Cell

A Bacterial Virulence Protein Promotes Pathogenicity by Inhibiting the Bacterium's Own F_1F_o ATP Synthase

Eun-Jin Lee,[1,2,3,4] Mauricio H. Pontes,[2,3] and Eduardo A. Groisman[1,2,3,*]
[1]Howard Hughes Medical Institute
[2]Department of Microbial Pathogenesis, Boyer Center for Molecular Medicine, Yale School of Medicine, 295 Congress Avenue, New Haven, CT 06536-0812, USA
[3]Yale Microbial Diversity Institute, P.O. Box 27389, West Haven, CT 06516, USA
[4]Department of Genetic Engineering, College of Life Sciences, Kyung Hee University, Yongin 446-701, South Korea
*Correspondence: eduardo.groisman@yale.edu
http://dx.doi.org/10.1016/j.cell.2013.06.004

SUMMARY

Several intracellular pathogens, including *Salmonella enterica* and *Mycobacterium tuberculosis*, require the virulence protein MgtC to survive within macrophages and to cause a lethal infection in mice. We now report that, unlike secreted virulence factors that target the host vacuolar ATPase to withstand phagosomal acidity, the MgtC protein acts on *Salmonella*'s own F_1F_o ATP synthase. This complex couples proton translocation to ATP synthesis/hydrolysis and is required for virulence. We establish that MgtC interacts with the *a* subunit of the F_1F_o ATP synthase, hindering ATP-driven proton translocation and NADH-driven ATP synthesis in inverted vesicles. An *mgtC* null mutant displays heightened ATP levels and an acidic cytoplasm, whereas *mgtC* overexpression decreases ATP levels. A single amino acid substitution in MgtC that prevents binding to the F_1F_o ATP synthase abolishes control of ATP levels and attenuates pathogenicity. MgtC provides a singular example of a virulence protein that promotes pathogenicity by interfering with another virulence protein.

INTRODUCTION

Many intracellular pathogens have the ability to survive within a membrane-bound acidic compartment inside macrophages (Kumar and Valdivia, 2009). Despite their phylogenetic distance, *Salmonella enterica*, *Mycobacterium tuberculosis*, and other bacterial intracellular pathogens rely on the MgtC protein to survive within acidic macrophage phagosomes and to cause a lethal infection in mice (Blanc-Potard and Groisman, 1997; Buchmeier et al., 2000; Grabenstein et al., 2006; Lavigne et al., 2005; Maloney and Valvano, 2006). The *mgtC* gene is often cotranscribed with the Mg^{2+} transporter-specifying *mgtB* gene (Blanc-Potard and Lafay, 2003; Snavely et al., 1991), and inactivation of the *mgtC* gene renders bacteria defective for growth in low Mg^{2+} (Blanc-Potard and Groisman, 1997; Buchmeier et al., 2000; Lavigne et al., 2005; Maloney and Valvano, 2006). However, the MgtC protein is not necessary for Mg^{2+} transport (Moncrief and Maguire, 1998; Tao et al., 1995), and its function has remained unknown. Unlike typical virulence factors, which are secreted and target host proteins, the *Salmonella* MgtC is an integral membrane protein (Rang et al., 2007), suggesting that it might function within the bacterium.

mgtC is the most highly induced horizontally acquired *Salmonella* gene when this pathogen is inside macrophages (Eriksson et al., 2003). This is due to the action of several signals and regulators. On the one hand, transcription initiation from the *mgtC* promoter depends on the PhoP/PhoQ system (Soncini et al., 1996), a major regulator of intramacrophage survival and virulence (Groisman, 2001). On the other hand, transcription elongation into the *mgtCBR* coding region is stimulated by an increase in cytosolic ATP levels detected by the leader portion of the polycistronic *mgtCBR* transcript (Lee and Groisman, 2012a). The ability to modify *mgtC* expression in response to changes in cytosolic ATP levels is required for *Salmonella* virulence (Lee and Groisman, 2012a). This suggests that *Salmonella* might need the MgtC protein to cope with excess cytosolic ATP generated as a result of phagosome acidification.

The F_1F_o ATP synthase is responsible for the synthesis of the majority of ATP in living cells (Harold and Maloney, 1996; Senior, 1990). A functional F_1F_o ATP synthase is critical for intracellular pathogens that remain within a membrane-bound acidic compartment, because inactivation of *atpB*, the gene encoding the F_o *a* subunit of the F_1F_o ATP synthase, attenuated *Salmonella* virulence in mice and chickens (Turner et al., 2003) and also because a novel anti-*M. tuberculosis* drug targets the F_1F_o ATP synthase (Andries et al., 2005).

In this paper, we reveal the mechanism of action of the MgtC virulence protein. We establish that, surprisingly, MgtC targets *Salmonella*'s own F_1F_o ATP synthase to enhance pathogen survival within macrophages. We demonstrate that the MgtC protein inhibits F_1F_o ATP synthase-promoted proton translocation and ATP synthesis to maintain physiological ATP levels and cytoplasmic pH. And we show that a single amino acid

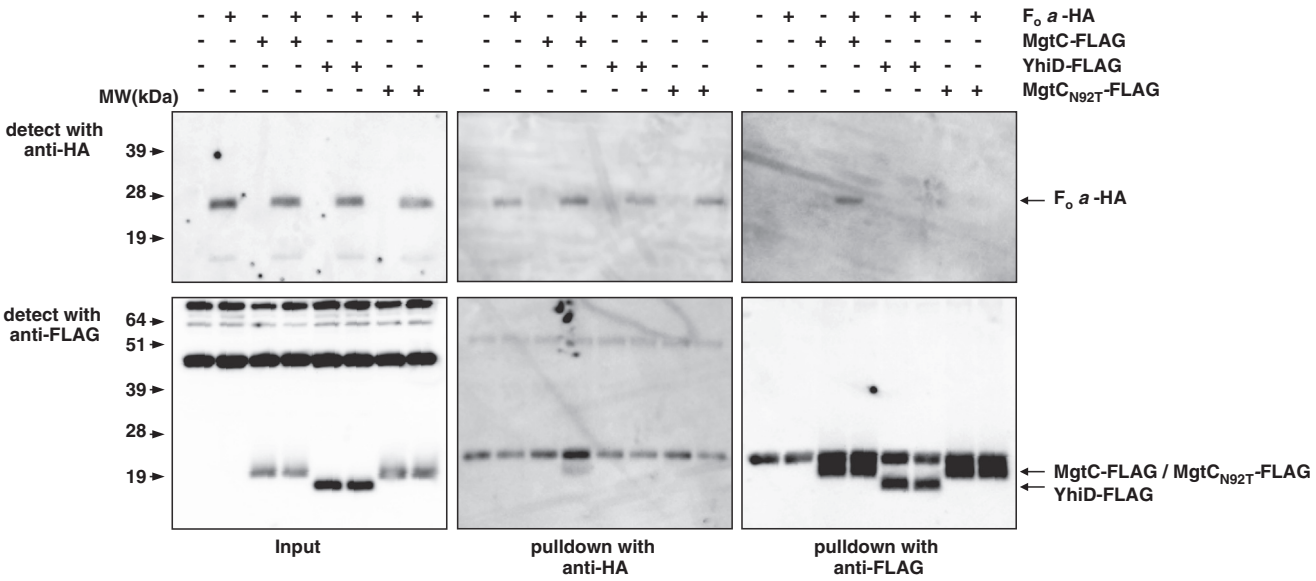

Figure 1. In Vitro Synthesized MgtC Interacts with ATP Synthase F_o *a* Subunit in Proteoliposomes

Western blot analysis of proteoliposomes reconstituted from in vitro synthesized F_1F_o ATP synthase containing F_o *a*-HA and in vitro synthesized MgtC-FLAG, YhiD-FLAG, or MgtC N92T-FLAG proteins. At the end of the reconstitution reaction, an aliquot (input) and fractions immunoprecipitated with either anti-HA or anti-FLAG antibodies were analyzed using anti-HA and anti-FLAG antibodies. Proteoliposomes were prepared as described in Experimental Procedures. The data are representative of two independent experiments, which gave similar results.

See also Figures S1, S2, and S3.

substitution in MgtC that attenuates *Salmonella* pathogenicity prevents MgtC from interacting with and inhibiting the F_1F_o ATP synthase. Our findings suggest that MgtC's virulence role is due, primarily, to its action on the F_1F_o ATP synthase. MgtC provides a singular example of a protein that inhibits the bacterium's own F_1F_o ATP synthase protein, thus differing from classical secreted virulence factors that target host proteins.

RESULTS

MgtC Interacts with the F_o *a* Subunit of the F_1F_o ATP Synthase

To identify potential partners of the MgtC protein, we used anti-FLAG antibodies to pull down proteins crosslinked by the cell permeable crosslinker dithiobis-(succinimidyl propionate) in a *Salmonella* strain deleted for the *mgtC* gene and harboring a plasmid that expressed a C-terminally FLAG-tagged MgtC protein from a derivative of the *lac* promoter. Bacteria were grown in low Mg^{2+} to activate the PhoP/PhoQ system (Groisman, 2001), so as to stimulate the expression of proteins that are normally produced when MgtC is made (Soncini et al., 1996). Three of the seven bands identified using mass spectrometry corresponded to the inner membrane protease FtsH, previously reported to promote MgtC degradation (Alix and Blanc-Potard, 2008), and to HflC and HflK, proteins known to associate with FtsH (Ito and Akiyama, 2005; Figure S1 available online). MgtC was also crosslinked to the Mg^{2+} transporter MgtB, which is normally encoded together with *mgtC* in the *mgtCBR* operon (Blanc-Potard and Groisman, 1997); to the DNA-binding proteins LacI (encoded in the multicopy number plasmid specifying the MgtC-FLAG protein) and PhoP (highly induced in low Mg^{2+} conditions) (Soncini et al., 1996); and to the F_o *a* subunit of the F_1F_o ATP synthase (Figure S1). We pursued the latter interaction because an increase in cytosolic ATP levels promotes transcription of the *mgtC* coding region (Lee and Groisman, 2012a) and because the F_1F_o ATP synthase governs ATP homeostasis (Harold and Maloney, 1996; Senior, 1990).

We verified the interaction between MgtC and the F_o *a* subunit in two sets of independent experiments. First, a C-terminally FLAG-tagged MgtC protein immunoprecipitated a C-terminally hemagglutin (HA)-tagged F_o *a* subunit in membrane extracts prepared from a strain, specifying these two proteins from their normal promoters and chromosomal locations (Figure S2A). Likewise, F_o *a*-HA immunoprecipitated MgtC-FLAG from the same extracts (Figure S2B). This interaction appears to be specific because F_o *a*-HA did not immunoprecipitate the inner membrane C-terminally FLAG-tagged MgtB (Figure S2).

Second, MgtC-FLAG immunoprecipitated F_o *a*-HA when synthesized along with all the subunits of the F_1F_o ATP synthase using the PURExpress in vitro transcription/translation system (Shimizu et al., 2001) and reconstituted into liposomes (Figure 1). Control experiments demonstrated that YhiD-FLAG failed to immunoprecipitate F_o *a*-HA (Figure 1), despite being the *Escherichia coli* protein most similar to the *Salmonella* MgtC protein. Moreover, MgtC-FLAG (but not YhiD-FLAG) immunoprecipitated F_o *a*-HA, even when F_o *a*-HA was expressed and incorporated into liposomes in the absence of other F_1F_o ATP synthase subunits (Figure S3). These experiments demonstrated that the integral membrane MgtC protein can bind to the integral membrane F_o *a* subunit of the F_1F_o ATP synthase.

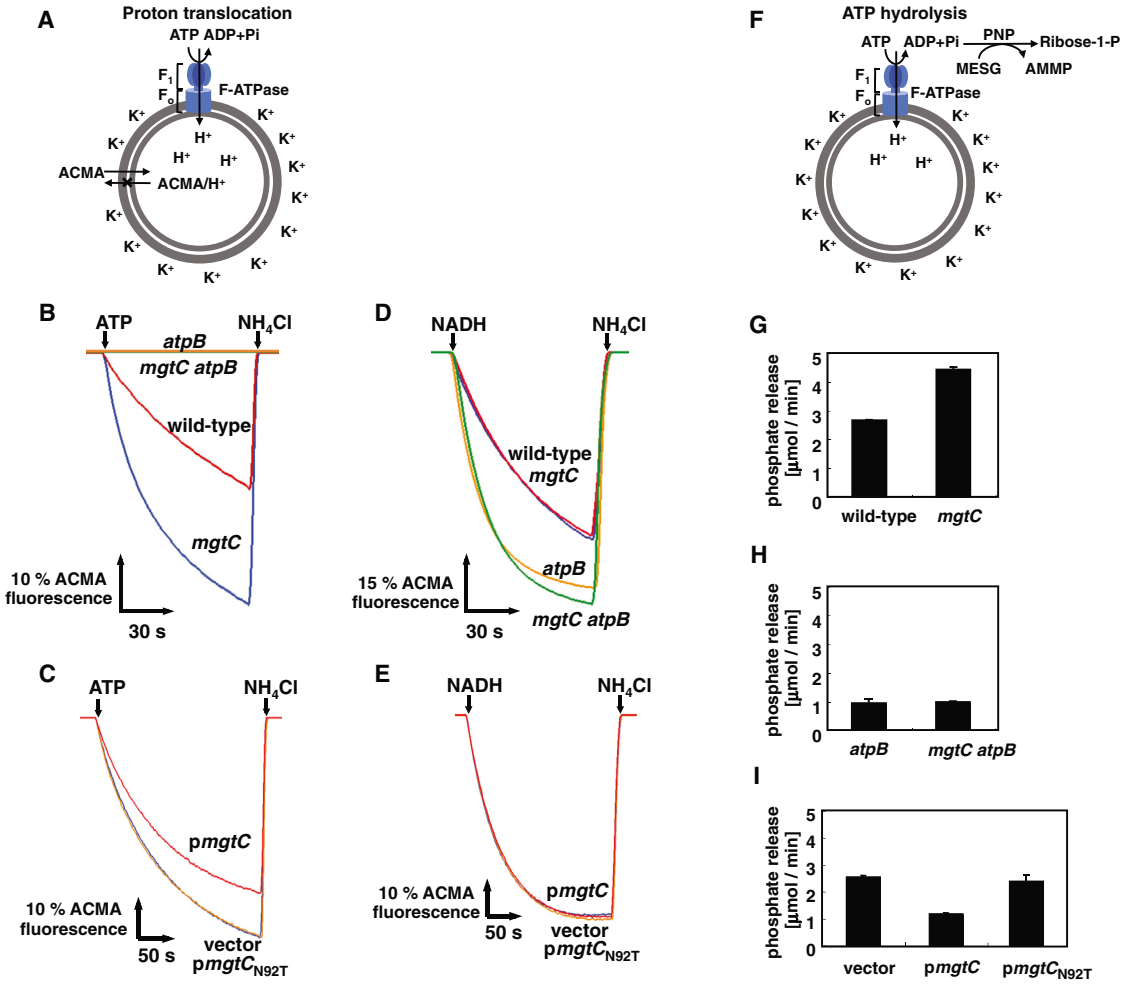

Figure 2. MgtC Inhibits ATP-Coupled Proton Translocation and ATP Hydrolysis in an *atpB*-Dependent Manner

(A) Schematic cartoon of the ATP-driven proton translocation assay (see text for details).

(B–E) Fluorescence quenching of the pH-dependent dye ACMA driven by ATP (B and C) or NADH (D and E) in inverted vesicles prepared from wild-type (14028s), *mgtC* (EL4), *atpB* (MP23), and *mgtC atpB* (MP25) *Salmonella* or wild-type *Salmonella* harboring a plasmid expressing either the wild-type *mgtC* gene or the $mgtC_{N92T}$ variant from a heterologous promoter (p*mgtC* or p$mgtC_{N92T}$) or the plasmid vector (pUHE21-2*lacI*^{q}). Proton-translocation was initiated by adding ATP and terminated by adding NH_4Cl as indicated by the arrow. Percent ACMA fluorescence corresponds to the relative change in fluorescence intensity before adding ATP or NADH when fluorescence was set to 100%.

(F) Schematic cartoon of the ATP hydrolysis assay (see text for details).

(G–I) ATP hydrolysis measured by phosphate release in inverted vesicles prepared from wild-type (14028s), *mgtC* (EL4) (G), *atpB* (MP23), and *mgtC atpB* (MP25) *Salmonella* (H) or wild-type *Salmonella* harboring a plasmid expressing the *mgtC* gene or its derivative from a heterologous promoter (p*mgtC* or p$mgtC_{N92T}$) or the plasmid vector (I). The reaction was initiated by adding ATP and monitored for 5 min, as described in Experimental Procedures.

For (B), (D), (G), and (H), vesicles were prepared from cells grown in 10 µM Mg^{2+} to induce *mgtC* expression from the normal chromosomal location (Soncini et al., 1996), and for (C), (E), and (I), vesicles were prepared from cells grown in 50 µM Mg^{2+} in the presence of 0.25 mM IPTG to induce *mgtC* expression from the heterologous promoter in the plasmid-borne *mgtC*. Data are represented as mean ± SEM.

See also Figures S4 and S5.

MgtC Inhibits the F_1F_o ATP Synthase

The F_1F_o ATP synthase couples the movement of protons (or Na^+ in certain cases) down an electrochemical gradient to the synthesis of ATP (Harold and Maloney, 1996; Senior, 1990), and it re-energizes the membrane by hydrolyzing ATP to pump protons (or Na^+) across the cytoplasmic membrane. Thus, we explored the possibility of the MgtC protein altering one or more activities of the F_1F_o ATP synthase.

We examined ATP-driven proton translocation in inside-out membrane vesicles using the membrane-permeable fluorescence dye 9-amino-6-chloro-2-methoxyacridine (ACMA). Fluorescence quenching occurs when protons pumped into the vesicles through the F_o subunit of the F_1F_o ATP synthase protonate ACMA, generating a nonfluorescent ACMA form (Figure 2A). Thus, the higher the proton translocation, the higher the quenching. Because the MgtC protein binds to the F_o *a* subunit, we

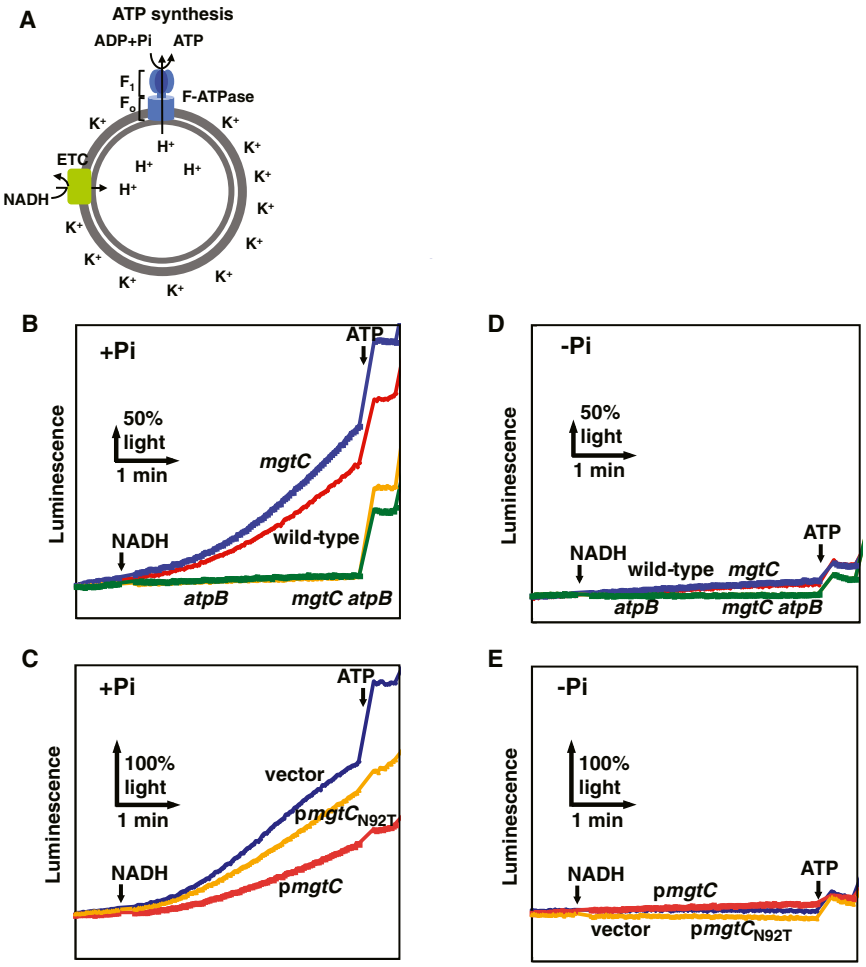

Figure 3. MgtC Inhibits NADH-Driven ATP Synthesis in an *atpB*-Dependent Manner

(A) Schematic cartoon of the NADH-driven ATP synthesis assay (see text for details).

(B–E) NADH-driven ATP synthesis assay in inverted membrane vesicles prepared from wild-type (14028s), *mgtC* (EL4), *atpB* (MP23), and *mgtC atpB* (MP25) *Salmonella* (B and D) or wild-type *Salmonella* harboring a plasmid expressing either the wild-type *mgtC* gene or the $mgtC_{N92T}$ variant from a heterologous promoter (p*mgtC* or p$mgtC_{N92T}$) or the plasmid vector (pUHE21-2*lacI*^{q}) (C and E) in the presence (+Pi) or absence (−Pi) of phosphate. The ATP synthesis reaction was initiated by adding NADH and monitored by the luciferase reaction for 210 s, as described in Experimental Procedures. Light (100% or 50%) corresponds to the initial luminescence intensity before adding NADH. Vesicles were prepared from cells grown as described in Figure 2.

See also Figures S4 and S5.

hypothesized that it might impact proton translocation. Vesicles prepared from the *mgtC* mutant quenched more fluorescence than those from wild-type *Salmonella* (Figure 2B). By contrast, vesicles from bacteria expressing the *mgtC* gene from a multicopy number plasmid quenched less fluorescence than those prepared from an isogenic strain carrying the plasmid vector (Figure 2C). Control experiments showed no proton translocation in vesicles prepared from both *atpB* and *atpB mgtC* mutants, both of which lack the F_o *a* subunit (Figure 2B). Furthermore, mutation of the *mgtC* gene or its expression from a multicopy number plasmid did not impact fluorescence when proton translocation was driven via the electron transport chain by the electron carrier nicotinamide adenine dinucleotide (NADH) (Figures 2D and 2E). Therefore, MgtC binding to the F_o *a* subunit of the F_1F_o ATP synthase compromises proton translocation by the F_1F_o ATP synthase.

Because proton pumping into the vesicles is coupled to ATP hydrolysis (Figure 2F), MgtC interaction with the F_o *a* subunit of the F_1F_o ATP synthase should also impact ATP hydrolysis mediated by the F_1 subunit. Indeed, vesicles prepared from wild-type *Salmonella* released less phosphate than those from the isogenic *mgtC* mutant strain (Figure 2G), and phosphate release was even lower in vesicles from wild-type *Salmonella* harboring the *mgtC*-expressing plasmid (Figure 2I). Control experiments demonstrated that the MgtC effect is dependent on the F_o *a* subunit, because there was no difference in phosphate release between vesicles prepared from an *atpB* single mutant and an *atpB mgtC* double mutant (Figure 2H).

Next, we investigated whether MgtC also inhibited NADH-driven ATP synthesis by monitoring ATP levels using the luciferase reaction (Figure 3A). Vesicles prepared from the *mgtC* mutant produced more ATP than those prepared from the wild-type strain (Figure 3B). By contrast, vesicles prepared from cells expressing the *mgtC* gene in a multicopy number plasmid produced less ATP than those prepared from an isogenic strain harboring the plasmid vector (Figure 3C). ATP synthesis was dependent on the F_1F_o ATP synthase because no ATP was generated in vesicles prepared from the *atpB* single mutant and the *atpB mgtC* double mutant (Figure 3B). As expected, control experiments showed no ATP synthesis when Pi was omitted from the reaction (Figures 3D and 3E). Given that the amount of F_o *a* present in membrane vesicles was not altered upon abrogating or elevating MgtC production (Figure S4), our data indicate that MgtC controls the activity but not the levels of the F_1F_o ATP synthase.

MgtC Is Required for *Salmonella* to Maintain Physiological ATP Levels and Cytosolic pH

To examine the in vivo consequences of MgtC action on the F_1F_o ATP synthase, we investigated a set of isogenic strains following incubation in low Mg^{2+} media to promote normal *mgtC* expression (Soncini et al., 1996). We determined that intracellular ATP levels were ~2.2-fold higher in the *mgtC* mutant than in wild-type *Salmonella* (Figure 4A). This phenotype is due to loss of *mgtC* function (as opposed to resulting from a polar effect on

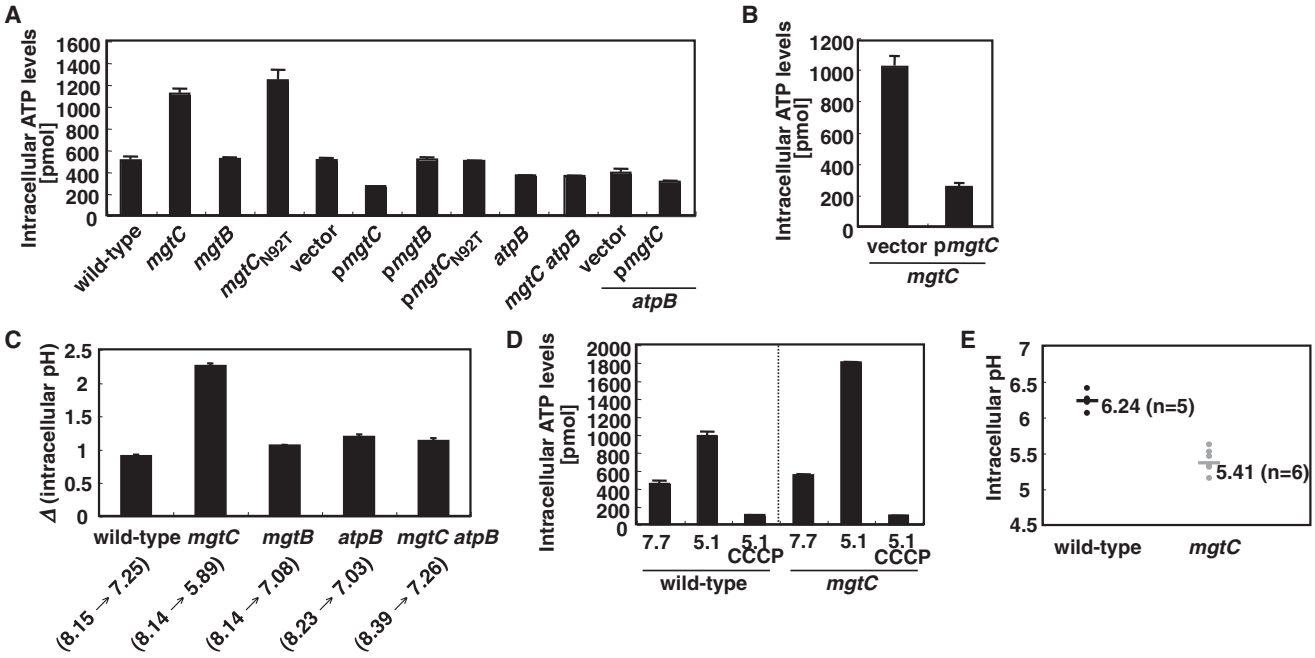

Figure 4. MgtC Controls Intracellular ATP Levels and the Proton Gradient in an *atpB*-Dependent Manner

(A) Intracellular ATP levels (determined at an OD_{600}: 0.1) of wild-type (14028s), *mgtC* (EL4), *mgtB* (EL5), and $mgtC_{N92T}$ (EL551) *Salmonella* or wild-type (14028s) *Salmonella* harboring the plasmid vector (pUHE21-2*lacI*^{q}) or derivatives with the *mgtC* (p*mgtC*), *mgtB* (p*mgtB*), or $mgtC_{N92T}$ (p$mgtC_{N92T}$) genes or an *atpB* mutant (EL515) and *mgtC atpB* (EL516) mutant *Salmonella* or *atpB* mutant (EL515) harboring either the plasmid vector or the *mgtC* gene. Bacteria were grown for 4 hr in N-minimal media pH 7.7 containing low Mg^{2+} (10 μM) and for plasmid-harboring strains IPTG (0.2 mM). Nucleic acids were extracted as described in Experimental Procedures. Intracellular ATP levels correspond to picomoles of ATP per ml of cells at given OD_{600}.

(B) Intracellular ATP levels of *mgtC* (EL4) *Salmonella* harboring the plasmid vector (pUHE21-2*lacI*^{q}) or a derivative with the *mgtC* coding region (p*mgtC*) determined at an OD_{600}: 0.177. Bacteria were grown as described in (A).

(C) Change in intracellular pH of wild-type (14028s), *mgtC* (EL4), *mgtB* (EL5), *atpB* (EL515), and *mgtC atpB* (EL516) *Salmonella* that had been grown at pH 7.7 and switched to pH 5.1 for 1 hr. Intracellular pH values in brackets are representative of two measurements, which gave similar results. Values for wild-type and *atpB* mutant are similar to those previously reported using a different method (Foster and Hall, 1991).

(D) Intracellular ATP levels of wild-type (14028s) and *mgtC* (EL4) *Salmonella* grown as described in (C). Control experiment was carried out at pH 5.1 in the presence of the uncoupler CCCP. Data in (A)–(D) are represented as mean ± SEM.

(E) Intracellular pH of wild-type (14028s) and *mgtC* (EL4) *Salmonella* inside the macrophage-like cell line J774A.1. Number represents the average pH of five independent replicates for wild-type (14028s) and six replicates for *mgtC* (EL4) *Salmonella*, which gave similar bacterial colony counts when plated on LB agar plates.

See also Figure S5.

the downstream *mgtB* gene; Snavely et al., 1991) because an *mgtB* mutant retained wild-type ATP levels (Figure 4A). In addition, the ATP levels of the *mgtC* mutant carrying a plasmid expressing the *mgtC* coding region from a heterologous promoter were 4-fold lower than those of an isogenic strain with the plasmid vector (Figure 4B). The *mgtC*-expressing plasmid reduced ATP levels, even in the wild-type strain, whereas an isogenic plasmid expressing the *mgtB* coding region behaved like the vector control (Figure 4A).

Because the F_1F_o ATP synthase mediates proton translocation, we reasoned that MgtC might affect cytosolic pH. Indeed, when bacteria were switched from pH 7.7 to pH 5.1 and incubated for 1 hr, the decrease in intracellular pH of the *mgtC* mutant was ~2 units, which is >1 unit greater than that experienced by wild-type *Salmonella* or the *mgtB* mutant (Figure 4C). These conditions resulted in higher ATP levels in the *mgtC* mutant than in wild-type *Salmonella* (Figure 4D). Next, we wondered whether the difference in intracellular pH between wild-type and *mgtC Salmonella* during growth in laboratory media (Figure 4C) was also observed when *Salmonella* is inside macrophage phagosomes, which display a mildly acidic pH (Rathman et al., 1996). We determined that the cytosolic pH of a *Salmonella mgtC* mutant was 0.8 units lower than that of the isogenic wild-type strain when investigated 6 hr postbacterial internalization by the J774A.1 macrophage-like cell line (Figure 4E). This result indicates that MgtC's action is manifested during infection of phagocytic cells.

A Single Amino Acid Substitution in the MgtC Protein that Compromises Binding to the F_1F_o ATP Synthase Prevents MgtC-Dependent Phenotypes

The asparagine residue at position 92 is conserved in the MgtC proteins from *S. enterica*, *M. tuberculosis*, *Yersinia pestis*, *Photorhabdus luminescens*, *Pseudomonas aeruginosa*, and *Brucella melitensis* (Rang et al., 2007). This residue is located in the fourth transmembrane helix, close to the cytoplasmic face (Rang et al.,

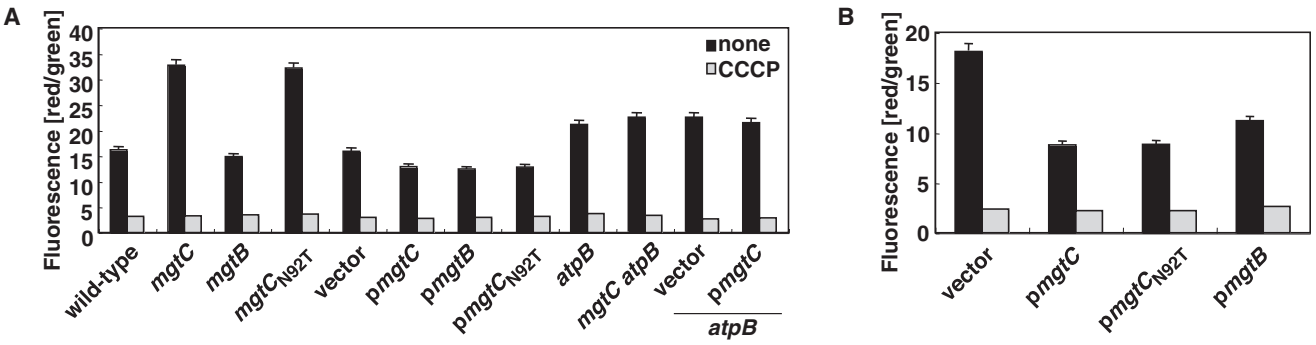

Figure 5. MgtC Affects Membrane Potential

(A) Membrane potential of *Salmonella* strains listed and grown as described in Figure 4A. Red/green fluorescence ratio was determined after incubation with the membrane potential-dependent dye $DiOC_2$ for 30 min in either the presence or absence of the uncoupler CCCP.

(B) Same as in (A), but bacteria were grown in N-minimal medium with 10 mM Mg^{2+}. Data are represented as mean ± SEM.

See also Figure S5.

2007). An MgtC protein variant with the asparagine at position 92 substituted by a threonine residue cannot restore the ability to survive inside macrophages to a *Salmonella mgtC*-null mutant (Rang et al., 2007).

We determined that in vitro synthesized MgtC N92T protein failed to interact with the F_1F_o ATP synthase in immunoprecipitation experiments (Figure 1). Moreover, vesicles prepared from wild-type *Salmonella* expressing a plasmid-encoded MgtC N92T protein did not inhibit proton translocation (Figure 2C) and ATP hydrolysis (Figure 2I), behaving like those harboring the vector control. In addition, in these vesicles, the MgtC N92T protein could not hinder NADH-driven ATP synthesis like the wild-type MgtC protein (Figures 3C and 3E). Furthermore, a *Salmonella* strain expressing the MgtC N92T protein from the normal *mgtC* chromosomal location and promoter harbored high ATP levels, similarly to the *Salmonella* mutant deleted for the *mgtC* gene (Figure 4A), and overexpression of the MgtC N92T protein in wild-type *Salmonella* did not decrease ATP levels, as observed upon overexpression of the wild-type MgtC protein (Figure 4A). Note that the MgtC N92T protein is produced in amounts similar to those of the wild-type MgtC protein in wild-type *Salmonella* (Figure S5A) and that a *Salmonella* strain that expresses the MgtC N92T protein from the normal promoter and chromosomal location is defective for survival inside macrophages (Figure S5B). Cumulatively, these results support the notion that the effect of the MgtC protein on bacterial energetics results from its ability to interact with the F_1F_o ATP synthase.

MgtC's Actions Are Largely Dependent on a Functional F_1F_o ATP Synthase

Because the in vitro activity of the MgtC protein is observed only in the presence of the F_o *a* subunit of the F_1F_o ATP synthase (Figures 2 and 3), we hypothesized that the in vivo phenotypes displayed by an *mgtC* mutant (Figure 4) would disappear in an *atpB* mutant background. Indeed, mutation of the *mgtC* gene had no effect on ATP levels (Figure 4A) and intracellular pH (Figure 4C) in a strain also lacking the *atpB* gene. Interestingly, the ATP levels present in the *atpB* mutant were similar to those exhibited by wild-type *Salmonella* carrying the *mgtC*-expressing plasmid (Figure 4A). These data support the notion that MgtC exerts its effect on intracellular pH and ATP levels by targeting the F_1F_o ATP synthase.

The ATP levels of the *atpB* mutant *Salmonella* expressing the *mgtC*-expressing plasmid were slightly lower than those of the strain expressing the plasmid vector (Figure 4A). Although the difference was not as pronounced as that observed in an *mgtC*$^+$ strain (Figure 4A), this raised the possibility of MgtC also affecting ATP levels by targeting a protein(s) other than the F_1F_o ATP synthase complex. This prompted us to explore the effect that MgtC had on membrane potential.

Given that MgtC hinders proton translocation in inside-out vesicles (Figures 2B and 2C), one would expect bacteria lacking MgtC to display a less polarized membrane than wild-type *Salmonella* (i.e., producing the MgtC protein). Surprisingly, the *mgtC* mutant accumulated the membrane potential-dependent fluorescent dye 3,3′-diethyloxacarbocyanine iodide ($DiOC_2$) ~2-fold more than wild-type *Salmonella* (Figure 5A) when comparing the same cell preparations used to determine intracellular ATP levels (Figure 4A). The strain expressing the MgtC N92T variant behaved like the strain deleted for the *mgtC* gene (Figure 5A), whereas the *mgtB* mutant displayed wild-type behavior (Figure 5A). As expected, membrane potential was collapsed when cells were incubated in the presence of the protonophore carbonyl cyanide 3-chlorophenyl hydrazone (CCCP) (Figure 5A).

The wild-type strain harboring a plasmid expressing *mgtC* from a heterologous promoter displayed slightly lower fluorescence levels than cells carrying the vector control (Figure 5A) when grown under *mgtC*-inducing (i.e., 10 μM Mg^{2+}) conditions. Isogenic strains expressing the *mgtC* N92T variant or the *mgtB* gene also exhibited a decrease in fluorescence (Figure 5A). The membrane depolarization phenotype was heightened when these strains were evaluated following growth in 10 mM Mg^{2+} (Figure 5B), which are noninducing conditions for the PhoP/PhoQ system (Groisman, 2001), the transcriptional activator of the *mgtCBR* operon (Soncini et al., 1996). The use of the latter growth conditions enables us to evaluate the activities of the MgtC and MgtB proteins in the absence of other PhoP/PhoQ-dependent products, which have been shown to reverse membrane potential in *E. coli* (Alteri et al., 2011). (Note that the *atpB* dependence of the membrane potential phenotypes

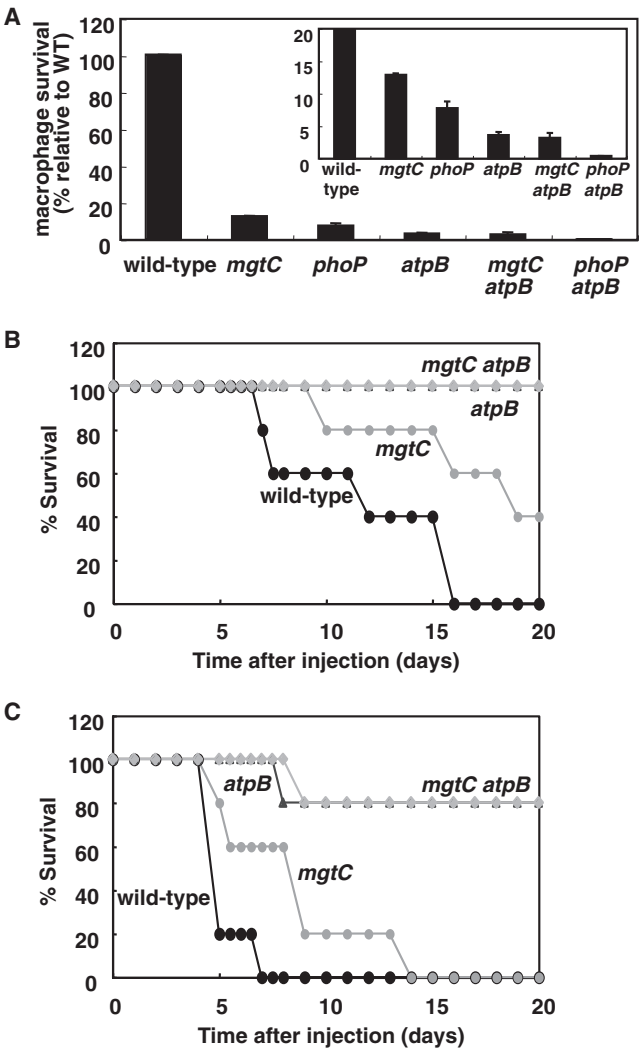

Figure 6. The Defect in Macrophage Survival and Virulence in Mice of the *mgtC* Mutant *Salmonella* Is *atpB*-Dependent

(A) Survival of wild-type (WT) (14028s), *mgtC* (EL4), *phoP* (MS7953s), *atpB* (EL515), *mgtC atpB* (EL516), and *phoP atpB* (EL535) *Salmonella* inside J774A.1 macrophages at 18 hr after infection. The inset shows survival values below 20%. Data are represented as mean ± SEM.

(B and C) Survival of C3H/HeN mice inoculated intraperitoneally with $\sim 10^4$ (B) or $\sim 10^5$ (C) colony-forming units of wild-type (14028s), *mgtC* (EL4), *atpB* (EL515), and *mgtC atpB* (EL516) *Salmonella*. The data are representative of two independent experiments, which gave similar results.

See also Figure S5.

resulting from deletion or overexpression of the *mgtC* gene [Figure 5A] can be ascribed to the F_1F_o ATP synthase being required to maintain membrane potential). Cumulatively, these results suggest that MgtC may have an additional target(s).

The Virulence Function of MgtC Is Largely Due to Its Ability to Inhibit the F_1F_o ATP Synthase

If the contribution of the MgtC protein to *Salmonella* pathogenicity results from its action on the F_1F_o ATP synthase, inactivation of the *mgtC* gene should not attenuate an *atpB* mutant any further. On the other hand, if an *atpB mgtC* double mutant is more attenuated than either *atpB* or *mgtC* single mutants, this would indicate that MgtC's participation in *Salmonella* pathogenicity goes beyond its effect on the F_1F_o ATP synthase. In agreement with the former possibility, the *atpB* single mutant and *atpB mgtC* double mutant exhibited comparable defects in their ability to survive within the macrophage-like cell line J774A.1 (Figure 6A). This is in contrast to a double mutant defective in both *atpB* and the regulatory gene *phoP*, which was more attenuated than either *atpB* or *phoP* single mutants (Figure 6A). Moreover, the *atpB* and *atpB mgtC* mutants were indistinguishable in their ability to cause disease following intraperitoneal injection of C3H/HeN mice (Figures 6B and 6C). Cumulatively, our findings support the notion that MgtC carries out its virulence function primarily through its action on the F_1F_o ATP synthase. Paradoxically, the F_1F_o ATP synthase is also required for virulence (Turner et al., 2003), and a *Salmonella* mutant lacking the corresponding genes can serve as a vaccine strain (Northen et al., 2010).

DISCUSSION

We have now established that the MgtC virulence protein targets *Salmonella*'s own F_1F_o ATP synthase and that this interaction is critical for MgtC's contribution to *Salmonella* pathogenicity. MgtC binds to the F_o *a* subunit of the F_1F_o ATP synthase (Figure 1), thereby altering the ability of the F_1F_o ATP synthase to translocate protons and to couple proton translocation to ATP synthesis (Figures 2 and 3). This action, in turn, enables *Salmonella* to maintain physiological ATP levels, cytosolic pH, and membrane potential (Figures 4 and 5). To our knowledge, MgtC provides a unique instance of a natural protein that inhibits the F_1F_o ATP synthase (Figures 2 and 3), as opposed to acting as chaperone for this complex (Yi et al., 2003).

MgtC's contribution to *Salmonella* survival inside macrophages and to *Salmonella*'s capacity to cause a lethal infection in mice appears to be dependent on its ability to alter the activity of the F_1F_o ATP synthase. This is because the virulence defect of an F_o *a* subunit mutant was unaltered upon additional inactivation of the *mgtC* gene. Our findings may apply to other intracellular pathogens that rely on the MgtC protein for pathogenicity (Buchmeier et al., 2000; Grabenstein et al., 2006; Lavigne et al., 2005; Maloney and Valvano, 2006). MgtC provides a singular example of a virulence protein that inhibits another protein required for virulence to enhance pathogenicity.

Intracellular Pathogens Utilize Different Strategies to Survive within Acidified Phagosomes

Intracellular pathogens have adopted two distinct strategies to survive within acidic macrophage phagosomes: they modify the phagosomal pH or deal with the consequences of phagosome acidification. For instance, *Legionella pneumophila* secretes an effector protein that targets the host vacuolar ATPase (Xu et al., 2010), thereby modifying the phagosome into a more hospitable locale (Figure 7B). *M. tuberculosis* also affects the host vacuolar ATPase to inhibit phagosome acidification (Wong et al., 2011). This is interesting because

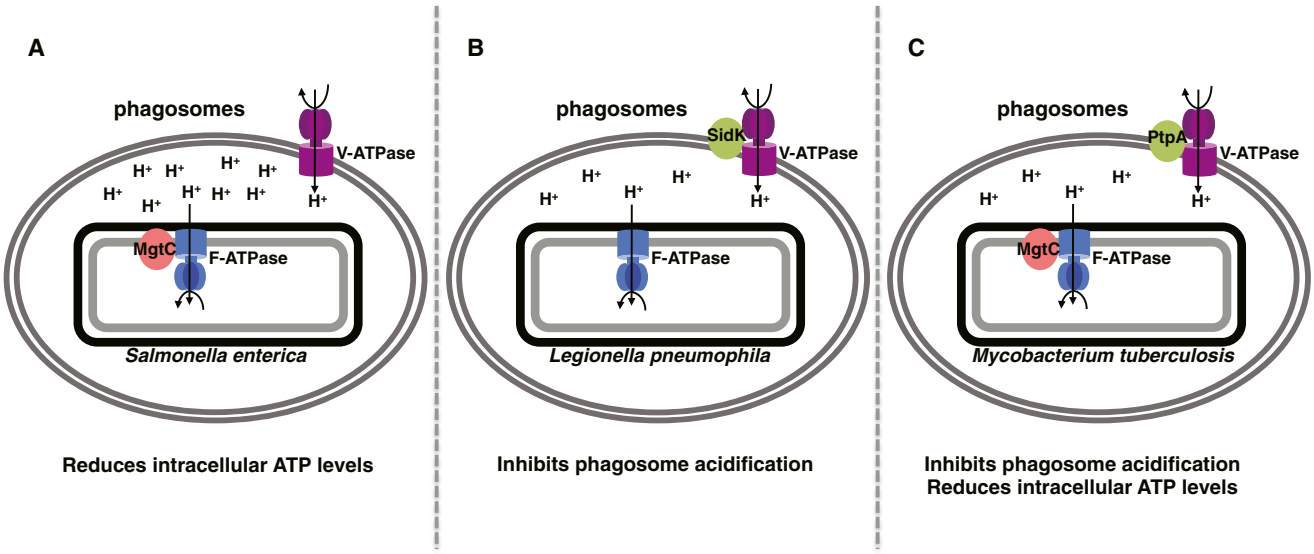

Figure 7. Intracellular Pathogens Utilize Different Strategies to Cope with Phagosome Acidification

(A) *S. enterica* harbors the inner membrane protein MgtC, which targets the bacterium's own F_1F_o ATPase to decrease intracellular ATP levels heightened by phagosomal acidic pH.

(B) *Legionella pneumophila* secretes the effector protein SidK to the host phagosomal membrane, where it inhibits phagosome acidification driven by the host vacuolar ATPase (Xu et al., 2010).

(C) *M. tuberculosis* secretes the PtpA protein, which affects the host vacuolar ATPase to hinder phagosome acidification (Wong et al., 2011) and harbors a functional *mgtC* gene (Buchmeier et al., 2000), which is expected to reduce intracellular ATP levels.

M. tuberculosis also harbors a functional MgtC protein (Buchmeier et al., 2000; Figure 7C).

We have now determined that *Salmonella* utilizes the MgtC protein to inhibit its own F_1F_o ATP synthase (Figure 7A). This multiprotein complex consists of the integral membrane F_o portion, which mediates proton translocation, and the F_1 portion, which drives ATP synthesis when protons enter the cytoplasm down an electrochemical gradient. The F_1 portion can also catalyze the reverse reaction: to pump protons across the cytoplasmic membrane using the energy derived from ATP hydrolysis. Phagosome acidification is predicted to increase the proton gradient across the *Salmonella* inner membrane. Thus, protons coming down the gradient via the F_1F_o ATP synthase would give rise to an increase in cytosolic ATP levels. By inhibiting the F_1F_o ATP synthase (Figures 2 and 3), the MgtC protein prevents the accumulation of ATP to nonphysiological levels (Figure 4A) and the acidification of the cytoplasm that are observed in the *mgtC* mutant both during growth in laboratory media (Figure 4C) and inside macrophages (Figure 4E).

It has been proposed that the MgtC protein activates the Na^+,K^+-ATPase in host cell membranes. This is because expression of the *Salmonella mgtC* gene in *Xenopus* oocytes depolarized the membrane and decreased ATP levels (Günzel et al., 2006). However, it is unclear how this would happen during a normal infection, given that MgtC is an integral membrane protein (Rang et al., 2007). In other words, for MgtC to have the opportunity to come in contact with a eukaryotic protein, it would have to leave the bacterial inner membrane, traverse the periplasm and outer membrane, and then gain access to the eukaryotic cell; or a proteolytic fragment that can reach the eukaryotic cell would have to be generated.

The MgtC protein appears to have a target(s) in addition to the F_1F_o ATP synthase. This is because, first, an *mgtC* deletion mutant as well as a strain expressing the MgtC N92T variant harbored a hyperpolarized membrane (Figure 5). However, expression of the wild-type MgtC, the MgtC N92T protein (which does not interact with the F_1F_o ATP synthase [Figure 1]), or the MgtB protein in wild-type *Salmonella* depolarized the membrane (Figure 5). Second, overexpression of the *mgtC* gene slightly reduced ATP levels in an *atpB* mutant background (Figure 4A).

Salmonella Limits Production of the MgtC Protein to Specific Conditions

That MgtC targets the F_1F_o ATP synthase might explain why *Salmonella* employs a variety of mechanisms to restrict the production of this virulence protein to particular conditions and for a limited period of time. On the one hand, *mgtC* transcription requires both the extracytoplasmic signals activating the PhoP/PhoQ regulatory system (Soncini et al., 1996) and the cytoplasmic signals detected by the *mgtCBR* messenger RNA (mRNA) leader (Lee and Groisman, 2012a, b). On the other hand, the PhoP protein also promotes transcription of AmgR, an antisense RNA that preferentially degrades the *mgtC* portion of the *mgtCBR* transcript (Lee and Groisman, 2010). In addition, the *mgtCBR* operon specifies the MgtR peptide, which brings about MgtC proteolysis (Alix and Blanc-Potard, 2008).

The accumulation of cytosolic ATP exhibited by the *mgtC* null mutant (Figure 4A) provides a physiological rationale for our previous finding that the *mgtC* mRNA leader responds to an

increase in cytoplasmic ATP levels by promoting transcription of the *mgtCBR* coding region (Lee and Groisman, 2012a). Thus, we would like to propose that, as discussed above, when *Salmonella* is present in an acidic phagosome, the cytoplasmic levels of ATP increase. Such an increase is detected by the *mgtCBR* leader mRNA, which enhances production of the MgtC protein. By binding to the F_1F_o ATP synthase, MgtC hinders proton translocation and ATP synthesis, thereby reducing the ATP concentration to physiological levels. This would then be sensed by the *mgtCBR* leader mRNA, which would promote a decrease in expression of the MgtC protein.

MgtC Links ATP Homeostasis and Pathogen's Persistence

Bacteria often exhibit the ability to tolerate (or persist within) changing environments by slowing growth and/or modulating their metabolic status. MgtC's mechanism of action highlights the critical role that ATP homeostasis plays in pathogen persistence. For example, *M. tuberculosis* requires the F_1F_o ATP synthase, even when present in a nonreplicating state (Gengenbacher et al., 2010; Rao et al., 2008), *Salmonella* needs the *mgtC* gene for long-term systemic infection (Lawley et al., 2006), and *E. coli*'s tolerance to aminoglycosides can be overcome by the generation of a proton-motive force (Allison et al., 2011), which drives ATP synthesis. Because a variety of conditions can increase intracellular ATP levels to promote MgtC expression (Lee and Groisman, 2012a) and because the MgtC protein modulates intracellular ATP levels (Figure 4A), the control of F_1F_o ATP synthase activity may help organisms cope with metabolic imbalances in addition to those resulting from phagosome acidification.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Oligodeoxynucleotides, and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table S1. All *Salmonella enterica* serovar Typhimurium strains are derived from the wild-type strain 14028s (Fields et al., 1986) and were constructed by phage P22-mediated transductions as described (Davis et al., 1980). DNA oligonucleotides used in this study are listed in Table S2. Bacteria were grown at 37°C in Luria-Bertani broth (LB), N-minimal media (pH 7.7) (Snavely et al., 1991) supplemented with 0.1% casamino acids, 38 mM glycerol, and the indicated concentrations of $MgCl_2$. When indicated, we used a modified N-minimal medium containing 0.2% glucose instead of 38 mM glycerol. *E. coli* DH5α was used as the host for preparation of plasmid DNA. Ampicillin was used at 50 μg ml^{-1}, chloramphenicol at 20 μg ml^{-1}, kanamycin at 20 μg ml^{-1}, and tetracycline at 10 μg ml^{-1}.

Measurement of Intracellular ATP in *Salmonella*

Experiments were performed using a luminometer (BioTek Synergy H1 reader) as described (Lee and Groisman, 2012a).

Measurement of Membrane Potential

We used the *Bac*Light bacterial membrane potential kit (Invitrogen) following the manufacturer's instruction with slight modifications. Briefly, bacteria were grown overnight in N-minimal media containing 10 mM Mg^{2+} and 0.2% glucose as a carbon source. One milliliter of the overnight culture was washed in the N-minimal media without Mg^{2+} and resuspended in 1 ml of the same media. One in one hundred dilution of bacteria was inoculated in 2 ml of N-minimal media containing either 10 μM Mg^{2+} or 10 mM Mg^{2+} and glycerol (with proper antibiotics if necessary) and grown for 4 hr. Cells were normalized by optical density at wavelength of 600 nanometers (OD_{600}) and resuspended in 150 μl of PBS containing 30 μM 3,3′-diethyloxacarbocyanine iodide ($DiOC_2$). As a control, to depolarize the membrane, we added CCCP at 5 μM. Samples were incubated at 37°C for 30 min, dispensed into a 96-well black microplate (PerkinElmer), and fluorescence was measured using the Synergy H1 plate reader (BioTek) at an excitation wavelength of 488 nm and an emission wavelength of 535 nm (for green) or 645 nm (for red). Measurements were normalized by the emission from $DiOC_2$ blank well.

Determining Intracellular pH in *Salmonella*

We measured intracellular pH using green fluorescence protein as described (Lee and Groisman, 2012a). For measuring *Salmonella*'s intracellular pH inside the macrophage-like cell line J774A.1, cells were seeded in 96-well black microplates (PerkinElmer) in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum at a density of 10^5 per well 1 day prior to infection with *Salmonella* harboring a plasmid containing the *gfp* gene expressed from a heterologous promoter (pfpv25.1). Bacteria were grown overnight in LB media with antibiotics at 37°C, washed with PBS, and used to infect macrophages at a multiplicity of infection of 50:1. At 6 hr postinfection, infected macrophages were resuspended in 150 μl of PBS or PBS buffer at different pH with 20 mM sodium benzoate for standard curve. Spectra were measured for four biological replicates at each pH for standard curve and 12 independent wells for measuring intracellular pH of wild-type or *mgtC Salmonella* inside macrophages. After measuring the spectra, infected macrophages were lysed by adding 0.1% Triton X-100 and bacteria were plated on LB plate with proper dilutions.

Preparing Inverted Membrane Vesicles

Inverted vesicles were prepared as described previously (Suzuki et al., 2007). Cells were grown overnight in N-minimal media containing 10 mM Mg^{2+}. One milliliter of the overnight culture was washed in the N-minimal media without Mg^{2+} and resuspended in 1 ml of the same media. One in one hundred dilution of bacteria was inoculated into 50 ml of N-minimal media containing 10 μM Mg^{2+} grown for 5 hr. Cells were resuspended in lysis buffer (10 mM 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES)/KOH (pH 7.5), 0.2 mM phenylmethylsulfonyl fluoride, 5 mM $MgCl_2$, and 10% glycerol) and disrupted by sonication. After removing cell debris, membranes were isolated by centrifugation for 1 hr at 40,000 × *g* at 4°C. The pellet was resuspended in the same buffer and used as the inverted membrane vesicles in this study. The protein concentration was determined using a BCA protein assay kit (Pierce) with bovine serum albumin as a standard.

Identification of MgtC-Interacting Proteins In Vivo

The whole-cell crosslinking experiments were performed with the *mgtC* deletion strain (EL4) harboring a plasmid encoding a C-terminally FLAG-tagged *mgtC* gene under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. As a control, we used a plasmid with the *mgtC* gene without a FLAG tag. 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. One in one hundred dilution of bacteria was inoculated in 50 ml of N-minimal media containing 10 μM Mg^{2+} and 0.5 mM IPTG to induce expression of MgtC-FLAG protein and grown for 5 hr. Cells were pelleted and washed twice in PBS buffer. Then, cells were incubated with 0.5 mM dithiobi(succinimidyl propionate) (DSP), a membrane-permeable crosslinker, at 37°C for 30 min. Control reaction lacking DSP were carried out in parallel. Crosslinking reactions were quenched by adding 40 mM Tris (pH 7.5) and incubated for 15 min. Cells were resuspended in lysis buffer (10 mM Tris-HCl [pH 7.5], 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM $MgCl_2$) and disrupted by sonication. Membranes were isolated by centrifugation for 1 hr at 40,000 × *g* and solubilized in Tris-buffered saline (TBS) buffer (50 mM Tris-HCl [pH 7.4], and 150 mM NaCl) containing 1% Triton X-100 by gentle agitation at room temperature for 1 hr. Insoluble materials were removed by centrifugation for 10 min at 40,000 × *g* and solubilized membrane proteins were incubated with EZview Red anti-FLAG M2 affinity gel (Sigma) overnight at 4°C according to manufacturer's instruction. After washing the beads, binding proteins were eluted in SDS sample buffer and separated on 4%–20% SDS-polyacrylamide gel and

analyzed by western blotting using anti-FLAG antibodies or by silver staining. Specific bands that were crosslinked by DSP were identified by MALDI-TOF analysis (Harvard Mass Spectrometry and Proteomics Facility).

MgtC-ATP Synthase F_o *a* Subunit Interaction by Immunoprecipitation Assay

Interaction between the MgtC protein and the ATP synthase F_o *a* subunit was investigated using a strain (EL481) expressing the FLAG-tagged *mgtC* gene and HA-tagged *atpB* gene at their 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 the N-minimal media without Mg^{2+} and resuspended in 1 ml of the same media. One in one hundred dilution of bacteria was inoculated in 25 ml of N-minimal media containing 10 μM Mg^{2+} grown for 5 hr. Membrane proteins were prepared as described above and incubated with EZview Red anti-HA affinity gel (Sigma) overnight at 4°C according to the manufacturer's instructions. After washing the beads, binding proteins were eluted in SDS sample buffer and separated on 12% SDS-polyacrylamide gel and analyzed by western blotting using anti-FLAG antibodies. We also incubated membrane vesicles with an anti-FLAG M2 affinity gel (Sigma) at 4°C overnight according to the manufacturer's instruction. After washing the beads, bound proteins were eluted in SDS sample buffer and separated on 12% SDS-polyacrylamide gel and analyzed by western blotting using anti-HA antibodies.

MgtC-ATP Synthase F_o *a* Subunit Interaction Using In Vitro Protein Synthesis and Reconstitution in Proteoliposomes

Proteoliposomes were prepared using soybean L-α-phosphatidylcholine (Sigma) and resuspended 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). We prepared the DNA templates harboring T7 promoter sequences by a two-step PCR process. In the first round of PCR, we amplified each of the templates using 14028s genomic DNA as a template and the following sets of primers: *atpB*-HA (W142/W169), *atpE* (W144/W145), *atpF* (W146/W147), *atpH* (W148/W149), *atpA* (W150/W151), *atpG* (W152/W153), *atpD* (W154/W156), *atpC* (W157/W158), *mgtC*-FLAG (W160/W162), and *yhiD*-FLAG (W172/W173). For $mgtC_{N92T}$-FLAG, we used the same primers as with *mgtC*-FLAG (W160/W162) and EL551 genomic DNA as a template. In the second round of PCR, we used the PCR products generated in the previous reactions as templates, which have identity with a T7 promoter containing universal primer (W141) and amplified final DNA fragments, with the T7 promoter using the universal primer and gene-specific reverse primers. DNA templates for the seven subunits of the F_1F_o ATP synthase (*atpE*, *atpF*, *atpH*, *atpA*, *atpG*, *atpD*, and *atpC*) were mixed together with DNA templates for *atpB*-HA, *mgtC*-FLAG, or *yhiD*-FLAG, and proteins were synthesized using cell-free PURExpress in vitro protein synthesis system (NEB) in the presence of 0.12 mg/ml proteoliposomes for 2 hr at 37°C. At the end of the reaction, samples were aliquoted for input and diluted in TBS buffer. Diluted reactions were split into two tubes and immunoprecipitated with either anti-HA or anti-FLAG antibodies. Then, immunoprecipitated samples were analyzed by western blotting using anti-HA or anti-FLAG antibodies as described above. For the experiment shown in Figure S3, the reaction was carried out as described above, except that the only F_1F_o ATP synthase DNA template was that corresponding to the F_o *a* subunit.

Measurement of Proton Translocating Activity of the F_1F_o ATP Synthase

ATP-driven proton translocating activity was determined by monitoring fluorescence quenching of ACMA, a pH-dependent dye, as described (Suzuki et al., 2007) with a modification. Membrane vesicles were diluted to a concentration of 30 μg or 56 μg of protein per ml of assay buffer (10 mM HEPES/KOH [pH 7.5], 100 mM KCl, 5 mM $MgCl_2$, and 0.3 μg/ml of ACMA). The proton translocating reaction was initiated by adding 0.5 mM ATP or NADH, and fluorescence quenching was monitored at room temperature with excitation at 410 nm and emission at 490 nm using LS-55 fluorescence spectrometer (PerkinElmer). The reaction was terminated by adding the uncoupler NH_4Cl (10 mM).

Measurement of ATPase Activity of the F_1F_o ATP Synthase

ATPase activity was measured as described (Suzuki et al., 2007) with the following modifications. The same vesicles (20 μg proteins/ml) prepared for the AMCA fluorescence quenching experiments were equilibrated with the assay buffer (10 mM HEPES/KOH [pH 7.5], 100 mM KCl, and 5 mM $MgCl_2$). The ATP hydrolysis reaction was initiated by adding 1 mM ATP and monitored phosphate release at absorbance 360 nm using EnzChek Phosphate Assay Kit (Invitrogen) according to manufacturer's instruction. Average hydrolysis rates in a time period from 1 to 5 min after initiation were calculated and presented as amount of phosphate (μmol) released per min. As a control, ATP was omitted from the reaction, which resulted in lack of phosphate release (data not shown).

Measurement of ATP Synthesis Activity of the F_1F_o ATP Synthase

ATP synthesis activity of the inverted membrane vesicles was measured as described with modification (Suzuki et al., 2007). Inverted vesicles were prepared from the same amount of cells normalized by OD_{600}, and 60 μg of membrane vesicles were mixed with assay buffer (20 mM HEPES pH 7.9, 100 mM potassium acetate, 5 mM magnesium acetate, 0.2 mM ADP, 40 μM di(adenosine-5′)pentaphosphate [Ap5A]) and 1/10 volume of the CLSII solution containing luciferin/luciferase (ATP bioluminescence kit, Roche) in the presence or absence of phosphate (5 mM Na_2HPO_4). The reaction was initiated by adding 0.8 mM NADH to generate proton gradient across the membrane. ATP production was monitored in real time by the light produced from the luciferase reaction for 210 s at 25°C using a luminometer (BioTek Synergy H1 reader). The amount of ATP synthesized was calibrated by adding 0.25 mM ATP at the end of the measurement.

Western Blot Analysis

Cells were grown for 5 hr in 10 ml of N-minimal medium containing 10 μM Mg^{2+}. Crude extracts were prepared in TBS buffer containing 0.1% Triton X-100 by sonication and analyzed as described (Lee and Groisman, 2010). The data are representative of two independent experiments, which gave similar results.

Examining Survival inside Macrophage

Macrophage survival assays were conducted with the macrophage-like cell line J774A.1 as described (Blanc-Potard and Groisman, 1997).

Mouse Virulence Assays

Six- to eight-week-old female C3H/HeN mice were inoculated intraperitoneally with $\sim 10^4$ or $\sim 10^5$ colony-forming units. Mouse survival was followed for 21 days. Virulence assays were conducted twice with similar outcomes, and data correspond to groups of five mice. All procedures were performed according to approved protocols by the Yale School of Medicine Committee on the Use and Care of Animals.

See Extended Experimental Procedures for more information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.06.004.

ACKNOWLEDGMENTS

We thank Dr. Xuanlin Tu for the crosslinking experiments, Dr. John Walker and Dr. Gregory Cook for comments on the manuscript, and anonymous reviewers for valuable suggestions. This work was supported, in part, by grant AI49561 from the National Institutes of Health to E.A.G., who is an investigator of the Howard Hughes Medical Institute.

Received: December 6, 2012
Revised: March 26, 2013
Accepted: June 3, 2013
Published: July 3, 2013

REFERENCES

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

Allison, K.R., Brynildsen, M.P., and Collins, J.J. (2011). Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature *473*, 216–220.

Alteri, C.J., Lindner, J.R., Reiss, D.J., Smith, S.N., and Mobley, H.L. (2011). The broadly conserved regulator PhoP links pathogen virulence and membrane potential in *Escherichia coli*. Mol. Microbiol. *82*, 145–163.

Andries, K., Verhasselt, P., Guillemont, J., Göhlmann, H.W., Neefs, J.M., Winkler, H., Van Gestel, J., Timmerman, P., Zhu, M., Lee, E., et al. (2005). A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. Science *307*, 223–227.

Blanc-Potard, A.B., and Groisman, E.A. (1997). The *Salmonella selC* locus contains a pathogenicity island mediating intramacrophage survival. EMBO J. *16*, 5376–5385.

Blanc-Potard, A.B., and Lafay, B. (2003). MgtC as a horizontally-acquired virulence factor of intracellular bacterial pathogens: evidence from molecular phylogeny and comparative genomics. J. Mol. Evol. *57*, 479–486.

Buchmeier, N., Blanc-Potard, A., Ehrt, S., Piddington, D., Riley, L., and Groisman, E.A. (2000). A parallel intraphagosomal survival strategy shared by *mycobacterium tuberculosis* and *Salmonella enterica*. Mol. Microbiol. *35*, 1375–1382.

Davis, R.W., Bolstein, D., and Roth, J.R. (1980). Advanced Bacterial Genetics (Cold Spring Harbor, NY: Cold Spring Harbor Lab).

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

Fields, P.I., Swanson, R.V., Haidaris, C.G., and Heffron, F. (1986). Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA *83*, 5189–5193.

Foster, J.W., and Hall, H.K. (1991). Inducible pH homeostasis and the acid tolerance response of *Salmonella* typhimurium. J. Bacteriol. *173*, 5129–5135.

Gengenbacher, M., Rao, S.P., Pethe, K., and Dick, T. (2010). Nutrient-starved, non-replicating *Mycobacterium tuberculosis* requires respiration, ATP synthase and isocitrate lyase for maintenance of ATP homeostasis and viability. Microbiology *156*, 81–87.

Grabenstein, J.P., Fukuto, H.S., Palmer, L.E., and Bliska, J.B. (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, E.A. (2001). The pleiotropic two-component regulatory system PhoP-PhoQ. J. Bacteriol. *183*, 1835–1842.

Günzel, D., Kucharski, L.M., Kehres, D.G., Romero, M.F., and Maguire, M.E. (2006). The MgtC virulence factor of *Salmonella enterica* serovar Typhimurium activates Na(+),K(+)-ATPase. J. Bacteriol. *188*, 5586–5594.

Harold, F.M., and Maloney, P.C. (1996). Energy transduction by ion currents. In *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, F.C. Neidhart, R. Curtiss, III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Scharchter, and H.E. Umbarger, eds. (Washington, DC: American Society for Microbiology), pp. 283–306.

Ito, K., and Akiyama, Y. (2005). Cellular functions, mechanism of action, and regulation of FtsH protease. Annu. Rev. Microbiol. *59*, 211–231.

Kumar, Y., and Valdivia, R.H. (2009). Leading a sheltered life: intracellular pathogens and maintenance of vacuolar compartments. Cell Host Microbe *5*, 593–601.

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

Lavigne, J.P., O'callaghan, D., and Blanc-Potard, A.B. (2005). Requirement of MgtC for *Brucella suis* intramacrophage growth: a potential mechanism shared by *Salmonella enterica* and *Mycobacterium tuberculosis* for adaptation to a low-Mg^{2+} environment. Infect. Immun. *73*, 3160–3163.

Lawley, T.D., Chan, K., Thompson, L.J., Kim, C.C., Govoni, G.R., and Monack, D.M. (2006). Genome-wide screen for *Salmonella* genes required for long-term systemic infection of the mouse. PLoS Pathog. *2*, e11.

Lee, E.J., and Groisman, E.A. (2010). An antisense RNA that governs the expression kinetics of a multifunctional virulence gene. Mol. Microbiol. *76*, 1020–1033.

Lee, E.J., and Groisman, E.A. (2012a). Control of a *Salmonella* virulence locus by an ATP-sensing leader messenger RNA. Nature *486*, 271–275.

Lee, E.J., and Groisman, E.A. (2012b). Tandem attenuators control expression of the *Salmonella mgtCBR* virulence operon. Mol. Microbiol. *86*, 212–224.

Maloney, K.E., and Valvano, M.A. (2006). The *mgtC* gene of *Burkholderia cenocepacia* is required for growth under magnesium limitation conditions and intracellular survival in macrophages. Infect. Immun. *74*, 5477–5486.

Moncrief, M.B., and Maguire, M.E. (1998). Magnesium and the role of MgtC in growth of *Salmonella* typhimurium. Infect. Immun. *66*, 3802–3809.

Northen, H., Paterson, G.K., Constantino-Casas, F., Bryant, C.E., Clare, S., Mastroeni, P., Peters, S.E., and Maskell, D.J. (2010). *Salmonella enterica* serovar Typhimurium mutants completely lacking the F(0)F(1) ATPase are novel live attenuated vaccine strains. Vaccine *28*, 940–949.

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

Rao, S.P., Alonso, S., Rand, L., Dick, T., and Pethe, K. (2008). The protonmotive force is required for maintaining ATP homeostasis and viability of hypoxic, nonreplicating *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA *105*, 11945–11950.

Rathman, M., Sjaastad, M.D., and Falkow, S. (1996). Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. Infect. Immun. *64*, 2765–2773.

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

Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K., and Ueda, T. (2001). Cell-free translation reconstituted with purified components. Nat. Biotechnol. *19*, 751–755.

Snavely, M.D., Miller, C.G., and Maguire, M.E. (1991). The *mgtB* Mg^{2+} transport locus of *Salmonella typhimurium* encodes a P-type ATPase. J. Biol. Chem. *266*, 815–823.

Soncini, F.C., García Véscovi, E., Solomon, F., and Groisman, E.A. (1996). Molecular basis of the magnesium deprivation response in *Salmonella typhimurium*: identification of PhoP-regulated genes. J. Bacteriol. *178*, 5092–5099.

Suzuki, T., Ozaki, Y., Sone, N., Feniouk, B.A., and Yoshida, M. (2007). The product of *uncI* gene in F_1F_o-ATP synthase operon plays a chaperone-like role to assist *c*-ring assembly. Proc. Natl. Acad. Sci. USA *104*, 20776–20781.

Tao, T., Snavely, M.D., Farr, S.G., and Maguire, M.E. (1995). Magnesium transport in *Salmonella* typhimurium: *mgtA* encodes a P-type ATPase and is regulated by Mg^{2+} in a manner similar to that of the *mgtB* P-type ATPase. J. Bacteriol. *177*, 2654–2662.

Turner, A.K., Barber, L.Z., Wigley, P., Muhammad, S., Jones, M.A., Lovell, M.A., Hulme, S., and Barrow, P.A. (2003). Contribution of proton-translocating proteins to the virulence of *Salmonella enterica* serovars Typhimurium, Gallinarum, and Dublin in chickens and mice. Infect. Immun. *71*, 3392–3401.

Wong, D., Bach, H., Sun, J., Hmama, Z., and Av-Gay, Y. (2011). *Mycobacterium tuberculosis* protein tyrosine phosphatase (PtpA) excludes host vacuolar-H^+-ATPase to inhibit phagosome acidification. Proc. Natl. Acad. Sci. USA *108*, 19371–19376.

Xu, L., Shen, X., Bryan, A., Banga, S., Swanson, M.S., and Luo, Z.Q. (2010). Inhibition of host vacuolar H^+-ATPase activity by a *Legionella pneumophila* effector. PLoS Pathog. *6*, e1000822.

Yi, L., Jiang, F., Chen, M., Cain, B., Bolhuis, A., and Dalbey, R.E. (2003). YidC is strictly required for membrane insertion of subunits *a* and *c* of the F(1)F(0)ATP synthase and SecE of the SecYEG translocase. Biochemistry *42*, 10537–10544.