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MINIREVIEW

Regulation and function of the *Salmonella* MgtC virulence protein

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***Salmonella enterica* serovar Typhimurium produces many virulence proteins to cause diseases. The *Salmonella* MgtC protein is one of such virulence proteins specially required for intracellular proliferation inside macrophages and mouse virulence. In this review, we will cover how the *mgtC* gene is turned on or off and what the signals required for *mgtC* expression are. Later in this review, we will discuss a recent understanding of MgtC function in *Salmonella* pathogenesis by identifying its target proteins.**

Keywords: *mgtC*, *Salmonella* virulence, ATP homeostasis

Introduction

MgtC is an inner membrane protein with five transmembrane helices at the N-terminus and one cytoplasmic domain at the C-terminus (Rang *et al.*, 2007). The *Salmonella mgtC* gene, encoding the MgtC virulence protein, was identified first as a part of *Salmonella* pathogenicity island 3 (SPI-3) and then determined as the gene required for survival inside macrophage-like J774.A1 cell lines among other genes located in SPI-3 (Blanc-Potard and Groisman, 1997). The *Salmonella mgtC* mutant has several defects for growth in low Mg^{2+} media and mouse virulence as well as intramacrophage survival (Blanc-Potard and Groisman, 1997). Later, it was found that MgtC proteins are conserved among several unrelated pathogens including *Salmonella enterica* (Blanc-Potard and Groisman, 1997; Blanc-Potard and Lafay, 2003; Retamal *et al.*, 2009), *Yersinia pestis* (Grabenstein *et al.*, 2006), *Mycobacterium tuberculosis* (Buchmeier *et al.*, 2000), *Brucella suis* (Lavigne *et al.*, 2005), *Burkholderia cenocepacia* (Maloney and Valvano, 2006). Interestingly, *mgtC* genes from all these pathogens are required both for survival inside macrophages and for growth in low Mg^{2+} media (Blanc-Potard and Groisman, 1997; Buchmeier *et al.*, 2000; Blanc-Potard and Lafay, 2003; Lavigne *et al.*, 2005; Grabenstein *et al.*, 2006; Retamal *et al.*, 2009), suggesting that a host environment where those pathogens reside could be Mg^{2+}-limiting. The *mgtC* genes are often cotranscribed with the *mgtB* gene encoding a Mg^{2+} transporter (Blanc-Potard and Lafay, 2003), leading to the idea that MgtC is a critical virulence factor possibly due to its ability to maintain Mg^{2+} homeostasis inside a Mg^{2+}-limiting host environment. However, it turns out that MgtC's ability to promote *Salmonella* pathogenicity depends on maintaining ATP homeostasis by inhibiting F_1F_o ATP synthase (Lee *et al.*, 2013). In this review, a recent advance in understanding of regulation and function of the MgtC protein will be discussed.

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Regulation of the *mgtCBR* operon during infection

In *Salmonella*, the *mgtC* gene is ahead of other two genes, *mgtB* and *mgtR* encoding the Mg^{2+} transporter and a regulatory peptide respectively, comprising the *mgtCBR* operon (Fig. 1). As expected from its role controlling a central enzyme for bioenergetics, MgtC is regulated at the multiple levels: transcription initiation, elongation, translation, mRNA stability, and protein degradation.

Regulation at the level of transcription initiation

Transcription initiation of the *mgtCBR* operon is controlled by the PhoP/PhoQ two component regulatory system (Soncini *et al.*, 1996). When *Salmonella* experiences low levels of Mg^{2+} ions (Garcia Vescovi *et al.*, 1996), mildly acidic pH (Prost *et al.*, 2007) or antimicrobial peptides (Bader *et al.*, 2005), PhoQ sensor kinase is phosphorylated at the histidine residue (His 277), and then transfers its phosphoryl group to PhoP response regulator at the aspartic residue (Asp 57) (Chamnongpol and Groisman, 2000; Chamnongpol *et al.*, 2003). Phosphorylated PhoP binds to the target promoters including the promoter in front of the *mgtC* gene (Shin and Groisman, 2005) and activates transcription initiation of the target genes (Zwir *et al.*, 2014)(Fig. 1). Although PhoP activates *mgtC* transcription initiation from a single promoter located in front of the *mgtC* gene, a computational approach found two other PhoP-binding sites, which are located downstream of the experimentally determined *mgtC* promoter (Zwir *et al.*, 2014). The effects of two other PhoP-binding sites on *mgtCBR* expression are currently unknown.

Regulation at the level of transcription elongation

Transcription elongation into the downstream *mgtC* gene is controlled by the 296-nt leader mRNA. The leader mRNA

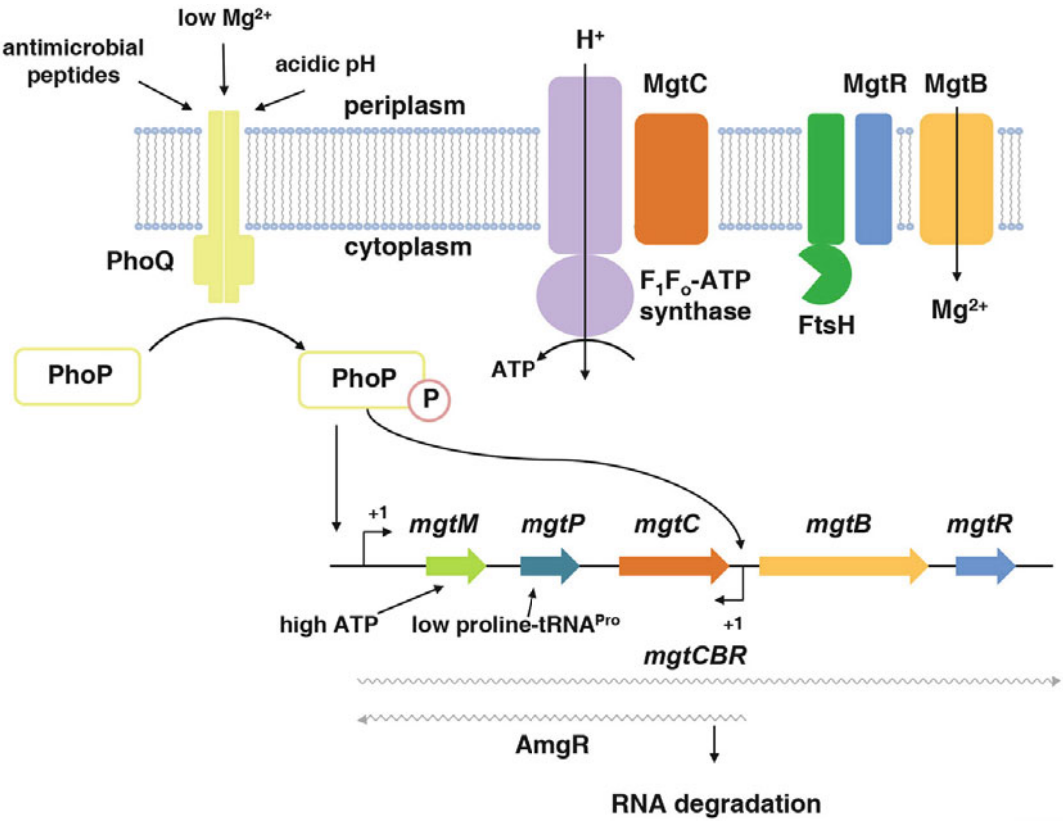

Fig. 1. Regulation of the *Salmonella mgtCBR* operon. When *Salmonella* experiences low Mg^{2+}, acidic pH, or antimicrobial peptides, the membrane-bound sensor kinase PhoQ protein phosphorylates the cytoplasmic DNA-binding protein PhoP. Then, phosphorylated PhoP binds to the promoter of the *mgtCBR* operon and activates transcription initiation. Transcription elongation is controlled by the long leader RNA harboring two open reading frames (ORFs), *mgtM* and *mgtP*. Coupling or uncoupling between transcription of the leader RNA and translation of short ORFs within the leader determines formation of one of two sets of alternative stem-loop structures. Depending on determined secondary structure, transcription elongation continues or discontinues into the downstream *mgtCBR* operon. PhoP also binds to the promoter region of the *amgR* with lower affinity and transcribes the AmgR antisense RNA, resulting in an RNase E-dependent degradation of the *mgtC* portion of the *mgtCBR* messages. The MgtC virulence protein binds to F_1F_o ATP synthase and maintains ATP homeostasis required for *Salmonella* pathogenesis during phagosome acidification. The MgtB Mg^{2+} transporter brings Mg^{2+} ions into the bacterium. The MgtR encodes a 30 aa-long peptide, which binds to MgtC protein resulting in MgtC proteolysis. The membrane-bound FtsH protease mediates MgtC degradation when it is accessible to MgtC protein (e.g., MgtC W226A).

harbors two short open reading frames (ORFs)-*mgtM* and *mgtP*-consisting of 12 aa and 17 aa respectively (Lee and Groisman, 2012a, 2012b) (Fig. 1). Each of ORFs is overlapped with sequences, which could form one of two alternative stem-loop structures. Therefore, translation of each ORFs determines formation of one of two alternative stem-loop structures and, depending on formed structure, it also determines whether or not the transcription continues into the first gene of the *mgtCBR* operon (Lee and Groisman, 2012a, 2012b). One thing to influence in this type of regulation is coupling/uncoupling of transcription of the leader mRNA and translation of short ORF located in the leader region. Because transcription and translation are usually coupled in bacteria (Martin and Koonin, 2006), tight coupling between transcription and translation allows the following ribosome to occupy 12–15 nt in the mRNA (Steitz, 1969) and thus favors formation of one distinctive set of stem-loop structures controlling transcription into the associated coding regions. However, a certain condition where transcription and translation could be uncoupled affects formation of alternative set of stem-loop structures, altering downstream gene expression. This is what is called transcription attenuation mechanism found in several biosynthetic operons (Henkin and Yanofsky, 2002). The *mgtCBR* operon is regulated by such a transcription attenuation-like mechanism (Lee and Groisman, 2012a, 2012b).

The presence of conserved A-nucleotides in *mgtM* and proline codons in *mgtP* (Fig. 2) enabled to identify two conditions that uncouple transcription and translation in the *mgtCBR* leader region. High levels of intracellular ATP uncouple transcription and translation at *mgtM* and promote formation of stem-loop A (Fig. 2), thereby allowing transcription to continue into the downstream leader region (Lee and Groisman, 2012a). Likewise, low levels of intracellular charged $tRNA^{Pro}$ uncouple transcription and translation at *mgtP* and favor formation of stem-loop E (Fig. 2), enhancing transcription of the *mgtC* gene (Lee and Groisman, 2012b). Interestingly, formation of stem-loop E could release ribosome binding site and the start codon of the *mgtC* gene sequestered in stem-loop D structure, further increasing MgtC translation. By contrast, either low levels of intracellular ATP or high levels of charged $tRNA^{Pro}$ tightly couple between tran-

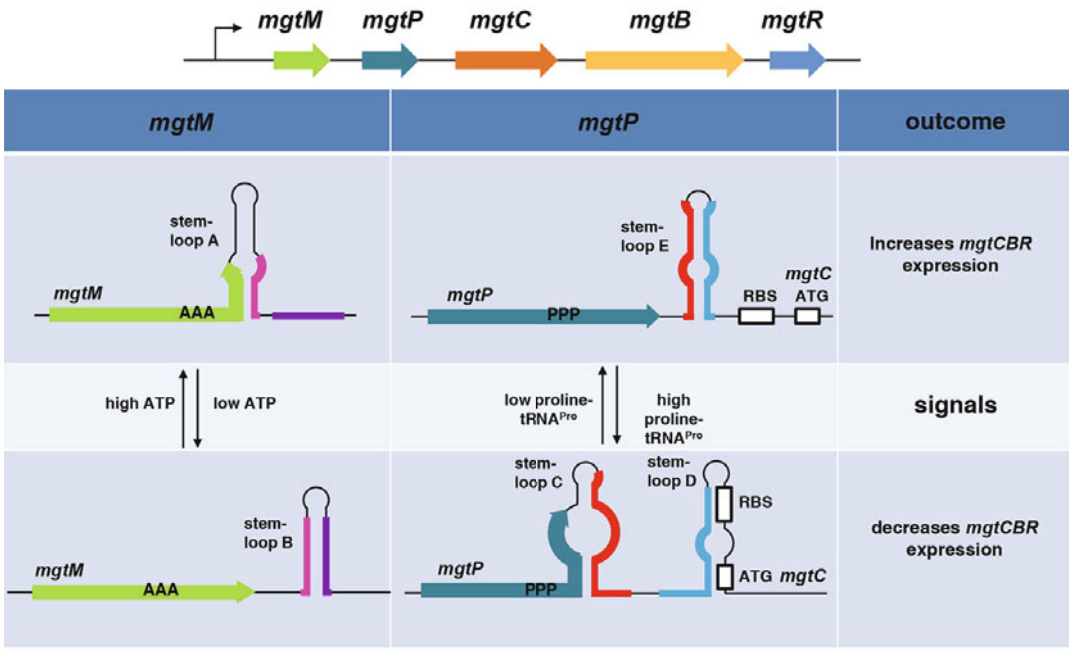

Fig. 2. Regulation of the *mgtCBR* operon by the ATP and proline-tRNA^{Pro}-sensing leader RNA. The *mgtCBR* leader RNA harbors the adenine nucleotides-rich *mgtM* and the proline codon-rich *mgtP*. Depending on intracellular ATP levels, coupling or uncoupling between transcription and translation controls formation of stem-loop A or B in the leader region associated with *mgtM*. Similarly, depending on intracellular charged-tRNA^{Pro} levels, coupling or uncoupling between transcription and translation controls formation of stem-loops C/D or E in the leader region associated with *mgtP*. Formation of stem-loop A or stem-loop E promotes transcription elongation into downstream regions, resulting in an increase in *mgtCBR* expression.

scription and translation, resulting in formation of stem-loop structures (stem-loop B and C/D respectively) that prevent transcription elongation into downstream *mgtCBR* operon (Lee and Groisman, 2012a, 2012b). Phagosome acidification creates the proton concentration across the bacterial inner membrane, hence stimulates ATP synthesis, resulting in an increase in intracellular ATP levels during infection (Lee and Groisman, 2012a). At the same time, low proline / hyperosmotic stress decreases proline levels available to charge tRNA^{Pro} (Lee *et al.*, 2014). Both high ATP and low charged tRNA^{Pro} levels contribute *mgtC* expression to be one of most highly expressed genes inside macrophages (Eriksson *et al.*, 2003). ATP and charged tRNA^{Pro} signals affect *mgtCBR* expression in an independent and additive manner (Lee and Groisman, 2012b). Nucleotide substitutions required for sensing intracellular ATP and charged tRNA^{Pro} levels attenuate *Salmonella* virulence in a mouse infection model (Lee and Groisman, 2012a; Lee *et al.*, 2014), indicating that both signals are critical for *Salmonella* virulence by controlling *mgtCBR* expression.

Regulation at the level of translation

Formation of stem-loop D structure was predicted by mfold software (Zuker, 2003) and determined by a chemical structure analysis such as in-line probing assay (Regulski and Breaker, 2008) using an *in vitro* synthesized *mgtCBR* leader RNA (Lee and Groisman, 2012b). Based on structural analysis, the RBS and the ATG translation start codon of the *mgtC* gene were occluded in the stem-loop D structure (Lee and Groisman, 2012b). Because stem-loop D would be formed whenever *Salmonella* experiences high levels of charged tRNA^{Pro} and thus transcription and translation are tightly coupled at *mgtP*, it needs to be addressed how MgtC could be translated in the presence of the stem-loop D structure.

Regulation at the level of mRNA stability

MgtC binds and inhibits F_1F_o ATP synthase (Lee *et al.*, 2013), which is a major machinery generating ATP in bacteria (Senior, 1990). Because of its unexpected role controlling a key enzyme in energy metabolism, *mgtC* expression itself is subject to multiple negative feedbacks at the posttranscriptional levels. mRNA stability control by the AmgR antisense RNA is one of them. When the PhoP/PhoQ two component system is activated by low Mg^{2+}, phosphorylated PhoP binds to the promoter region of the *mgtCBR* operon and transcribes *mgtCBR* messenger RNAs (Soncini *et al.*, 1996; Shin and Groisman, 2005). At the same time, PhoP also binds to the *amgR* promoter located in the intergenic region between the *mgtC* and *mgtB* genes and transcribes AmgR RNA towards the *mgtC* coding region (Lee and Groisman, 2010). Because AmgR has a perfect complementarity with the *mgtC* message, AmgR preferentially degrades the *mgtC* part of the *mgtCBR* messenger RNAs in an RNase E-dependent fashion (Lee and Groisman, 2010). PhoP binds to both promoters for the *mgtCBR* operon and the *amgR* with different affinities, the former higher and the latter lower, resulting in an initial increase but a subsequent decrease in MgtC protein levels relative to MgtB protein levels in a PhoP-inducing condition such as low Mg^{2+} media (Lee and Groisman, 2010). The *amgR* mutant has a hypervirulent phenotype in a mouse

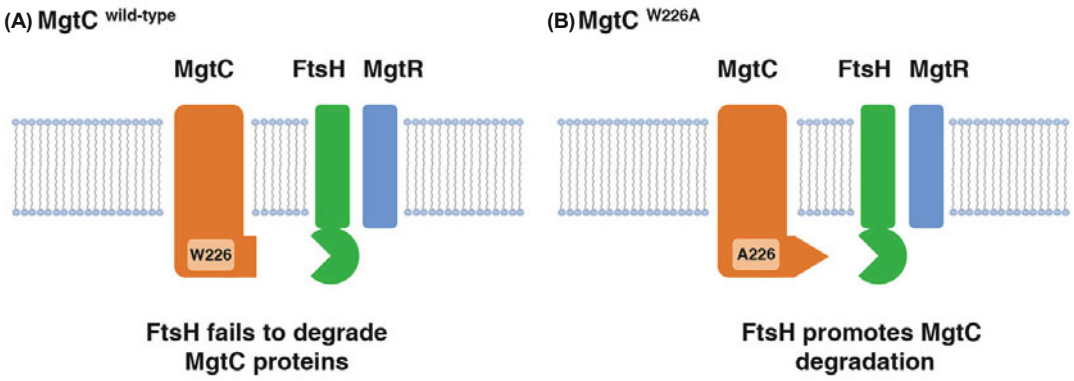

Fig. 3. Regulation of MgtC degradation by the FtsH protease. (A) In the wild-type MgtC protein, Trp226 protects from proteolysis by the membrane-bound FtsH protease. (B) The Trp226 to Ala substitution at the C-terminus of the MgtC protein accelerates the FtsH-mediated proteolysis. Even in the wild-type MgtC, MgtR overexpression promotes MgtC degradation independently of the MgtC Trp 226 residue.

infection model (Lee and Groisman, 2010), suggesting that establishing a certain ratio between MgtC and MgtB proteins is required for *Salmonella* virulence.

Regulation at the level of protein stability

i) MgtR regulatory peptide

The *mgtCBR* operon encodes a regulatory peptide MgtR in its third position (Alix and Blanc-Potard, 2008). The MgtR consists of 30 amino acids with one transmembrane helix (Alix and Blanc-Potard, 2008). Overexpression of the MgtR by a multicopy plasmid resulted in a decrease in MgtC protein levels, indicating that elevated levels of the MgtR peptides promote MgtC degradation. Interaction between the MgtR and MgtC proteins was demonstrated by the bacterial two-hybrid system (Karimova *et al.*, 1998). Because two amino acids identified as critical residues for MgtC degradation were located in the transmembrane domain (Leu15 and Ala24)(Alix and Blanc-Potard, 2008), it was suggested that interaction between the transmembrane domain of the MgtR and the fourth transmembrane domain (TM4) of the MgtC protein is important for MgtR-mediated MgtC down-regulation. Using an *Escherichia coli ftsH* temperature-sensitive mutant, it was proposed that the MgtR regulatory peptides promote MgtC degradation by the membrane-bound FtsH protease (Alix and Blanc-Potard, 2008). Although the FtsH protease is anchored by two transmembrane domains at its N-terminus, the catalytic active site for proteolysis is located in the cytoplasmic domain. Therefore, it is still unclear how interaction between MgtR and MgtC through transmembrane domains mediates MgtC proteolysis by the catalytic domain of the FtsH protease (Fig. 3). The *mgtR* mutant has a marginal defect in survival inside macrophages (Alix and Blanc-Potard, 2008).

ii) Counteraction by the W226 residue of the MgtC protein against the FtsH-mediated proteolysis

MgtC proteins are conserved in intracellular pathogens, all of which replicate within host macrophages during course of infection (Alix and Blanc-Potard, 2007). Several amino acid residues are conserved in all those pathogens and were analyzed by a complementation study (Rang *et al.*, 2007). Among them, the W226 residue is located at the C-terminus of the MgtC protein and the *mgtC* gene with substitution of tryptophan at position 226 to alanine (W226A) failed to complement a growth defect of the *mgtC* deletion mutant in low Mg^{2+} media (Rang *et al.*, 2007). It turned out that growth failure of the W226A substitution mutant in low Mg^{2+} media is because the W226A substitution accelerates MgtC proteolysis by the FtsH protease (Choi *et al.*, 2015). By acquiring an aromatic side chain at the position 226 such as tryptophan, the *Salmonella* MgtC protein seems to resist proteolytic degradation by the FtsH protease during infection because the tryptophan to phenylalanine substitution behaves like the wild-type MgtC protein (Choi *et al.*, 2015). The W226A substitution promotes MgtC proteolysis independently of MgtR's regulatory action (Choi *et al.*, 2015). Therefore, it still remains as a question how MgtR overexpression mediates proteolysis of the wild-type MgtC even in the presence of the W226 residue. Interestingly, macrophage survival of the *mgtC* mutant with the W226A substitution is comparable to that of wild-type *Salmonella* (Rang *et al.*, 2007; Choi *et al.*, 2015), suggesting that the FtsH protease might be less active inside macrophages.

Function of the MgtC virulence protein during infection

Maintaining ATP homeostasis during infection

In *Salmonella*, *mgtC* is the first gene in the *mgtCBR* operon. The *mgtC* gene often constitutes an operon with the *mgtB* gene, encoding the Mg^{2+} transporter (Blanc-Potard and Groisman, 1997; Lee and Groisman, 2010). Thus, it was initially proposed that MgtC protein could have a regulatory role in transporting Mg^{2+} into the bacterium or inserting MgtB into the membrane. Low Mg^{2+}-induced *mgtC* expression by the PhoP/PhoQ two component system and the growth defect of the *mgtC* mutant in low Mg^{2+} media seemed to be in agreement with the proposed function (Soncini *et al.*, 1996; Blanc-Potard and Groisman, 1997). However, all the data do not support the proposal. MgtC does not affect Mg^{2+} uptake for the MgtB transporter when investigated by $^{63}Ni^{2+}$ uptake or topology of the MgtB protein (Tao *et al.*, 1995). It was also suggested that MgtC activates host Na^+, K^+ ATPase by an

electrochemical study using a heterologous expression system in *Xenopus* oocytes (Gunzel *et al.*, 2006). And again, it seemed unlikely for MgtC as an innermembrane protein with five transmembrane helices to traverse outer membrane to activate host ATPase (Rang *et al.*, 2007). A recent biochemical approach has thrown light on a possible role of the MgtC protein in *Salmonella* pathogenesis. *In vivo* crosslinking experiment using a membrane-permeable crosslinker revealed that MgtC binds to a membrane-embedded F_o subunit of the F_1F_o ATP synthase, which drives ATP synthesis in bacteria (Lee *et al.*, 2013). MgtC's interaction to F_o subunit blocks proton translocation through F_o subunit across the innermembrane, thus inhibits ATP synthesis via F_1 subunit (Lee *et al.*, 2013). Whenever *Salmonella* experiences high ATP condition, the *mgtCBR* leader induces *mgtC* expression (Lee and Groisman, 2012a) and then MgtC protein inhibits ATP synthesis by targeting F_1F_o ATP synthase (Lee *et al.*, 2013), maintaining intracellular ATP levels back to physiological levels. Because *Salmonella* resides within an acidified vacuole compartment inside macrophages, which creates a large proton concentration gradient across the bacterial innermembrane, MgtC seems to promote *Salmonella*'s survival inside macrophages by preventing too much ATP being generated during phagosome acidification.

Repressing cellulose production during infection

The *mgtC* mutant has a distinctive growth phenotype in low Mg^{2+} media (Blanc-Potard and Groisman, 1997). It grows similar to wild-type until it reaches up to a certain optical density and then has a sharp decrease in optical density. This is because the *mgtC* mutant adheres together at the surface of a glass tube, constituting biofilms. The nature of exopolysaccharides holding the mutant cells together turned out to be cellulose (Pontes *et al.*, 2015). The *mgtC* mutant accumulates ATP levels and subsequently increases c-di-GMP levels, which activate the *bcsA* gene encoding a cellulose synthase (Pontes *et al.*, 2015). This means that *Salmonella* represses cellulose production during infection by expressing MgtC proteins. Interestingly, the *bcsA* mutant has a hypervirulent phenotype in a mouse infection model, indicating that cellulose acts as an antivirulence factor during *Salmonella* infection (Pontes *et al.*, 2015).

Conclusions

Classical virulence proteins are usually toxins, or secreted effectors targeting host cells or modulating host physiology to accommodate pathogen's fitness during infection. MgtC is an atypical virulence protein because it modulates bacterium's own physiology to promote *Salmonella* pathogenicity. Specially, MgtC targets one of a central enzyme in energy metabolism and thus controls bacterium's energy currency-ATP. Such an alteration in ATP levels impacts *Salmonella*'s entire physiology to promote pathogenicity and to repress antivirulence traits during infection. At the same time, a number of signals act on various levels of MgtC expression, thus fine-tuning MgtC production during *Salmonella* infection.

Author Contributions

E.L. wrote the paper. J.L. made the figures. All authors read the paper and contributed to its final form.

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