCAGAAAATATGACGCCGCAGGAATACATAGG
15209	AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTACTTGTCATCGTCGTCCTTGTAGTCATGCTCTTCGGACGCCATCGACAGATAG

was amplified from strain MS7953s using primers 14721/14722. Then, the PCR product was used to electroporate strain 14028s harboring plasmid pKD46. The resulting strain containing the *tetRA* cassette was kept at 30°C. Then, the *tetRA* cassette in the *cigR* promoter region was replaced by annealed oligonucleotides (14723/14724). These oligonucleotides were used to electroporate strain p*cigR::tetRA* harboring pKD46, and the bacterial suspension was plated on media containing fusaric acid and incubated at 42°C to select against the *tetRA* genes (Maloy & Nunn, 1981).

Plasmids expressing CigR and CigR derivatives were constructed as follows: The *cigR* gene was amplified using primers 15088/15089 for *cigR* and 15088/14718 for *cigR*-FLAG and then introduced between the *BamH*I and *Hind*III sites of plasmid pUHE21-2*lacI*^{q} (Soncini *et al*, 1995).

Western blot analysis

Cells were grown in N-minimal medium containing 10 μM or 10 mM Mg^{2+}. Crude extracts were prepared in B-PER reagent (Pierce) with 100 μg/ml lysozyme and EDTA-free protease inhibitor (Roche). Samples were loaded onto 4–12% NuPAGE gels (Life Technologies). Then, samples were analyzed by Western blotting using anti-HA or anti-FLAG antibodies at 1:2,000 dilution. Mouse anti-GroEL or anti-DnaK antibodies (Abcam) at 1:5,000 dilution were used as control. Secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse antisera (GE healthcare) were used at 1:5,000 dilution. The blots were developed with the Amersham ECL Western Blotting Detection Reagents (GE Healthcare) or SuperSignal West Femto Chemiluminescent system (Pierce).

Pull-down assay with proteins synthesized using an *in vitro* transcription/translation system

Proteins were produced using the cell-free PURExpress *in vitro* protein synthesis system (NEB) in the presence of 60 μg/ml proteoliposomes at 37°C for 2 h. Proteoliposomes were prepared using soybean L-α-phosphatidylcholine (Sigma) in buffer (20 mM Tricine, 20 mM succinic acid, 80 mM NaCl, and 0.6 mM KOH, adjusted to pH 8.0) to a concentration of 32 mg/ml as described (Kuruma *et al*, 2012). DNA templates for *mgtC*-FLAG, *cigR*-HA, *atpB*-HA, and *cigR*-HA domain mutants were used to synthesize the corresponding proteins according to the manufacturer's instructions. DNA templates were made with primers 14057/14058 for *mgtC*-FLAG, 14300/14301 for *atpB*-HA, and 14065/14066 for *cigR*-HA. Each protein was synthesized separately. At the end of the reaction, samples were diluted in TBS (Tris-buffered saline) buffer 20-fold. Diluted samples were mixed in 500 μl TBS and incubated at 4°C for 2 h. Then, samples were immunoprecipitated with antibodies directed to the HA or FLAG epitopes at 4°C for 2 h and analyzed by Western blotting using the same antibodies.

In vivo pull-down assay

The interaction between the MgtC and CigR proteins was investigated using strain JY92, which expresses the FLAG-tagged *mgtC* gene and HA-tagged *cigR* gene from the normal chromosomal locations. Cells were grown overnight in N-minimal media containing 10 mM Mg^{2+}. One milliliter of the overnight culture was washed in N-minimal media without Mg^{2+} and resuspended in 1 ml of the same media. 1/100 dilution of bacteria was used to inoculate 25 ml of N-minimal media containing 10 μM Mg^{2+} and grown for 5 h. Crude extracts were prepared as described above and incubated with anti-HA magnetic beads (Pierce) or anti-FLAG magnetic beads (Sigma) at 4°C overnight. After washing the beads, bound proteins were eluted in 100 μl SDS sample buffer and separated on 4–12% SDS–polyacrylamide gel and analyzed by Western blotting using antibodies directed to the HA or FLAG epitopes.

In vitro and *in vivo* competition assays

CigR-HA, AtpB-HA, and MgtC-FLAG proteins were synthesized using the PURExpress *in vitro* synthesis system (Promega) per the manufacturer's protocol. To measure the concentrations of the CigR-HA, AtpB-HA, and MgtC-FLAG proteins, known amounts of purified CigR-HA, AtpB-HA, and MgtC-FLAG proteins were run on the same gel and used as standards. Standard curves calculated from purified proteins in the same blots used for calculation of molecular concentrations and binding affinities of the CigR-HA, AtpB-HA, and MgtC-FLAG proteins. All proteins were dissolved in 500 μl TBS and incubated at room temperature for 2 h. Then, samples were pulled down with antibodies directed to the HA or FLAG epitopes at room temperature for 2 h. Proteins were then electrotransferred onto nitrocellulose membrane (iBlot; Life Technologies) following the manufacturer's protocol, detected by immunoblotting using monoclonal antibodies directed to the HA or FLAG epitopes, and a secondary antibody, horseradish peroxidase-conjugated anti-mouse IgG fragment (GE). All proteins were visualized by the Supersignal West Femto Chemiluminescent Substrate (Thermo Scientific) and LAS-4000 (FujiFilm). K_m and IC_{50} were calculated using Prism (Graphpad, ver. 7). The densities of protein bands were determined by quantification using the ImageJ program (NIH, ver. 1.49u). The amounts of CigR-HA, AtpB-HA, and MgtC-FLAG proteins were then calculated from the standard curve derived from serial dilutions of purified protein standards run on the same gel (see Fig EV3A and F). For the *in vivo* competition assay, bacteria were grown in N-minimal media pH 7.7 containing 10 μM $MgCl_2$ and IPTG (0.01, 0.1, and 1 mM) for 6 h. Samples were analyzed immunoblotting using antibodies directed to the HA or FLAG epitopes.

Intramacrophage survival assay

The murine-derived macrophage cell line J774A.1 was cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% FBS (Life Technologies) at 37°C under 5% CO_2. Confluent monolayers were prepared in 24-well tissue culture plates. Each well of a 24-well plate was seeded with 5×10^5 cells suspended in DMEM/10% FBS and incubated at 37°C under 5% CO_2 for 20 h. Bacteria were grown in Luria-Bertani (LB) media at 37°C for 16 h. Bacterial cells were washed two times with PBS, suspended in pre-warmed DMEM, and then added to the cell monolayer at a multiplicity of infection (MOI) of 10. Following 30-min incubation, the wells were washed three times with pre-warmed Dulbecco's phosphate-buffered saline (DPBS; Life Technologies) to get rid of extracellular bacteria and then incubated with pre-warmed

medium supplemented with 120 μg/ml gentamicin for 1 h to kill the remaining extracellular bacteria. Then, the wells were washed three times with DPBS incubated with pre-warmed medium supplemented with 10 μg/ml gentamicin. Bacteria were collected at 5 and 20 h post-infection. Wells were washed two times with DPBS to remove extracellular bacteria. Then, 1 ml DPBS and 0.1% Triton X-100 was added to the wells, and following a 10-min incubation, serial dilutions of bacteria were plated on LB agar plates and incubated at 37°C overnight to determine the number of colony forming units.

Measurement of ATP levels

Adenosine triphosphate levels were measured using a microplate reader (Tecan, Infinite M1000 PRO) with modification of a described protocol (Pontes *et al*, 2015). Briefly, bacteria were grown in N-minimal media containing 10 mM Mg^{2+} overnight. One milliliter of the overnight culture was washed three times in N-minimal medium without Mg^{2+} and resuspended in 1 ml of the same media. Diluted (1/50) bacteria were inoculated in 1 ml of N-minimal media containing 10 μM Mg^{2+} and grown for 4 h. Cells were normalized by OD_{600} and heated at 70°C for 10 min. Intracellular ATP was measured using the BacTiter-Glo Microbial Cell Viability Assay Kit (Promega) according to the manufacturer's instructions. ATP levels (picomoles per milliliter of cells at given OD_{600}) were obtained using as reference standards of known concentration.

Measurement of ATP hydrolysis

Inverted vesicles were prepared as described (Suzuki *et al*, 2007). The ATP hydrolysis reaction was started by adding 1 mM ATP and phosphate release monitored at absorbance 360 nm using EnzChek Phosphate Assay Kit (Life Technologies) according to the manufacturer's instructions. Average of hydrolysis rates in a time period from 1 to 5 min after initiation was calculated and presented as amount of phosphate (μmol) released per min. As a control, ATP was omitted from the reaction.

Measurement of *Salmonella* pH when inside macrophages

pH was measured with green fluorescent protein as an indicator (Kneen *et al*, 1998; Olsen *et al*, 2002; Wilks & Slonczewski, 2007). Bacteria harboring a plasmid containing the *gfp* gene expressed from a vector promoter (pFPV25.1) were grown in N-minimal media pH 7.7 containing 10 μM $MgCl_2$ for 5 h. Cells were normalized by OD_{600} values and resuspended in 150 μl of PBS in a 96-well black microplate (PerkinElmer). Excitation spectra were measured at 30°C in 500 nm (slit width, 10 nm), using an emission wavelength of 545 nm (slit width, 10 nm) by a microplate reader (Tecan, Infinite M1000 PRO). To measure *Salmonella*'s pH when inside J774A.1 macrophages, cells were seeded in 96-well black microplates (PerkinElmer) in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum at a density of 5×10^5 per well 24 h prior to infection with *Salmonella* harboring plasmid pFPV25.1. Bacteria were grown overnight in LB broth at 37°C, washed with PBS, and used to infect macrophages at a multiplicity of infection of 50:1. At 6 h post-infection, infected macrophages were resuspended in 150 μl of PBS with 20 mM sodium benzoate for standard curve. A standard curve was determined for green fluorescent protein by measuring fluorescence of samples resuspended in the same buffer at pH 4.8, 5.8, 6.5, 7.5, or 7.7 with addition of 20 mM sodium benzoate, an acid that equilibrates cytoplasmic pH with external pH (Wilks & Slonczewski, 2007). After measuring the spectra, infected macrophages were lysed by adding 0.1% Triton X-100 and serial dilutions of bacteria were plated on LB agar plates and incubated at 37°C overnight.

Quantitative RT–PCR

To measure mRNA amounts, bacteria were grown in N-minimal medium containing 10 μM or 10 mM Mg^{2+} for 4 h. Total RNA was purified by using RNeasy Kit (Qiagen) with on-column DNase treatment, and cDNA was synthesized by using VILO Super Mix (Life Technologies). 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 mRNA was determined using a standard curve obtained by PCR with serially diluted genomic DNA, and results were normalized to the amounts of the *rpoD* gene. The mRNA amounts of the *mgtC*, *cigR,* and *rpoD* genes were measured using the following primer pairs (*mgtC*, 7530/7531; *mgtB*, 7763/7764; *cigR*, 15171/15172; and *rpoD*, 4149/4150). Data shown are an average from at least two independent experiments.

RNA polymerase ChIP-sequencing

Wild-type *Salmonella* (14028s) was grown overnight in N-minimal medium (pH 7.7) supplemented with 10 mM $MgCl_2$. Cultures were washed three times with N-minimal medium without $MgCl_2$ and resuspended in a volume to give the same initial optical density (OD_{600}). Samples were diluted 1:50 in N medium (pH 7.7) containing 10 or 50 μM $MgCl_2$ and grown in a shaking water bath at 250 rpms and 37°C for 4 h ($OD_{600} \approx 0.34$–0.4). ChIP assays were performed as described (Shin & Groisman, 2005; Pontes & Groisman, 2018), with the following modifications. Following formaldehyde cross-linking, samples were quenched with 220 mM glycine. Immunoprecipitation was performed with MagnaChip Protein A/G Magnetic Beads (Millipore) complexed with a monoclonal antibody directed against RpoC, the β' subunit of the *E. coli* RNA polymerase (RNAP; NeoClone). Purified immunoprecipitated DNA samples were submitted for library construction and high-throughput sequencing at the Yale Center for Genomic Analysis. Assembly of sequenced fragments was performed using CLC Genomics Workbench software.

Reverse transcriptase–PCR

An overnight culture of wild-type *Salmonella* was diluted 1:50 into 1 ml of N-minimal medium (pH 7.7) containing 10 μM, 50 μM, or 10 mM $MgCl_2$ and grown at 37°C for 4 h. Total RNA was isolated using the RNeasy Kit (Qiagen) with on-column DNase treatment. Reverse transcription was performed with the ThermoScript™ RT–PCR System (Life Technologies) following the manufacturer's instructions. Primers 15106/15107 were used for amplification of *mgtC_cigR*, 15108/15109 for *mgtB_cigR*, 15110/15111 for *cigR*, and 15106/15112 for *mgtC_mgtB*. PCR was performed as follows: 5 min

initial denaturation at 95°C, 27 repetitions of 30 s at 95°C, 0.5~5 min at 57°C, 30 s at 68°C, and a final amplification of 5 min at 68°C. To exclude the possibility of genomic DNA contamination contributing to the measured values, control reactions were performed without reverse transcriptase.

Amounts of *Salmonella* proteins inside macrophages

The murine-derived macrophage-like cell line J774A.1 was cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% FBS (Life Technologies) at 37°C under 5% CO_2. Confluent monolayers for infection with bacteria were prepared in 12-well tissue culture plates. Each well was seeded with 10^6 (12-well plate) cells suspended in DMEM/10% FBS and incubated at 37°C under 5% CO_2 for 5 or 20 h. Bacterial cells were washed two times with DPBS, suspended in pre-warmed DMEM, and then added to the cell monolayer at a multiplicity of infection (MOI) of 10. After washing with DPBS, cells were lysed with 1 ml/well of cell lysis solution [0.1% (w/v) SDS, 1% (v/v) acidic phenol, and 19% (v/v)] ethanol in double distilled water for 30 min. The cell lysates from two plates were pooled and centrifuged at 5,000 × *g* for 20 min. Pellets were washed twice with DPBS and resuspended in 100 μl of 100 mM NH_4 HCO_3, pH 8.4. Recovered *Salmonella* were subsequently analyzed by Western blot (Shi *et al*, 2006).

Bacterial two-hybrid analysis to examine protein–protein interactions

We used the BACTH system (Battesti & Bouveret, 2012) with the following constructs: the *mgtC, cigR, pmrB, mgtC* derivatives, and domain mutated *cigR* genes were PCR-amplified, and the PCR fragments were cloned between the *Xba*I and *Kpn*I sites of the pUT18/pUT18C vectors and pKT25 to generate genes specifying the corresponding fusion proteins. Recombinant plasmids carrying the *mgtC* and *cigR* genes were co-transformed into strain BTH101. Transformants were plated on LB agar plates containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml) and incubated at 30°C for 24 h. To quantify the interaction between hybrid proteins, bacteria were grown at 30°C overnight as recommended in the BACHT protocol (Battesti & Bouveret, 2012) in LB Amp Kan liquid medium supplemented with 0.5 mM IPTG. All samples were spotted onto LB agar plates or measured β-galactosidase activity supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), X-Gal (40 μg/ml), and IPTG (0.5 mM). Values of β-galactosidase activity are normalized by absorbance (OD_{595}).

Subcellular localization of the MgtC and CigR proteins

Wild-type (14028s), MgtC-N92T (EL551), CigR-HA (JY6), and wild-type harboring pUHE-*cigR*-FLAG, pUHE-D3*cigR*-FLAG, and pUHE-W133A*cigR*-FLAG were grown in N-minimal medium, pH 7.7, with 10 mM $MgCl_2$ overnight. Then, bacteria were washed three times with N-minimal medium, pH 7.7, without $MgCl_2$. Cells were diluted 1:100 in 25 ml of N-minimal medium, pH 7.7, with 10 μM $MgCl_2$, and grown at 37°C with/without 100 μM IPTG for 5 h. Cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C. Cells were washed once with PBS and resuspended in 4 ml of PBS containing sucrose (20%) and lysozyme (100 μg/ml). The supernatant fraction was filtered using a 0.22-μm PVDF filter and then concentrated by precipitation with tricloroacetic acid. After 30 min on ice, cells were disrupted by sonication or prench press. Cell debris was removed by centrifugation at 4,000 × *g* for 15 min, and the whole-cell lysate was loaded on top of a sucrose gradient made with 4 ml each of 60 and 70% sucrose in a Beckman Ultra-Clear centrifuge tube followed by centrifugation in an SW40 rotor at 250,000 × *g* at 4°C for 20 h. Bands between 20 and 60% (upper, reddish band) and between 60 and 70% (lower, white band) sucrose, corresponding to the inner and outer membranes, respectively, were collected and dialyzed against PBS. Protein concentrations were determined by a BCA method, with bovine serum albumin used as a standard protein. NADH oxidase activity was used as a marker for inner membrane purity. Inner and outer membrane preparations (10 μg of protein each) were run in 4–12% NuPAGE gel (Life Technologies), transferred onto a nitrocellulose membrane, and developed with an anti-HA antibody (Sigma), an anti-MgtC, an anti-FLAG (Sigma), an anti-rabbit immunoglobulin G horseradish peroxidase-linked antibody (GE Healthcare), and the ECL detection system (Amersham Biosciences).

Determination of MgtC, CigR, and AtpB proteins amounts

An overnight culture of wild-type (14028s) and *cigR* promoter mutant (JY372) *Salmonella* were grown in N-minimal medium at pH 7.7 with 10 mM $MgCl_2$, washed twice with N-minimal medium containing no Mg^{2+}, and used to inoculate 50 ml of N-minimal medium containing 10 μM $MgCl_2$ at a 1:50 dilution. 0.5 ml of each culture was taken at the indicated time points, except for the 2.5 h samples (when 1.5 ml was taken), and 100 μl B-PER solution was added. Triton X-100 (final 0.5%; Sigma) was added to the total cell lysates to solubilize the membrane, and the lysates were incubated at room temperature for 30 min. Known amounts of purified MgtC-FLAG, CigR-FLAG, and AtpB-FLAG proteins were run on the same SDS–PAGE and used as standards.

For purification of the MgtC-FLAG and CigR-FLAG proteins, overnight cultures of EG16539 or wild-type *Salmonella* (14028s) harboring pUHE-CigR-FLAG were used to inoculate N-minimal medium at pH 7.7 with 10 mM $MgCl_2$ media. Cells were grown at 37°C to logarithmic phase ($OD_{600} \approx 0.5$), and the CigR protein was induced by addition of 0.2 mM of IPTG at 30°C for an additional 5 h. Cells were collected, washed once with 10 mM Tris–HCl (pH 8.0), and resuspended in a solution containing 20 mM Tris–HCl (pH 8.0), 20% sucrose, 5 mM M EDTA, 20 mM $MgCl_2$ (final concentration), and 150 μg/ml lysozyme. After a 30-min incubation at 4°C, cells were centrifuged at 18,000 × *g* at 4°C for 20 min. Cells were resuspended in 10 mM Tris–HCl (pH 8.0) and 10 mM $MgCl_2$, subjected to sonication, and membranes were collected by centrifugation for 1 h at 25,000 × *g* at 4°C. Isolated membranes were solubilized in 1× binding buffer [50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 0.1% n-dodecyl α-D-maltoside (DM; Sigma)] on ice for 1 h. Solubilized proteins were recovered by centrifugation at 25,000 × *g* at 4°C for 1 h and applied to FLAG® M Purification Kit (Sigma) per the manufacturer's protocol. Finally, the eluate was exchanged with TKM buffer [10 mM KCl, 10 mM Tris–Cl (pH 7.5), 1 mM $MgCl_2$], followed by TKM buffer containing 50% glycerol, and concentrated using Amicon Ultra-3 (MW 3,000; Millipore). For purification of the

AtpB-FLAG protein, the AtpB-FLAG was produced from a DNA template (primers 15208/15209) using the PURExpress system (Promega) per the manufacturer's protocol. Purification steps were the same for the MgtC-FLAG and CigR-FLAG proteins.

Proteins were electrotransferred onto a nitrocellulose membrane (iBlot; Life Technologies) following the manufacturer's protocol, detected by immunoblotting using antiserum against MgtC or CigR and the secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG fragment (GE). Both MgtC and CigR proteins were visualized by the Supersignal West Femto Chemiluminescent Substrate (Thermo Scientific) using LAS-4000 (FujiFilm). The densities of protein bands were determined by quantification with ImageJ program (NIH, ver. 1.49u). The amounts of MgtC, CigR, and AtpB proteins were then calculated from the standard curve derived from a serial dilution of the purified MgtC-FLAG, CigR-FLAG, and AtpB-FLAG protein standards run on the same gel (see Figs 4E and EV5D).

Macrophage cell death assay

The percentage of macrophage death was determined by measuring the release of host cytoplasmic lactate dehydrogenase (LDH). At 20 h after infection, 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 multi-detector (Tecan, Infinite M1000 PRO), 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 by 2% Triton X-100.

Expanded View for this article is available online.

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Author contributions
JY and EAG designed the experiments. JY performed the majority of the experiments. MHP performed the RNA polymerase ChIP-sequencing experiment. JC performed the *in vivo* co-immunoprecipitation experiment. JY and EAG analyzed the data, and JY and EAG discussed the data. JY and EAG wrote the manuscript, and MHP commented on the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

References

Alix E, Blanc-Potard A-B (2008) Peptide-assisted degradation of the *Salmonella* MgtC virulence factor. *EMBO J* 27: 546 – 557

Alpuche Aranda CM, Swanson JA, Loomis WP, Miller SI (1992) *Salmonella typhimurium* activates virulence gene transcription within acidified macrophage phagosomes. *Proc Natl Acad Sci USA* 89: 10079 – 10083

Battesti AL, Bouveret E (2012) The bacterial two-hybrid system based on adenylate cyclase reconstitution in *Escherichia coli*. *Methods* 58: 325 – 334

Belon C, Gannoun-Zaki L, Lutfalla G, Kremer L, Blanc-Potard A-B (2014) *Mycobacterium marinum* MgtC plays a role in phagocytosis but is dispensable for intracellular multiplication. *PLoS One* 9: e116052

Bijlsma JJE, Groisman EA (2005) The PhoP/PhoQ system controls the intramacrophage type three secretion system of *Salmonella enterica*. *Mol Microbiol* 57: 85 – 96

Blanc-Potard AB, Groisman EA (1997) The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival. *EMBO J* 16: 5376 – 5385

Blanc-Potard AB, Solomon F, Kayser J, Groisman EA (1999) The SPI-3 pathogenicity island of *Salmonella enterica*. *J Bacteriol* 181: 998 – 1004

Bogan AA, Thorn KS (1998) Anatomy of hot spots in protein interfaces. *J Mol Biol* 280: 1 – 9

Brown NA, Urban M, Hammond-Kosack KE (2016) The trans-kingdom identification of negative regulators of pathogen hypervirulence. *FEMS Microbiol Rev* 40: 19 – 40

Buchmeier NA, Heffron F (1991) Inhibition of macrophage phagosome-lysosome fusion by *Salmonella typhimurium*. *Infect Immun* 59: 2232 – 2238

Chamnongpol S, Groisman EA (2002) Mg^{2+} homeostasis and avoidance of metal toxicity. *Mol Microbiol* 44: 561 – 571

Choi J, Groisman EA (2013) The lipopolysaccharide modification regulator PmrA limits *Salmonella* virulence by repressing the type three-secretion system Spi/Ssa. *Proc Natl Acad Sci USA* 110: 9499 – 9504

Choi J, Groisman EA (2016) Acidic pH sensing in the bacterial cytoplasm is required for *Salmonella* virulence. *Mol Microbiol* 101: 1024 – 1038

Coburn B, Grassl GA, Finlay BB (2007) *Salmonella*, the host and disease: a brief review. *Immunol Cell Biol* 85: 112 – 118

Conlon BP, Rowe SE, Gandt AB, Nuxoll AS, Donegan NP, Zalis EA, Clair G, Adkins JN, Cheung AL, Lewis K (2016) Persister formation in *Staphylococcus aureus* is associated with ATP depletion. *Nat Microbiol* 1: 16051

Cunningham ML, Titus RG, Turco SJ, Beverley SM (2001) Regulation of differentiation to the infective stage of the protozoan parasite *Leishmania major* by tetrahydrobiopterin. *Science* 292: 285 – 287

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: 6640 – 6645

Davis RW, Botstein D, Roth JR (1980) *Advanced bacterial genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory

Eriksson S, Lucchini S, Thompson A, Rhen M, Hinton JCD (2003) Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol Microbiol* 47: 103 – 118

Fabrega A, Vila J (2013) *Salmonella enterica* Serovar Typhimurium skills to succeed in the host: virulence and regulation. *Clin Microbiol Rev* 26: 308 – 341

Fields PI, Groisman EA, Heffron F (1989) A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science* 243: 1059 – 1062

Fields PI, Swanson RV, Haidaris CG, Heffron F (1986) Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci USA* 83: 5189 – 5193

Gerdes K, Christensen SK, Løbner-Olesen A (2005) Prokaryotic toxin-antitoxin stress response loci. *Nat Rev Microbiol* 3: 371 – 382

Grabenstein JP, Fukuto HS, Palmer LE, Bliska JB (2006) Characterization of phagosome trafficking and identification of PhoP-regulated genes important for survival of *Yersinia pestis* in macrophages. *Infect Immun* 74: 3727 – 3741

Groisman EA, Chiao E, Lipps CJ, Heffron F (1989) *Salmonella typhimurium phoP* virulence gene is a transcriptional regulator. *Proc Natl Acad Sci USA* 86: 7077 – 7081

Guo L, Lim KB, Gunn JS, Bainbridge B, Darveau RP, Hackett M, Miller SI (1997) Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. *Science* 276: 250 – 253

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 166: 557 – 580

Ionescu M, Baccari C, Da Silva AM, Garcia A, Yokota K, Lindow SE (2013) Diffusible signal factor (DSF) synthase RpfF of *Xylella fastidiosa* is a multifunction protein also required for response to DSF. *J Bacteriol* 195: 5273 – 5284

Jones BD, Falkow S (1996) Salmonellosis: host immune responses and bacterial virulence determinants. *Annu Rev Immunol* 14: 533 – 561

Karimova G, Pidoux J, Ullmann A, Ladant D (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci USA* 95: 5752 – 5756

Kidwai AS, Mushamiri I, Niemann GS, Brown RN, Adkins JN, Heffron F (2013) Diverse secreted effectors are required for *Salmonella* persistence in a mouse infection model. *PLoS One* 8: e70753

Kneen M, Farinas J, Li Y, Verkman AS (1998) Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys J* 74: 1591 – 1599

Kröger C, Colgan A, Srikumar S, Händler K, Sivasankaran SK, Hammarlöf DL, Canals R, Grissom JE, Conway T, Hokamp K, Hinton JCD (2013) An infection-relevant transcriptomic compendium for *Salmonella* enterica Serovar Typhimurium. *Cell Host Microbe* 14: 683 – 695

Kuruma Y, Suzuki T, Ono S, Yoshida M, Ueda T (2012) Functional analysis of membranous Fo-a subunit of F1Fo-ATP synthase by *in vitro* protein synthesis. *Biochem J* 442: 631 – 638

Lee EJ, Groisman EA (2010) An antisense RNA that governs the expression kinetics of a multifunctional virulence gene. *Mol Microbiol* 76: 1020 – 1033

Lee EJ, Groisman EA (2012) Control of a *Salmonella* virulence locus by an ATP-sensing leader messenger RNA. *Nature* 486: 271 – 275

Lee EJ, Pontes MH, Groisman EA (2013) A bacterial virulence protein promotes pathogenicity by inhibiting the bacterium's own F1Fo ATP synthase. *Cell* 154: 146 – 156

Maloy SR, Nunn WD (1981) Selection for loss of tetracycline resistance by *Escherichia coli*. *J Bacteriol* 145: 1110 – 1111

Marcus SL, Brumell JH, Pfeifer CG, Finlay BB (2000) *Salmonella* pathogenicity islands: big virulence in small packages. *Microbes Infect* 2: 145 – 156

Miller SI, Kukral AM, Mekalanos JJ (1989) A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. *Proc Natl Acad Sci USA* 86: 5054 – 5058

Mouslim C, Hilbert F, Huang H, Groisman EA (2002) Conflicting needs for a *Salmonella* hypervirulence gene in host and non-host environments. *Mol Microbiol* 45: 1019 – 1027

Niemann GS, Brown RN, Gustin JK, Stufkens A, Shaikh-Kidwai AS, Li J, McDermott JE, Brewer HM, Schepmoes A, Smith RD, Adkins JN, Heffron F (2011) Discovery of novel secreted virulence factors from *Salmonella enterica* Serovar Typhimurium by proteomic analysis of culture supernatants. *Infect Immun* 79: 33 – 43

Niemann GS, Brown RN, Mushamiri IT, Nguyen NT, Taiwo R, Stufkens A, Smith RD, Adkins JN, McDermott JE, Heffron F (2013) RNA type III secretion signals that require Hfq. *J Bacteriol* 195: 2119 – 2125

Olsen KN, Budde BB, Siegumfeldt H, Rechinger KB, Jakobsen M, Ingmer H (2002) Noninvasive measurement of bacterial intracellular pH on a single-cell level with green fluorescent protein and fluorescence ratio imaging microscopy. *Appl Environ Microbiol* 68: 4145 – 4147

Parkhill J, Dougan G, James KD, Thomson NR, Pickard D, Wain J, Churcher C, Mungall KL, Bentley SD, Holden MT, Sebaihia M, Baker S, Basham D, Brooks K, Chillingworth T, Connerton P, Cronin A, Davis P, Davies RM, Dowd L *et al* (2001) Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature* 413: 848 – 852

Pontes MH, Groisman EA (2018) Protein synthesis controls phosphate homeostasis. *Genes Dev* 32: 79 – 92

Pontes MH, Lee EJ, Choi J, Groisman EA (2015) *Salmonella* promotes virulence by repressing cellulose production. *Proc Natl Acad Sci USA* 112: 5183 – 5188

Pontes MH, Yeom J, Groisman EA (2016) Reducing ribosome biosynthesis promotes translation during low Mg^{2+} stress. *Mol Cell* 64: 480 – 492

Prost LR, Daley ME, Le Sage V, Bader MW, Le Moual H, Klevit RE, Miller SI (2007) Activation of the bacterial sensor kinase PhoQ by acidic pH. *Mol Cell* 26: 165 – 174

Rang C, Alix E, Felix C, Heitz A, Tasse L, Blanc-Potard A-B (2007) Dual role of the MgtC virulence factor in host and non-host environments. *Mol Microbiol* 63: 605 – 622

Rasmussen A, Rasmussen T, Edwards MD, Schauer D, Schumann U, Miller S, Booth IR (2007) The role of tryptophan residues in the function and stability of the mechanosensitive channel MscS from *Escherichia coli*. *Biochemistry* 46: 10899 – 10908

Retamal P, Castillo-Ruiz M, Mora GC (2009) Characterization of MgtC, a virulence factor of *Salmonella enterica* Serovar Typhi. *PLoS One* 4: e5551

Sato Y, Takaya A, Yamamoto T (2011) Meta-analytic approach to the accurate prediction of secreted virulence effectors in gram-negative bacteria. *BMC Bioinformatics* 12: 442

Schumacher MA, Balani P, Min J, Chinnam NB, Hansen S, Vulić M, Lewis K, Brennan RG (2015) HipBA-promoter structures reveal the basis of heritable multidrug tolerance. *Nature* 524: 59 – 64

Senior AE (1990) The proton-translocating ATPase of *Escherichia coli*. *Annu Rev Biophys Chem* 19: 7 – 41

Shan Y, Brown Gandt A, Rowe SE, Deisinger JP, Conlon BP, Lewis K (2017) ATP-dependent persister formation in *Escherichia coli*. *mBio* 8: e02267–16

Shi L, Adkins JN, Coleman JR, Schepmoes AA, Dohnkova A, Mottaz HM, Norbeck AD, Purvine SO, Manes NP, Smallwood HS, Wang H, Forbes J, Gros P, Uzzau S, Rodland KD, Heffron F, Smith RD, Squier TC (2006) Proteomic analysis of *Salmonella enterica* serovar typhimurium isolated from RAW 264.7 macrophages: identification of a novel protein that contributes to the replication of serovar typhimurium inside macrophages. *J Biol Chem* 281: 29131 – 29140

Shi Y, Cromie MJ, Hsu F-F, Turk J, Groisman EA (2004) PhoP-regulated *Salmonella* resistance to the antimicrobial peptides magainin 2 and polymyxin B. *Mol Microbiol* 53: 229 – 241

Shimono N, Morici L, Casali N, Cantrell S, Sidders B, Ehrt S, Riley LW (2003) Hypervirulent mutant of *Mycobacterium tuberculosis* resulting from disruption of the mce1 operon. *Proc Natl Acad Sci USA* 100: 15918 – 15923

Shin D, Groisman EA (2005) Signal-dependent binding of the response regulators PhoP and PmrA to their target promoters *in vivo*. *J Biol Chem* 280: 4089 – 4094

Snavely MD, Miller CG, Maguire ME (1991) The *mgtB* Mg^{2+} transport locus of *Salmonella typhimurium* encodes a P-type ATPase. *J Biol Chem* 266: 815 – 823

Soncini FC, Garcia Vescovi E, Solomon F, Groisman EA (1996) Molecular basis of the magnesium deprivation response in *Salmonella typhimurium*: identification of PhoP-regulated genes. *J Bacteriol* 178: 5092 – 5099

Soncini FC, Vescovi EG, Groisman EA (1995) Transcriptional autoregulation of the *Salmonella typhimurium phoPQ* operon. *J Bacteriol* 177: 4364 – 4371

Sory MP, Boland A, Lambermont I, Cornelis GR (1995) Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the *cyaA* gene fusion approach. *Proc Natl Acad Sci USA* 92: 11998 – 12002

Suzuki T, Ozaki Y, Sone N, Feniouk BA, Yoshida M (2007) The product of *uncI* gene in F1Fo-ATP synthase operon plays a chaperone-like role to assist c-ring assembly. *Proc Natl Acad Sci USA* 104: 20776 – 20781

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: 367 – 378

Wilks JC, Slonczewski JL (2007) pH of the cytoplasm and periplasm of *Escherichia coli*: rapid measurement by green fluorescent protein fluorimetry. *J Bacteriol* 189: 5601 – 5607

Wösten MM, Kox LF, Chamnongpol S, Soncini FC, Groisman EA (2000) A signal transduction system that responds to extracellular iron. *Cell* 103: 113 – 125

Yeom J, Wayne KJ, Groisman EA (2017) Sequestration from protease adatpor confers differential stability to N-end rule substrate. *Mol Cell* 66: 234 – 246

Yin J, Xia J, Tao M, Xu L, Li Q, Geng S, Jiao X (2016) Construction and characterization of a *cigR* deletion mutant of *Salmonella enterica* serovar Pullorum. *Avian Pathol* 45: 569 – 575