THE EFFECTS OF THE H-NS PROTEIN ON PHOP-DEPENDENT TRANSCRIPTIONAL REGULATION OF THE *mgtCBRU-cigR* OPERON IN *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

by

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I would like to dedicate this work to all my friends and family that supported and encouraged me throughout my graduate studies. I would also like to thank my advisor, Dr. Laszlo N. Csonka, for his faith in my scientific abilities.

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ABSTRACT

PhoQP is a two-component system that regulates the transcription of ~5% of the genes of *Salmonella enterica*. The membrane-bound PhoQ protein is phosphorylated in response to low extracellular Mg^{2+} concentration, acid pH, and a number of antimicrobial peptides. The inorganic phosphate bound to PhoQ is transferred to PhoP, which according to the classical model, acts as a typical transcriptional activator of its target genes. However, Will et al. (doi.org/10.1038/ncomms6270) proposed an alternate "counter-silencing" model, according to which genes in the PhoP regulon that were acquired by *Salmonella* via horizontal transfer are repressed by the generalized DNA-binding protein H-NS at high [Mg^{2+}] and are induced at low [Mg^{2+}] because the phosphorylated PhoP displaces the H-NS from the promoters and lifts repression. We evaluated this model by examining the transcriptional regulation of the *mgtCBRU-cigR* operon, which encodes the virulence protein MgtC and the Mg^{2+} transport protein MgtB and is in the SPI-3 pathogenesis island that has been acquired by *Salmonella* via horizontal transfer. Our main finding was that in the non-pathogenic strain of *S.* Typhimurium (LT2), induction of the *mgtCBRU-cigR* operon by Mg^{2+} limitation requires a functional PhoP protein, regardless of the presence or absence of H-NS. Interestingly, the pathogenic strain of *S.* Typhimurium (ATCC 14028s) revealed PhoP-independent transcription in the absence of H-NS, but only under inducing conditions. Thus, our results do not support the counter-silencing model and are consistent with the canonical view that PhoP is needed as a transcriptional activator of genes in the PhoP regulon.

CHAPTER 1. INTRODUCTION

1.1 Overview

The *Salmonella enterica* serovars are Gram-negative, rod-shaped, facultatively anaerobic bacteria that belong to the *Enterobacteriaceae* family. This species is infamous for causing typhoidal and nontyphoidal enteric diseases that are clinically manifested as gastroenteritis or systemic illness such as typhoid fever (Boyle et al., 2007; Nielsen et al., 2013). Many of the serotypes are a major concern for health organizations and food manufacturers as they lead to high morbidity and mortality rates amongst humans and their broad host ranges makes their containment more difficult (Boyle et al., 2007). Humans become infected when they consume contaminated food or water. Pathogenesis by *Salmonella enterica* involves invasion of intestinal epithelium cells and causing localized infection or disseminating throughout the body through "hiding" in macrophages and evading the host immune response (Falkow, 1996). To perform its various mechanisms of evasion and adaptation to the host environment, like many bacterial pathogens, *Salmonella* possesses numerous variety of virulence genes to permit its survival.

Genes that contribute to the pathogenesis of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium hereafter) tend to be clustered in what are referred to as pathogenicity islands, or more specifically, *S.* Typhimurium pathogenicity islands (SPIs) (Gerlach & Hensel, 2007). These horizontally acquired group of genes are regulated in response to environmental signals such as pH, osmolarity, temperature, antimicrobial peptides, as well as other signals so that cells can continue proliferation when they encounter environmental stressors. The host's immune system can create an unfavorable host environment by using many defense mechanisms that are detrimental to the survival of a foreign microbe, one of which being various phagocytes. *S.* Typhimurium, however, is an intracellular pathogen and it uses several virulence factors to survive

and maintain its own protective compartment termed the *Salmonella*-containing vacuole (SCV) to avoid death via the endocytic pathway in macrophages (Abrahams & Hensel, 2006; Gerlach & Hensel, 2007). Numerous virulence genes belonging to different SPIs are activated to promote pathogenesis but are primarily under the control of two-component regulatory systems (TCS), which are signal transduction systems involving membrane-bound sensor kinases responding to environmental changes coupled with transcriptional regulators that either induce or repress virulence genes within their regulon (Bijlsma & Groisman, 2003).

Though it can avoid acidification within a phagolysosome, *Salmonella* experiences environmental stressors within the SCV, including antimicrobial peptides, reactive oxygen species, acidic pH, and low nutrient/metabolite availability, which trigger the PhoQP two-component regulatory system to regulate gene expression to respond to those stressors (Abrahams & Hensel, 2006; Véscovi et al., 1996). In this system, the sensor kinase PhoQ has two transmembrane regions with a cytoplasmic C-terminal domain containing a histidine residue where autophosphorylation occurs when PhoQ detects environmental changes via the periplasmic region of the protein (Groisman, 2001). The inorganic phosphate molecule is then transferred to the transcriptional regulator PhoP, which according to the classical model enables it to bind to the promoters of various genes within its regulon (Figure 1.1). PhoP is responsible for regulating ~5% of the genes in *S.* Typhimurium and does so by binding to a direct repeat motif, or the PhoP box, near the promoter region (Heithoff et al., 1999; Zwir et al., 2012). PhoP's binding to its specific locations promotes transcription by RNA polymerase. Alternatively, when there are no environmental changes and conditions are optimal, expression of virulence genes is down-regulated as phosphorylated PhoP is dephosphorylated by PhoQ (Groisman, 2001). One of the activating

signals for the PhoQP TCS is low external concentration of Mg^{2+} and is needed for the adaptation to the intracellular concentration of Mg^{2+} in the host (Groisman, 1998; Rychlik & Barrow, 2005).

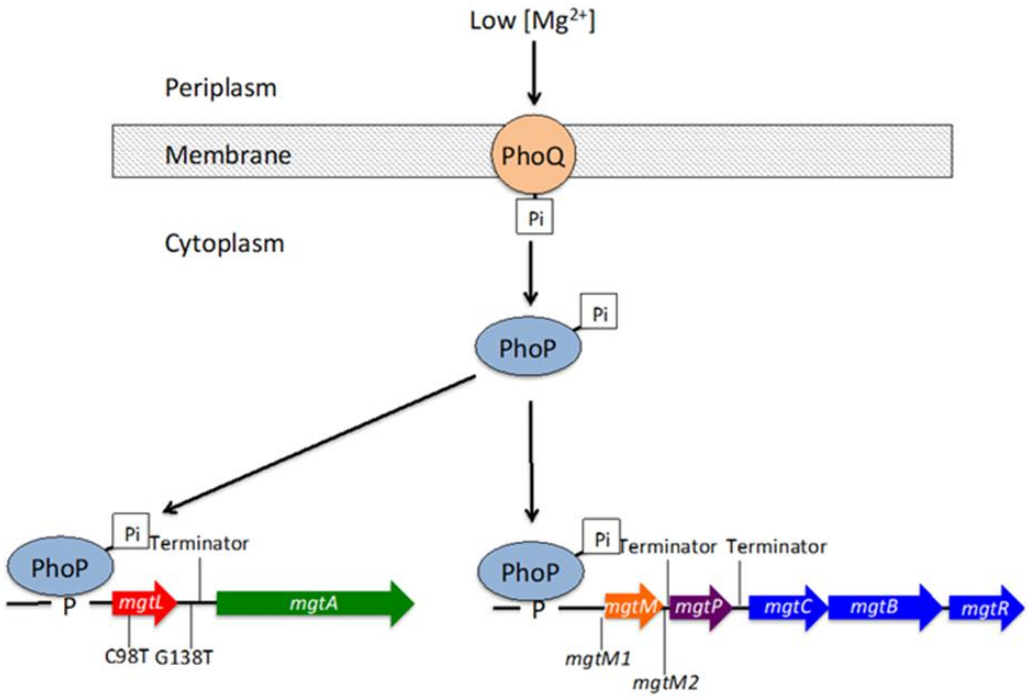


Figure 1.1: **Transcriptional control of the *mgtA* gene and *mgtCBRU-cigR* operon.** The PhoQP two-component system activates transcription from the promoter (P) of the *mgtA* gene and the *mgtCBRU-cigR* operon at low external [Mg^{2+}], acid pH, or some antimicrobial peptides. The 5' leader of the mRNA of *mgtA* encodes a 17-amino acid ORF *mgtL* and 5' leader of the *mgtCBRU-cigR* mRNA encodes a 15-amino acid ORF *mgtM* and a 17-amino acid ORF *mgtP* while also possessing a Rho-dependent terminator sequence.

Mg^{2+} is essential for various biological functions in all organisms. It is imperative for efficient nucleoside triphosphate-dependent phosphorylations, catalysis of multiple metabolic processes, and stabilization of the inner and outer membranes (Silver, 1996; Papp-Wallace & Maguire, 2008). Because this divalent cation plays such a key role, proper influx and efflux is necessary for magnesium homeostasis. Proteins responsible for magnesium homeostasis in *S.* Typhimurium are CorA and MgtB and MgtA (Kehres & Maguire, 2002). CorA is a constitutively expressed, bi-directional channel that is the primary Mg^{2+} transporter, and the P-type ATPases MgtA and MgtB are responsible for additional uptake when Mg^{2+} is limited in the environment (Papp-Wallace & Maguire, 2008). MgtA in *S.* Typhimurium shares 91% homology with MgtA in

E. coli, but MgtB is only present in few species within the *Enterobacteriaceae* family, suggesting that it was horizontally-acquired (Papp-Wallace & Maguire, 2008). Mutations that cause high-level, constitutive expression in MgtA and MgtB have been shown to enhance thermotolerance in *S.* Typhimurium (Gall et al., 2018; O'Connor et al., 2009).

The PhoQP TCS regulates the expression of genes *mgtA* and *mgtB*. The *mgtB* gene is in the *mgtCBRU-cigR* operon, in which the *mgtC* gene encodes an inner membrane protein that is required for intramacrophage survival, virulence in mice, and proliferation in low Mg^{2+} conditions. This protein, which is not involved in Mg^{2+} transport directly, has been proposed to be an inhibitor of the F_0F_1 ATPase (J.-W. Lee & Lee, 2015) but its function is not fully known (Lee , & Groisman, 2010; Papp-Wallace & Maguire, 2008). MgtR is a 30 amino acid-long peptide that aids the turnover of MgtC, MgtA, and MgtB by the protease FtsH (Choi et al., 2012; E.-J. Lee & Groisman, 2010; Papp-Wallace & Maguire, 2008; Yeom et al., 2020). The 28-amino acid long MgtU has been proposed to protect MgtB from FtsH-mediated proteolysis (Yeom et al., 2020). The *cigR* gene, which encodes an inner membrane-bound inhibitor of the degradation of the F_0F_1 ATPase by MgtC, is transcribed from the *mgtCBRU-cigR* promoter as well as its own promoter (Yeom et al., 2018).

In addition to regulation by PhoP, the 5' leader regions of the *mgtA* and *mgtCBRU-cigR* mRNAs contain short open reading frames (ORFs): *mgtL* (for *mgtA*), and *mgtM* and *mgtP* (for *mgtCBRU-cigR*) that contribute to the transcriptional regulation of *mgtA* and *mgtCBRU-cigR* (Gall et al., 2018). The short ORF *mgtL* encodes a proline-rich leader peptide whose translation is sensitive to the Mg^{2+} concentration and controls the efficiency of progression of transcription into the *mgtA* gene in response to the availability of Mg^{2+} (Gall et al., 2016). The two tandem ORFs *mgtM* and *mgtP* transcriptionally attenuate *mgtCBRU-cigR* expression in an analogous manner. The short ORFs of *mgtA* and *mgtCBRU -cigR* provide a secondary level of control that is analogous

to the functioning of the regulatory leader transcripts of the *his* and *trp* operons where, contingent on nutrient availability, alternative stem loop structures form to either permit or terminate read-through of the structural genes (Gall et al., 2018; Kolter & Yanofsky, 1982). The function of *mgtP* is not as well-characterized as that of *mgtM* (Gall et al., 2018). Between the latter two ORFs there is a terminator sequence that is recognized by an RNA helicase termed Rho and stops transcription when Mg^{2+} is high (Gall et al., 2018; Hollands et al., 2012).

Another component of *mgtCBRU-cigR*'s regulation has been suggested to be the histone-like nucleoid structuring protein H-NS, which is a DNA-binding, global transcriptional repressor that has been proposed to be involved in "xenogenic" silencing of many horizontally acquired genes, including *mgtCBRU-cigR*, to help integrate them into *S.* Typhimurium's established regulatory network (Navarre, 2006; Will et al., 2014). It is well accepted that virulence factors were acquired through horizontal transfer, but the caveat is that inordinate transcription of such newly acquired genes may place excessive drain on energy and reduce the fitness of the bacteria (Navarre et al., 2007). To combat this, H-NS recognizes AT-rich sequences and silences transcription by polymerization that bridges or bends target DNA regions, forming loops, or increasing the rigidity of DNA (Navarre et al., 2007; Will et al., 2014). In regard to the PhoQP TCS, newly acquired foreign genes within this regulon can be repressed by H-NS. Transcription of *mgtCBRU-cigR* has been proposed to be typically repressed by H-NS in high Mg^{2+} conditions, and induction at limiting Mg^{2+} is brought about by phosphorylated PhoP displacing H-NS and thereby relieving repression (Will et al., 2014).

1.2 Statement of the problem

Previous researchers in this lab discovered that high-level transcription of the *mgtA* or *mgtCBRU-cigR* operon genes confers enhanced heat tolerance in *S.* Typhimurium (O'Connor et al., 2009). Although we do not know how Mg^{2+} homeostasis is connected to heat tolerance, this discovery is what led the lab to tackle the elucidation of the mechanism of regulation of the Mg^{2+} transport genes. This investigation is significant in that it can enable us to close the gap in understanding the relationship between Mg^{2+} homeostasis and enhanced heat tolerance, yielding information that can be used to prevent increased thermotolerance of *S.* Typhimurium during food preparation. The aim of my project was to study the transcriptional regulation of the *mgtCBRU-cigR* operon (Figure 1.2), and within that objective, our interests lie in the effect that the DNA-binding H-NS protein has on the transcriptional regulation of the operon. As stated previously, PhoP is a known transcriptional activator of a regulon that consists of over 100 genes, including the *mgtCBRU-cigR* operon, *mgtA*, *pagC*, and *phoP* itself.

Contrary to the accepted PhoP model according to which phosphorylated PhoP assists the binding of RNA polymerase to PhoP-regulon promoters, Will et al. suggested that this protein acts as a "counter-silencer" against H-NS during induction of *pagC* expression by displacing H-NS from the promoter of this gene at low Mg^{2+} conditions (Will et al., 2014) (Figure 1.3). Based on bioinformatic analysis, they argued this to be the case also for the *mgtCBRU-cigR* operon, although they did not provide enough supportive experimental evidence. This conclusion seems unlikely for *mgtCBRU-cigR* regulation since, based on our lab's own observations, PhoP has a key role in regulation that is beyond solely antagonizing H-NS. To assess the effect of H-NS, I evaluated the model proposed by Will et al. by investigating the transcriptional regulation of the *mgtCBRU-cigR* operon by measuring mRNA levels via qRT-PCR.

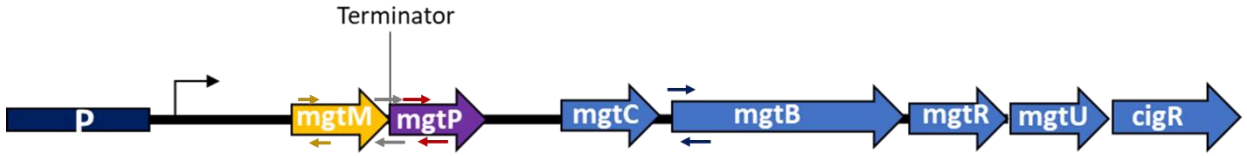


Figure 1.3: **Schematic of the organization of the *mgtCBRU-cigR* operon.** The small arrows represent locations of forward and reverse primers that were used to amplify *mgtM*, the Rho-dependent terminator sequence, *mgtP*, and *mgtB* for qRT-PCR. The box containing the letter "P" represents the promoter and the large black arrow indicates the transcription start site and its orientation

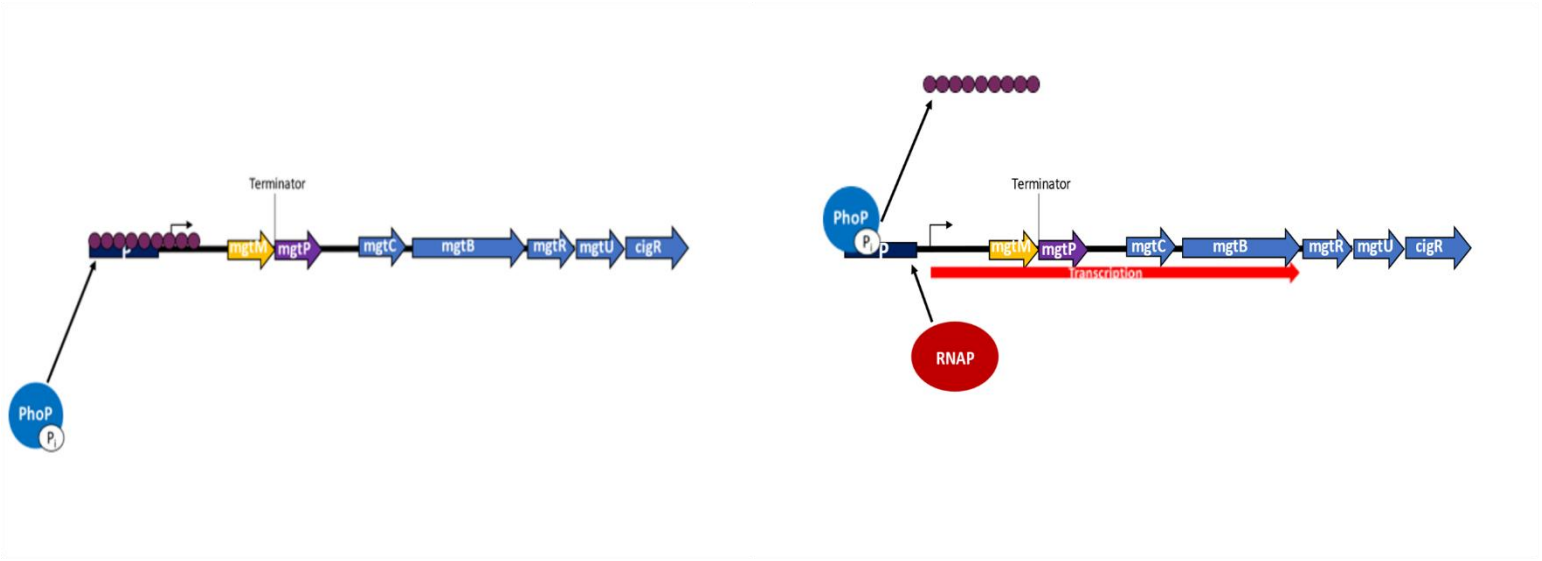


Figure 1.2: **Counter-silencing mechanism of PhoP as proposed by Will et. al at the *mgtCBRU-cigR* promoter.** At horizontally-acquired genes such as *mgtCBRU-cigR*, H-NS (purple) is bound to the promoter and represses its transcription. At low Mg^{2+} conditions, phosphorylated PhoP binds to the PhoP boxes near the promoter and displaces H-NS, allowing RNA polymerase to bind and initiate transcription.

CHAPTER 2. MATERIALS AND METHODS

2.1 Media and growth conditions

Cells were grown in Luria-Bertani/Lysogeny Broth (LB) (Davis et al., 1980), and potassium morpholinopropane sulfonate (MOPS) media (Neidhardt et al., 1974), and SOB media (Sambrook et al., 1982). Green plates used for isolation of phage P22-free, P22-sensitive transductants were made up according to (Davis et al., 1980). Solid media were made by the addition of 20 g/ l agar (Fisher Scientific Co.). As needed, media were supplemented with following antibiotics: chloramphenicol at 15µg/ml, kanamycin at 75µg/ml, tetracycline at 20µg/ml, ampicillin at 100 µg/ml (*& 50µg/ml). Media was supplemented with 2mM or 10 mM glucose and/or 0.05% casamino acids. Strains were grown aerobically at *30℃ or 37℃.

2.1.1 Growth in LB, MOPS media, and SOB media

Strains were grown in 2ml of LB liquid medium until stationary phase overnight. Then, cultures were diluted 1:100 in 5ml of MOPS medium containing either low (0.016mM) or high (2mM) $MgSO_4$, supplemented with 10 mM glucose and 0.05% casamino acids and grown overnight. After the overnight growth, cells were diluted to an OD_{600} of ~0.1 in their respective MOPS media containing 0.016 mM or 2mM $MgSO_4$ and supplemented with 10 mM glucose and 0.05% casamino acids. The diluted cells were grown to mid-logarithmic phase to stationary phase growth (OD_{600}= 0.5 to 2). Then, the RNA isolation procedure followed.

*To make electrocompetent cells for transformation, cultures were grown in 2ml of SOB + ampicillin at 50µg/ml at 30℃ overnight. The following day, cultures were diluted 100-fold in 50ml SOB + ampicillin at 50µg/ml and 10mM L-arabinose for approximately 3 hours at 30℃.

* denotes information relevant to the data in the Appendix

Cells were then harvested and washed three times in ice-cold 10% glycerol. The linear DNA transformation protocol followed.

2.2 Strain construction

2.2.1 P22 phage transduction

Protocol was adapted from Davis et al. (1980).

2.2.2 *Linear DNA transformation

Protocol was adapted from Datsenko & Wanner (2000). The GibcoBRL® Cell Porator® Electroporation System, 230 V and the GibcoBRL® Cell Porator® Voltage Booster, 115V was used to electroporate cells with 500ng of PCR product containing the chloramphenicol resistance gene.

2.3 RNA isolation and qRT-PCR protocol

RNA isolation began with transferring cells grown to mid-logarithmic or stationary phase into 4ml of RNAprotect Bacteria Reagent (Qiagen, Inc.) and performed as suggested by the manufacturer's protocol. All strains were harvested in triplicate from independent colonies. Pelleted cells were stored overnight at -70°C. On the following day, the pelleted cells were thawed at room temperature and subsequent steps were performed based on the RNeasy Mini Kit manufacturer's protocol (Qiagen, Inc.). DNase digestion using the RNase-Free DNase Set (Qiagen, Inc.) was performed during the RNA on-column isolation. The RNA concentration of each sample was measured using the NanoDrop2000 (Thermo Fisher Scientific, Inc.). Next, the RNA samples were treated with DNase again using the Turbo-DNA free kit (Invitrogen, Inc.). RNA

* denotes information relevant to the data in the Appendix

concentration was measured again, and RNA integrity was observed through 1% agarose gel electrophoresis using 1µg of RNA.

To prepare for qRT-PCR, 1µg of each RNA sample underwent cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, Inc.). Next, cDNA and control samples without reverse transcriptase were diluted to a concentration of 5ng/µl. QRT-PCR was performed using LightCycler® SYBR Green I Master Mix (Roche LifeSciences, Inc.) and the LightCycler® 96 thermocycler following manufacturer's instructions.

Data analysis involved calculating fold-change expression of each gene using the $2^{-\Delta\Delta Ct}$ method as described by Livak & Schmittgen, 2001. The internal control gene used was *gnd*, which specifies the pentose phosphate pathway enzyme gluconate-6-phosphate dehydrogenase that is expressed constitutively in all conditions (Chen et al., 2010; Isturiz & Wolf, 1975). The calibrator used to normalize the mRNA level of each gene was the respective level of expression of the gene in the wild-type strain in high Mg^{2+} conditions.

2.4 Bacterial strains

Table 2.1: List of bacterial strains

Name	Genotype[1]	Source of construction
***Salmonella enterica* serovar Typhimurium**		
TL 1	wild-type *Salmonella enterica* serovar Typhimurium LT2, attenuated	From J. L. Ingraham
TL 6566	*hns-1*::IS*10 zde-1710::Tn10*, LT2 background	L. Csonka lab
TL4299	*phoP102::Tn10d-Cam*, LT2 background	L. Csonka lab
TL6698	*phoP102::Tn10d-Cam hns-1*::IS*10 zde-1710::Tn10*, LT2 background	This study
ATCC 14028s	wild-type *Salmonella enterica* serovar Typhimurium 14028s, pathogenic	From S. Porwollik
TLa6558	*hns-1*::IS*10 zde-1710::Tn10*, ATCC 14028s background	L. Csonka lab
TLa6731	*phoP102::Tn10d-Cam*, ATCC 14028s background	This study
TLa6733	*phoP102::Tn10d-Cam hns-1*::IS*10 zde-1710::Tn10*, ATCC 14028s background	This study
***TL4733**	TL155 / pKD46, LT2 background; used to insert Cm^R sequence between 4A and 5T in the *mgtCBR-cigR* mRNA 5' leader region (refer to Datsenko & Wanner)	From K. Datsenko
***TLa6793**	Cm^R: JLM1, where JLM1 is the name for the Cm^R insertion between nucleotides 4 and 5 of the *mgtCBR* mRNA 5' leader region, ATCC 14028s background	This study

* denotes information relevant to the data in the Appendix

Name	Genotype[1]	Source of construction
***Salmonella enterica* serovar Typhimurium**		
***TLa6797**	Cm^R*:* JLM 1, *hns-1*::IS*10 zde-1710::Tn10,* ATCC 14028s background	This study
***TLa6801**	Cm^R*:* JLM 1, *phoP*::Kan, ATCC 14028s background	This study
***TLa6803**	Cm^R*:* JLM 1, *phoP*::Kan, *hns-1*::IS*10 zde-1710*::Tn*10,* ATCC 14028s background	This study

[1]The *hns-1*::IS*10* mutation, formerly called *osmZ1* (Higgins et al., 1988) is an insertion of IS*10* after codon 87 of the 137-codon *hns* gene.

phoP102::Tn*10d-Cam* insertion is located approximately 0.46 kbp downstream of the translation start site of the 0.672 kbp *phoP* gene (Miller et al., 1989).

2.5 Primer list

Table 2.2: Primers used in this work

Name	Sequence (5' to 3')	Comments
mgtB	AAACCGGAAACTCAATCGCC	
	CGTTCGCTGGCCTCTTCTAT	
mgtA	TTGTGCATATGATCCGTACCC	
	ATCAGCCACGGGAAATAGC	
phoP	TTCCACATCGAAGAGGTAATGG	
	GCGTTTCCATAATGGTGTATTCG	
pagC	TGAATCCGCTGGAGAATATCG	
	TCAGAAACGGTATCCAACCC	
mgtM	ATTCATGCAGGAGTAATATGTTGG	
	GCTGCCCACCGGTTC	
term	ACGTAAATCATCTGGCAAGT	Binds to Rho-dependent terminator sequence between *mgtM* and *mgtP* (Gall et al., 2018)
	TGAACATGACGATTACCTCG	
mgtP	ATAATGTTACAACACGCGCA	
	CGACAAATTTCTGCCCTGA	
gnd	CTGGTCACTACGTCAAGATGG	Internal control gene for qRT-PCR; encodes gluconate-6-phosphate dehydrogenase
	GTTCGCCAACTCTTCATTGC	
***JLM 12 FW and JLM 13 RV**	CACTTTACGGAACATATTGGCTGACTATAATAAGCGCAAAGAAATAAGATCACTACCGGG	Amplifies chloramphenicol resistance gene and used to insert it between nucleotides 3,965,515 and 3,965,516 upstream of *mgtM*
	TTACGTAAAAGTGACTGTCCAACATATTACTCCTGCATGAAAAAATTACGCCCCGC	
***LC 20 and LC 21**	CTATCGCCGGTATTAAGCAG	Used to check if chloramphenicol insertion is at the correct location; amplifies sequences between nucleotides 3,964,162 and 3,965,651
	AGTGTCTCCTCCGGGCTATG	
***Cm^R FW and RV**	TCAGGAGCTAAGGAAGCTAA	Amplifies inserted chloramphenicol sequence for qRT-PCR
	CTGAACGGTCTGGTTATAGG	

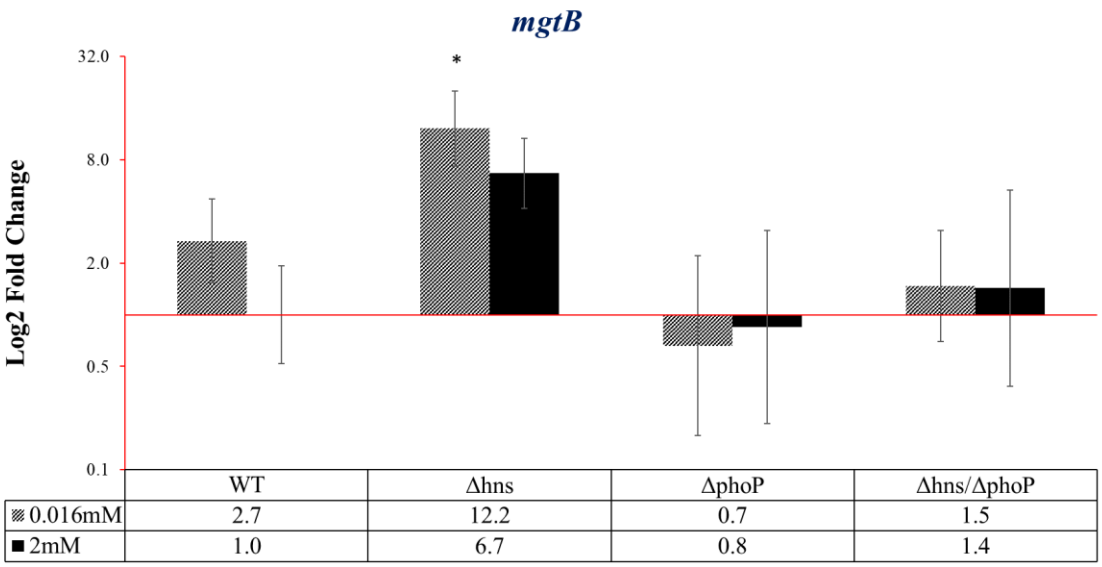
* denotes information relevant to the data in the Appendix

CHAPTER 3. THE EFFECTS OF H-NS ON THE REGULATION OF THE *mgtCBRU-cigR* OPERON

3.1 Inactivation of *hns*, *phoP*, or both in *S.* Typhimurium strain LT2

3.1.1 PhoP regulon genes

To investigate potential effects H-NS may have on the transcription of PhoP regulon genes, we looked for changes in the expression of the PhoP-dependent genes *mgtB*, *mgtA*, *phoP*, and *pagC* in strains that carry *hns*, *phoP* single mutations or *hns phoP* double mutations by performing qRT-PCR to measure gene expression. All cultures were grown to mid-logarithmic phase (OD_{600} = ~0.6) in MOPS medium with either low concentration (0.016 mM; inducing) or high concentration (2 mM; non-inducing) of Mg^{2+}. Despite previous reports of *hns* mutants being unviable in the otherwise wild-type background (Will et al., 2014), we were able to isolate transductants with the *hns-1*::IS*10* (*osmZ1*) mutation. The single *phoP* and double *hns phoP* mutants were constructed by transduction with the *phoP102*::Tn*10d*-Cam mutation. In the wild-type background, modest 2.7-fold up-regulation was observed under the inducing condition of 0.016 mM Mg^{2+} compared to that seen in control cells grown with 2 mM Mg^{2+} (Figure 3.1), a result that is consistent with previous reports of the regulation of *mgtCBRU-cigR* regulation (Snavely et al., 1991; Lee and Groisman, 2012; Gall et al. 2018). In accord with data presented by Will et. al (2014), inactivation of *hns* caused an up-regulation in the transcription of *mgtB* in both inducing (0.016 mM Mg^{2+}) and non-inducing (2 mM Mg^{2+}) conditions, and inactivation of *phoP* in the *hns*$^{+}$ strain reduced expression to low, background level. However, our data suggests that PhoP is necessary for *mgtB*'s transcription, even in the absence of H-NS, due to the *hns phoP* double mutant strain exhibiting similar low induction in both low and high [Mg^{2+}] conditions as the *phoP* single mutant.



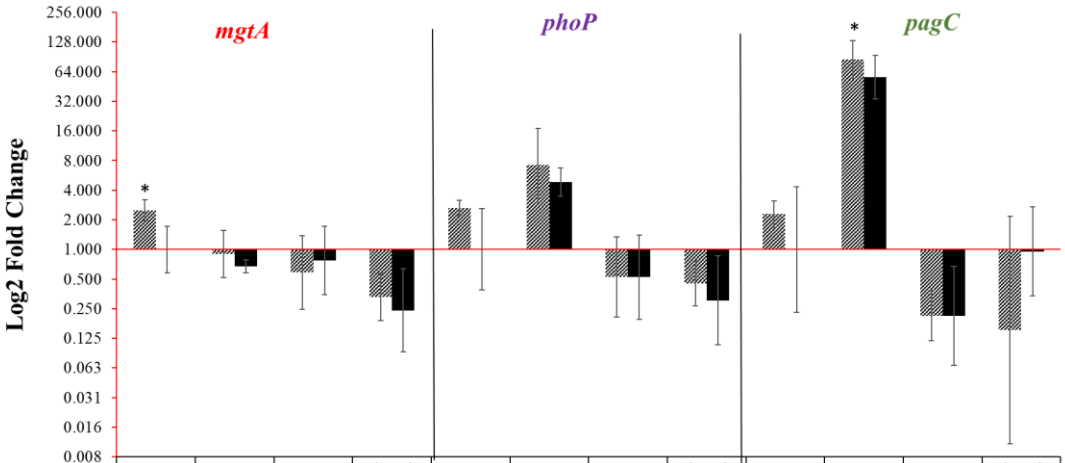
	WT	Δhns	ΔphoP	Δhns/ΔphoP
▨ 0.016mM	2.7	12.2	0.7	1.5
■ 2mM	1.0	6.7	0.8	1.4

Figure 3.1: **Fold-change expression levels of *mgtB* in *hns*::IS*10, phoP,* and *hns*:: IS*10 phoP* strains in the LT2 background grown to mid-log phase at low (0.016 mM) and high (2 mM) Mg^{2+} concentrations.** Expression was normalized to the internal control constitutive gene *gnd* (gluconate-6-phosphate dehydrogenase) and then normalized again to the wild type at high Mg^{2+} concentrations.

For other PhoP regulon genes, *phoP*, *pagC*, and *mgtA*, a similar trend was observed (Figure 3.2). The absence of H-NS significantly enhanced the expression of *pagC* in both low and high [Mg^{2+}], in accord with a result reported by Will et al. (2014). However, I observed that the expression of *pagC* was reduced to background levels in both low and high [Mg^{2+}] in the *hns phoP* double mutant. It should be noted that this result is inconsistent with the model proposed by Will et al. because if PhoP were an antagonist of the repression *pagC* by H-NS, then *pagC* would be expressed at constitutively high levels in *hns* mutants under all conditions. Up-regulation by the *hns* mutation was not observed for *mgtA* but Will et. al (2014) suggested that this gene may be H-NS independent because it is a core gene in *Enterobacteriaceae*, and H-NS regulates horizontally transferred foreign DNA in that family.

We also investigated the regulation of expression of *phoP* in the wild-type and in the three mutant backgrounds. It should be noted that the *phoP102*::Tn*10d-Cam* insertion that we used is

located approximately 0.46 kbp downstream of the translation start of the gene (Miller et al., 1989) whereas the primers we employed in the qRT-PCR analysis hybridized to nucleotides 322 to 343 and 474 to 496 of *phoP*, and therefore we were able to monitor the transcription of this gene in the absence of functional PhoP protein. *phoP* is a core gene as well, but H-NS plays only a modest role in its regulation. This could suggest that *phoP* may require additional regulatory measures to prevent unnecessary induction. Levels of expression in the *phoP* mutant at both Mg^{2+} concentrations were down-regulated, but not as drastically as expected. Nonetheless, these results demonstrate that these genes follow the canonical model of PhoP promoter activation, irrespective of the presence or absence of H-NS.



	WT	Δhns	ΔphoP	Δhns/ΔphoP	WT	Δhns	ΔphoP	Δhns/ΔphoP	WT	Δhns	ΔphoP	Δhns/ΔphoP
▨ 0.016mM	2.5	0.9	0.6	0.3	2.6	7.2	0.5	0.5	2.3	85.2	0.2	0.2
■ 2mM	1.0	0.7	0.8	0.2	1.0	4.8	0.5	0.3	1.0	56.5	0.2	1.0

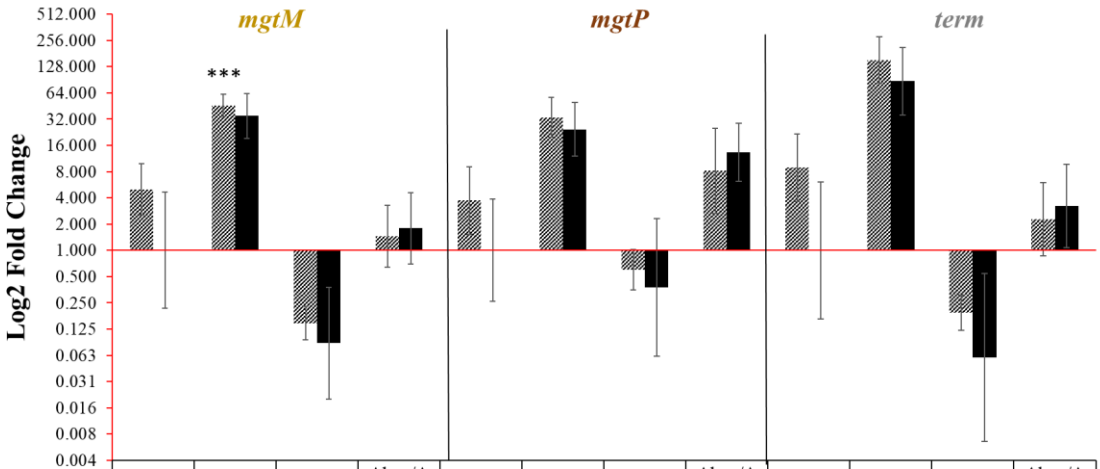
Figure 3.2: **Fold-change expression levels of other PhoP regulon genes in *hns*::IS*10*, *phoP*, and *hns*:: IS*10* *phoP* strains in the LT2 background grown to mid-log phase at low (0.016 mM) and high (2 mM) Mg^{2+} concentrations.** Expression was normalized to the internal control constitutive gene *gnd* (gluconate-6-phosphate dehydrogenase) and then normalized again to the wild type at high Mg^{2+} concentrations**.**

3.1.2 Open-reading frame sequences in the 5' leader region of *mgtCBRU-cigR* mRNA

How the 5' leader region of the *mgtCBRU-cigR* operon that contains the *mgtM* and *mgtP* regulatory ORFs behaved when either *hns*, *phoP*, or both were missing was also investigated. In

accord with the results observed with *mgtB*, inactivation of *hns* resulted in up-regulation of the mRNA sequences containing these ORFs at low and high [Mg^{2+}] (Figure 3.3), demonstrating that H-NS represses expression upstream of the regulatory ORFs. Inactivation of H-NS had the greatest effect on the terminator sequence. Interestingly, loss of H-NS did not cause as great an increase in transcription of *mgtB* as it did in the transcription of the upstream regulatory ORFs (Figure 3.1).

Another interesting finding was that the transcription of these genetic elements was higher in the *hns phoP* double mutant than the seen in the *phoP* single mutant at both Mg^{2+} concentrations, initially suggesting that the anti-silencer model of Will et al. is possible. However, the *hns* single mutant exhibited more elevated expression at both conditions than the *hns phoP* double mutant instead of similar levels after de-repression for *pagC* described by Will et al. (2014) in the *hns* single mutant and the *hns phoP* double mutant. My data show that PhoP is required for the transcription of *mgtB*, *mgtM*, *mgtP*, and *term* at low and high [Mg^{2+}] even in the absence of H-NS.

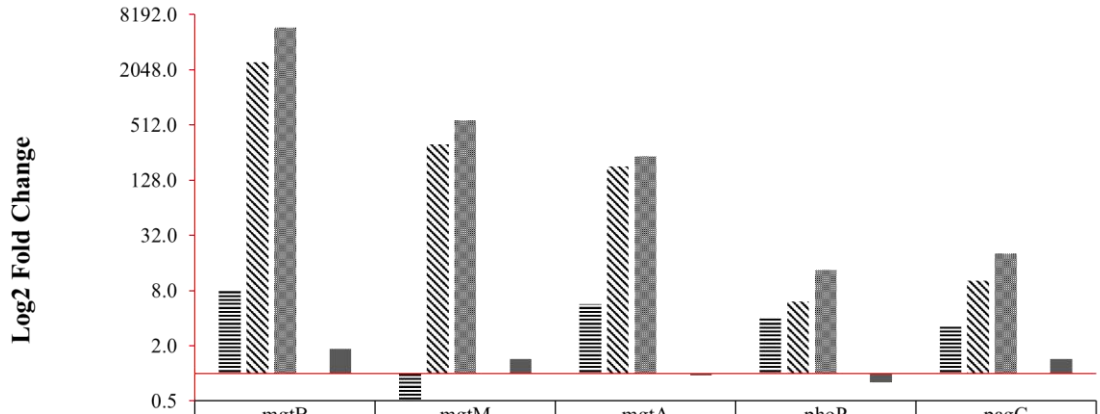


	WT	Δhns	ΔphoP	Δhns/ΔphoP	WT	Δhns	ΔphoP	Δhns/ΔphoP	WT	Δhns	ΔphoP	Δhns/ΔphoP
▨ 0.016mM	5.0	46.0	0.1	1.5	3.7	33.4	0.6	8.2	8.9	152.9	0.2	2.3
■ 2mM	1.0	34.8	0.1	1.8	1.0	24.4	0.4	13.2	1.0	87.0	0.1	3.2

Figure 3.3: **Fold-change expression levels of ORFs in the 5' mRNA leader region of the *mgtCBRU-cigR operon* in *hns*::IS*10*, *phoP*, and *hns*::*IS10 phoP* strains grown to mid-log phase at low (0.016 mM) and high (2 mM) Mg^{2+} concentrations.**

3.1.3 Extreme Mg^{2+} limitation is required for full induction of PhoP regulon genes

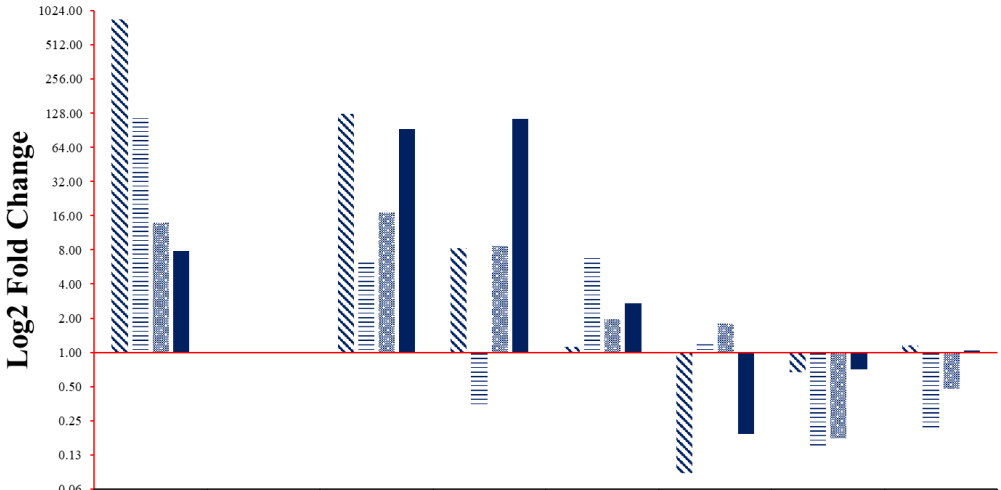
The modest induction of all PhoP regulon genes at a low (0.016 mM) concentration of Mg^{2+} (Figure 3.1) caused concern about our ability to measure the level of transcription of these genes correctly because of the previous reports demonstrating how the PhoP regulon is induced at low [Mg^{2+}] (Groisman, 1998). Additionally, we realized that *mgtCBRU-cigR* and *mgtA* are transcribed when conditions mimic Mg^{2+} starvation in intramacrophage environments, so the cells needed to be grown to stationary phase to observe full induction of these genes. Therefore, we grew wild-type cells in 0.016 mM Mg^{2+} to late logarithmic/early stationary phase under conditions in which growth was limited by the [Mg^{2+}]. Genes *mgtA*, *mgtB* and the ORF *mgtM* were expressed at increasingly high levels as the cells progressed from late logarithmic phase (OD_{600} = 0.9) to stationary phase (OD_{600} = 1.5-2) representing Mg^{2+} depletion, fully inducing promoter activation of the Mg^{2+} transporters. At the same time, *phoP* and *pagC* exhibited modest induction.



	mgtB	mgtM	mgtA	phoP	pagC
≡ 0.960OD 0.016mM	7.9	0.5	5.7	4.1	3.2
⩙ 1.672OD 0.016mM	2486.7	315.2	181.0	6.1	10.2
▒ 1.985OD 0.016mM	5873.5	576.0	233.9	13.4	20.4
■ 0.967OD 2mM	1.0	1.0	1.0	1.0	1.0
■ 2.012OD 2mM	1.8	1.4	0.9	0.8	1.4

Figure 3.4: **Preliminary data of relative expression levels of PhoP regulon genes in LT2 wild-type cells grown from late logarithmic phase to early stationary phase cells .**

Subsequently, we measured induction of the PhoP regulon genes in the aforementioned three mutant strains at low and high [Mg^{2+}] but grown to stationary phase (Figure 3.5). As expected, the PhoP regulon genes were more induced when Mg^{2+} availability depleted. *mgtA* and *mgtB* exhibited the largest induction in low Mg^{2+} because of the Mg^{2+}-sensing ORFs permitting strong expression. The data also suggest that even under these conditions of extreme Mg^{2+} limitation, the expression of all of these genes was brought down to background levels in both the *phoP* single and *hns phoP* double mutant. Demonstrated by our previous data, induction at low and high Mg^{2+} was enhanced by the inactivation of *hns* in all PhoP regulon genes investigated except for *mgtA*. However, *mgtA* in the *hns* single mutant did not show enhanced expression at low and high [Mg^{2+}] (Figure 3.5). This result could be attributed to the inactivation of H-NS causing pleotropic effects affecting the robustness of cell growth in addition to cells being harvested at stationary phase.



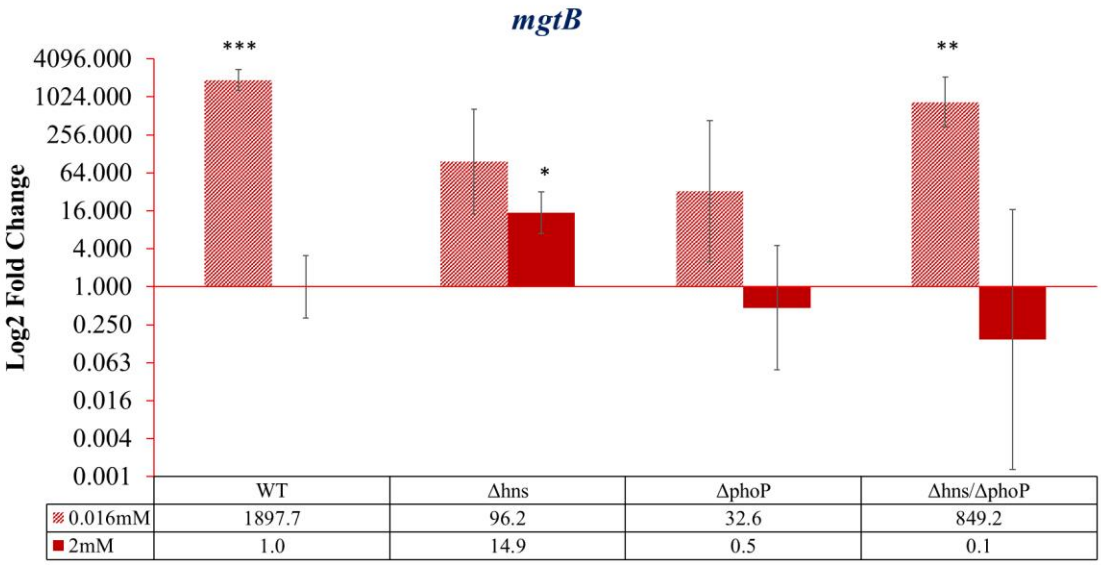
	0.016mM	2mM	0.016mM	2mM	0.016mM	2mM	0.016mM	2mM
	WT		Δhns		ΔphoP		ΔhnsΔphoP	
mgtB	861.1	1.0	126.2	8.3	1.1	0.1	0.7	1.1
mgtA	116.2	1.0	6.5	0.3	7.2	1.2	0.1	0.2
phoP	13.8	1.0	17.0	8.6	2.0	1.8	0.2	0.5
pagC	7.8	1.0	93.1	113.8	2.7	0.2	0.7	1.0

Figure 3.5: **Preliminary data of fold-change expression levels of PhoP regulon genes in *hns*::IS*10, phoP*, and *hns*::*IS10 phoP* LT2 strains at low (0.016 mM) and high (2mM) Mg^{2+} concentrations grown through late logarithmic phase to early stationary phase.**

3.2 Inactivation of *hns*, *phoP*, or both in *S.* Typhimurium strain ATCC 14028s

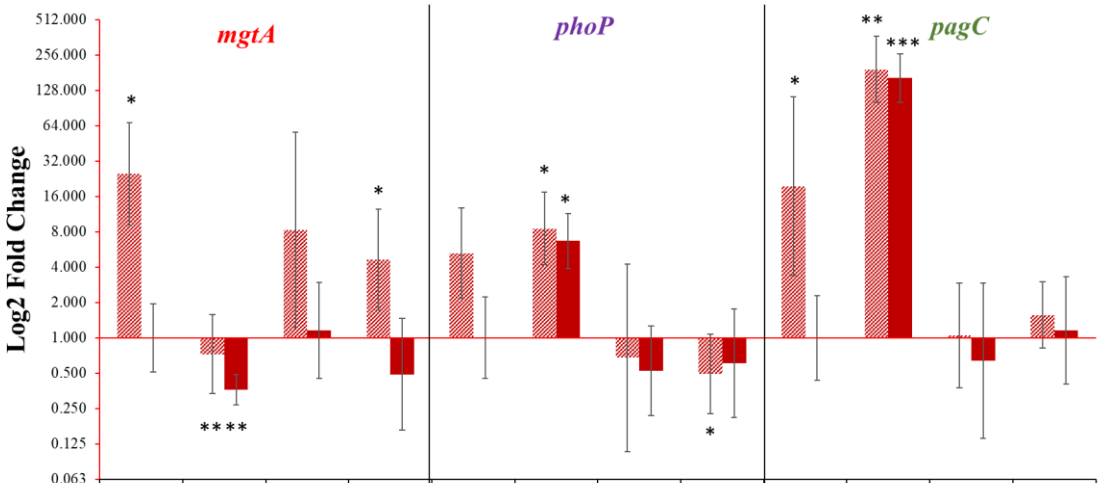
3.2.1 PhoP regulon genes

In our initial experiments, we studied the expression of PhoQP-regulon genes in the non-pathogenic strain LT2, whereas Will et. al (2014) assessed behavior of such genes upon the inactivation of *hns* and/or *phoP* in strain ATCC 14028s, which is the pathogenic version of *S.* Typhimurium. To determine if our findings are consistent in the pathogenic version, wild-type ATCC 14028s and *hns*, *phoP* single and double mutant derivatives were grown at low (0.016 mM) and high (2mM) Mg^{2+} to stationary phase (OD_{600}= 1.5-2) to fully induce the Mg^{2+} transporter genes. Much greater up-regulation of expression of *mgtB* was observed in *hns phoP* double mutant of strain ATCC 14028s at low [Mg^{2+}] than in the LT2 counterpart, revealing a difference in regulation between the non-pathogenic and pathogenic isolates (Figure 3.6). However, up-regulation in the double mutant was not observed in non-inducing conditions, also contradicting the anti-silencing model of Will et al. Another interesting finding was that low Mg^{2+} conditions still induced expression of *mgtB*, and to a lesser extent of *mgtA* in a PhoP-independent manner (Figure 3.7). Ultimately, these results suggest that *mgtCBRU-cigR* and *mgtA* can be expressed at a low level from a promoter that is PhoP-independent. The residual regulation of these genes by the Mg^{2+} concentration could be the due to the attenuation function of the regulatory ORFs in the mRNA leader sequences. The other PhoP-regulated genes not responsible for Mg^{2+} uptake, *phoP* and *pagC*, do not share this same trend in the *phoP* single and *hns phoP* double mutant backgrounds and behave similarly to what is observed in the non-pathogen.



	WT	Δhns	ΔphoP	Δhns/ΔphoP
0.016mM	1897.7	96.2	32.6	849.2
2mM	1.0	14.9	0.5	0.1

Figure 3.6: **Fold-change expression levels of *mgtB* genes in *hns*::IS*10*, *phoP*, and *hns*:: IS*10 phoP* strains in the ATCC 14028s background grown to stationary phase at low (0.016 mM) and high (2 mM) Mg^{2+} concentrations (OD_{600}=~1.5-2).**

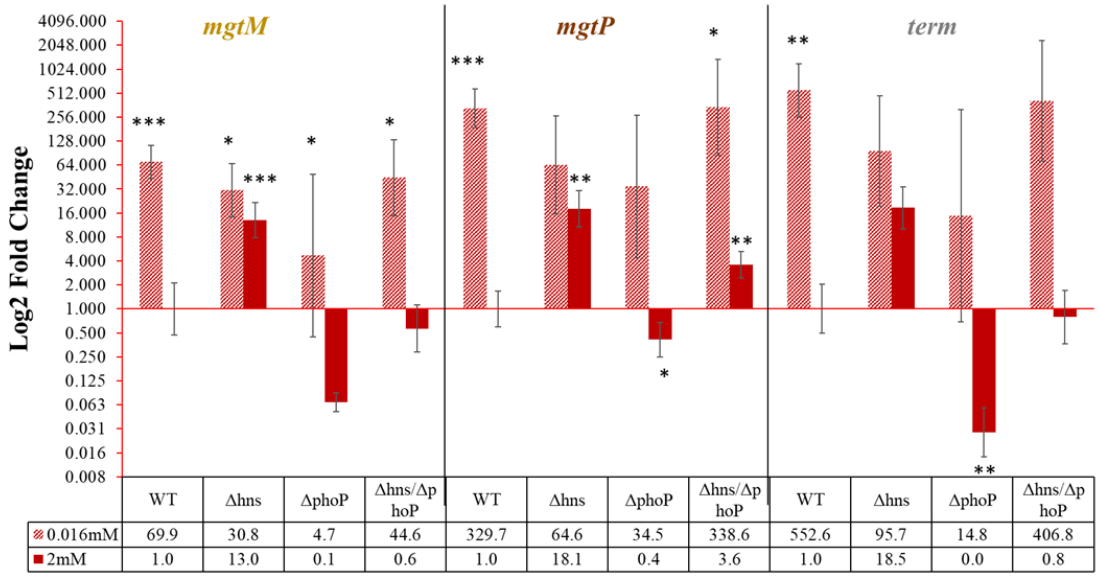


	WT	Δhns	ΔphoP	Δhns/ΔphoP	WT	Δhns	ΔphoP	Δhns/ΔphoP	WT	Δhns	ΔphoP	Δhns/ΔphoP
0.016mM	24.9	0.7	8.3	4.6	5.3	8.5	0.7	0.5	19.5	193.1	1.0	1.6
2mM	1.0	0.4	1.2	0.5	1.0	6.7	0.5	0.6	1.0	163.0	0.6	1.2

Figure 3.7: **Fold-change expression levels of PhoP regulon genes in *hns*::IS*10*, *phoP*, and *hns*:: IS*10 phoP* strains in the ATCC 14028s background grown to stationary phase at low (0.016 mM) and high (2 mM) Mg^{2+} concentrations (OD_{600}=~1.5-2).**

3.2.2 Open-reading frame sequences in the 5' leader region of *mgtCBRU-cigR* mRNA

I investigated the behavior of the regulatory ORFs and the Rho-dependent terminator sequence in the ATCC 14028s background at stationary phase (Figure 3.8) and found that those sequences share similar trends with *mgtB* in exhibiting high induction at low Mg^{2+}, up-regulation of expression in the *hns phoP* double mutant , and PhoP-independent activity .



	WT	Δhns	ΔphoP	Δhns/ΔphoP	WT	Δhns	ΔphoP	Δhns/ΔphoP	WT	Δhns	ΔphoP	Δhns/ΔphoP
0.016mM	69.9	30.8	4.7	44.6	329.7	64.6	34.5	338.6	552.6	95.7	14.8	406.8
2mM	1.0	13.0	0.1	0.6	1.0	18.1	0.4	3.6	1.0	18.5	0.0	0.8

Figure 3.8: **Fold-change expression levels of ORFs in the 5' mRNA leader region in *hns*::IS*10*, *phoP*, and *hns*:: IS*10 phoP* strains in the ATCC 14028s background grown to stationary phase at low (0.016 mM) and high (2 mM) Mg^{2+} concentrations (OD_{600}=~1.5-2).**

CHAPTER 4. DISCUSSION

Our goal was to determine the role of H-NS in the transcriptional regulation of the *mgtCBRU-cigR* operon. Using qRT-PCR, we measured differential expression of this operon in cells grown at low (0.016 mM, inducing) or high (2 mM, non-inducing) Mg^{2+} conditions with mutations inactivating H-NS, PhoP, or both. This was performed in both the non-pathogen *S.* Typhimurium LT2 and the pathogenic ATCC 14028s to observe if there were differences in regulation of *mgtCBRU-cigR*.

We saw that in LT2, PhoP-dependent genes were induced only when PhoP was present, irrespective of H-NS repression. This observation supports our opposing argument against Will et. al's proposed model of PhoP solely antagonizing H-NS to activate the transcription of the *mgtCBRU-cigR* operon. The *mgtCBRU-cigR* promoter has proven to be H-NS dependent, as shown by the significant de-repression in in both inducing and non-inducing conditions in mutants lacking this protein (Figure 3.1). That the transcription of *phoP* itself was also dependent on H-NS (Figure 3.2) causes us to question whether the PhoQ/PhoP two component regulatory system was horizontally acquired. Another PhoP regulon gene, *pagC*, exhibited greater de-repression in the *hns* single mutant background in both in low and high [Mg^{2+}] than either *mgtB* or *phoP* (Figure 3.2). As predicted, inactivation of H-NS had no effect on the transcription of *mgtA*, further confirming that the latter is a core gene (Figure 3.2). Each gene exhibits down-regulation or no change in expression in the *hns phoP* double mutant in both inducing and non-inducing conditions, suggesting that PhoP is required for activation at each promoter despite H-NS's absence. H-NS may be imperative for the silencing of foreign genes, but it does not mask the key role of PhoP in promoter activation. It could be true that, in the non-pathogen, the mutation of PhoP disrupts the

robustness of intracellular activity. This characteristic was noticed when cells were subjected to growth under extremely limited Mg^{2+} conditions.

We also investigated the regulation of transcription of the 5' leader of the *mgtCBRU-cigR* operon, which contains the short regulatory ORF's *mgtM* and *mgtP* and a Rho-dependent transcription terminator *term*. The transcription of these genetic elements was also H-NS and PhoP-dependent (Figure 3.3), but the two ORFs and the Rho-dependent terminator show more elevated expression at low and high Mg^{2+} than *mgtB*. This observation can be attributed to the regulatory ORFs continuing to regulate efficiency of transcription of downstream sequences. Furthermore, there was some PhoP-independent transcription of these sequences in the *hns phoP* mutant at both low and high Mg^{2+}. We hypothesize that these genetic elements might be transcribed from a low-level promoter that does not require PhoP but are repressed by H-NS. The role for an additional promoter could be to slightly enhance efficiency of transcription in extremely low Mg^{2+} conditions without requiring PhoP to recruit RNA polymerase. Locating this putative low-level promoter would be a next step in further understanding the *mgtCBRU-cigR* operon. Also, determining where H-NS binds on the *mgtCBRU-cigR* promoter(s) is essential and should be considered a future direction.

Maximal expression of the PhoP-dependent genes was only observed when cells were in Mg^{2+} starvation mode at or near stationary phase growth (Figure 3.4 and 3.5). The small up-regulation in low Mg^{2+} conditions for the PhoP regulon gene under investigation (Figure 3.1-3.3) lead us to determine how expression would appear as cells progressed through the growth cycle. Interestingly, expression increased as the cell density (OD_{600}) increased or when cells reached stationary phase in low but not in high Mg^{2+}, implying that the induction in low Mg^{2+} is due to the exhaustion of this nutrient as cells propagate, rather than to stationary phase per se. This

phenomenon seems reasonable as one would expect there to be elevated expression as more PhoP proteins are being phosphorylated in low Mg^{2+} conditions. Down-regulation or a minute change observed in the *hns phoP* double mutant compared to the *phoP* single mutant in both conditions further suggests that, in LT2, PhoP is necessary for the activation of the promoter irrespective of the presence of H-NS (Figure 3.5).

In the pathogenic *S.* Typhimurium ATCC 14028s, *mgtA* and *mgtB* could be induced independently of PhoP in low Mg^{2+} (Figures 3.6 and 3.7). We subjected this strain to extremely low Mg^{2+} conditions by allowing the cells to grow until they reached an OD_{600} between 1.5-2 based to our previous findings and discovered unexpected expression behavior of the Mg^{2+} transporter genes. Figures 3.6 and 3.7 depict that in the *phoP* and *hns phoP* double mutant these genes were expressed at higher levels at low Mg^{2+} than at high Mg^{2+}. Alternatively, in non-inducing conditions, expression of these genes was repressed. Repression of *mgtB* and *mgtA* in non-inducing conditions can be explained by the functioning regulatory ORFs terminating transcription of the structural genes. Aside from what occurs in non-inducing conditions, this result differs from the expression observed in LT2 (Figure 3.1 and 3.2). This PhoP-independent transcription in the pathogen suggests the presence of a low-level promoter upstream of the 5' mRNA leader region of *mgtA* and *mgtCBRU-cigR*, as previously suggested. Surprisingly, the regulatory ORFs and the Rho-dependent terminator sequence show PhoP-independent activity in the pathogen and, to a lesser extent, in the non-pathogen. (Figure 3.3 and 3.8). We have already suggested the cells are recruiting RNA polymerase more immediately by allowing it to bind more efficiently to an additional promoter without PhoP to circumvent the inability to express Mg^{2+} importers. However, because this activity is primarily observed under extremely low Mg^{2+} conditions, the combination of the environmental pressure and deficiency in phosphorylated PhoP

could exacerbate PhoP-independent behavior. Studying the effects of extreme Mg^{2+} starvation on *mgtL*, the regulatory ORF upstream of *mgtA*, could contribute to this conclusion. Additionally, we can speculate that in the pathogen, a compensatory mechanism somehow may alleviate the stress that the double mutation causes so that the cell continues to import Mg^{2+}. We may assume that another regulatory protein, one that also controls expression of SPI-III genes, plays a role. Determining what other proteins bind to the promoter may prove to be informative.

In an Mg^{2+}-depleted environment, only *phoP* and *pagC* exhibit H-NS-dependent behavior (Figure 3.7). There was a difference in the effect of loss of H-NS on the expression of *mgtB* at low and high Mg^{2+}: the gene was induced by low Mg^{2+} in the *hns* mutant, but to only about 50% of the level of the wild type. However, the gene was expressed at ~15-fold higher level at high Mg^{2+} in the *hns* mutant in comparison to the wild type (Figure 3.6). Reduced levels of expression of the *mgtB* in the *hns* mutant grown in low Mg^{2+} conditions could be attributed to pleotropic effects of the *hns* mutation in addition to Mg^{2+} starvation and other nutrient limiting conditions, causing fragile growth.

Overall, the regulation of transcription of *pagC* and *phoP* is similar in the non-pathogen and pathogen (Figure 3.2 and 3.7). The apparent difference between the regulation of *mgtA* and *mgtB* on one hand and that of *pagC* and *phoP* other hand is that latter two genes do not possess a regulatory ORF upstream from them, shortening the distance between the promoter and the transcription start site. This minimizes the possibility that there would be an additional low-level promoter and establishes their transcription as being solely dependent on regulation via PhoP at the promoter.

The role of H-NS to suppress transcription of foreign genes must be met with a method of de-repression to activate foreign genes that will positively affect the survival of *Salmonella*.

Response regulators such as SsrB, a protein that regulates expression of SPI-2 genes, has been shown to counter H-NS-mediated silencing and promote activation of the promoters of effector proteins within its regulon (Walthers et al., 2011). Determining how this phenomenon translates to the activation of PhoP regulon genes in SPI-3 could expand our understanding of how these genes are being regulated. The regulation of transcription of foreign DNA acquired by lateral transfer by H-NS is not exclusive to *S*. Typhimurium but has been noted in other organisms within *Enterobacteriaceae*, as well as species in other families. *Pseudomonas aeruginosa*, a Gram-negative, opportunistic bacterium that can be lethal to cystic fibrosis patients, also contains nucleoid-associated proteins MvaT and MvaU that fall into the H-NS family (Castang et al., 2008). Like H-NS in enteric bacteria, they control the expression of virulence genes, conferring resistance to antibiotics and promote biofilm formation (Castang et al., 2008). Additionally, despite its low sequence similarity to H-NS, the Lsr2 protein of *Mycobacterium tuberculosis* functions similarly to H-NS in, regulating virulence genes involved in antibiotic resistant and cell wall lipid biosynthesis (Gordon et al., 2010). The prevalence of H-NS and related proteins indicates that transcriptional repression of horizontally acquired genes is imperative to the maintenance of genetic regulatory circuits and robustness of cell growth.

Our introduction into this subject was motivated by the data published by the aforementioned Will et al. group proposing the model that suggested PhoP's role in regulation was to solely antagonize H-NS at a subset of the promoters under its control. Their research did not exclusively suggest that PhoP does not mediate activation on its own at some of the promoters, but rather the canonical model of PhoP-mediated activation requires a specific promoter architecture (Will et al., 2014). They classified various PhoP targets and found that those possessing a single PhoP binding site overlapping the -35 box exhibit PhoP-mediated activation. PhoP targets with this architecture

include *mgtA* and *phoP*. From our observations, *mgtA* appears to have PhoP-mediated activation at the promoter, however, H-NS has an influence on the activation of *phoP*, contradicting Will et al's claim that a certain promoter architecture is indicative of PhoP's only role in promoter activation (Figure 3.2 and 3.7). *pagC* and *mgtCBRU-cigR* are both categorized as promoters that not only have a PhoP-binding site upstream of the -35 box, but at variable locations and orientations (Will et al., 2014). Also, their category of promoters has additional binding sites at farther distances. Will et al. have determined that the *pagC* promoter undergoes PhoP-mediated counter-silencing based on biochemical analyses revealing that PhoP and SlyA (another regulatory protein) are not required for optimal expression of the *pagC* promoter when H-NS is absent. Additionally, in an *in vitro* system they saw that increasing concentrations of purified unphosphorylated or phosphorylated, PhoP protein had no effect on the transcription levels of *pagC* or *mgtC*, suggesting the dispensable role of PhoP for their promoter structures. Our findings are inconsistent with theirs, as we found that both *pagC* and *mgtB* were required PhoP for transcriptional activation in LT2 (Figure 3.1 and 3.2). In ATCC 14028s, our qRT-PCR data for *pagC* supports our argument that its activation is PhoP-dependent (Figure 3.7). At first glance, our result that *mgtB* was induced to high level in the *hns phoP* double mutant of the pathogen is consistent with the anti-silencing model of Will et al. However, the high-level expression of *mgtB* in the *phoP* mutant under low Mg^{2+} conditions (Figure 3.6) implies that the PhoP-independent activity is not repressed by H-NS.

Previously, Kong et al. (2008) also investigated how H-NS controls PhoP-dependent gene regulation in *Salmonella* ATCC 14028s. They assessed *phoP* and *pagC* promoter regulation but did not examine *mgtCBR-cigR* or *mgtA*. Their data revealed elevated levels of expression in the *hns phoP* double mutant that was comparable to that seen in the *hns* single mutant at both low and

high Mg^{2+}. They concluded that both *phoP* and *pagC* are repressed by H-NS and that PhoP has dual functions as an anti-repressor of H-NS and an activator of the *phoP* promoter, whereas it acts solely as an anti-repressor of H-NS (in concert with SlyA) at the *pagC* promoter. The question is why our data contrast from both Will et al (2014) and Kong et al (2008), and there could be some factors that may be due to those differences. We attributed the differences between our data in LT2 and their results to the attenuated virulence. Attenuated virulence may indirectly or directly inhibit the cell's ability to compensate for the loss of PhoP, being a major regulatory protein, and H-NS, rendering it unable to activate expression of *mgtB* and other PhoP regulon genes. The discrepancy between our ATCC 14028s data and those of Will et al. and Kong et al. might also be due to differences in growth conditions and genetic backgrounds of the strains used. There is a critical issue concerning the viability of *hns* mutants. We were readily able to transduce the *hns*::IS*10* and an *hns*::*kan* mutation into both our LT2 and ATCC 14028s, as have Kong et al. However, Will et al. reported that they could not construct a simple *hns* mutant from their ATCC 14028s, but they needed a compensatory *rpoS* mutation to be present in the strain to be able to introduce an *hns* mutation. For that reason, all of the experiments of Will et al. were conducted in a *rpoS hns* double mutant. Because *rpoS* encodes a central transcriptional activator of ~400 genes in the stationary phase regulon, weakening the production of RpoS protein could lead to pleiotropic effects, potentially affecting the typical genetic regulatory circuit. The differences in genetic background of the ATCC 14028s strains may influence the differences observed in *pagC* expression. Also, we investigated the mRNA expression in ATCC 14028s in cells that were grown to stationary phase due to Mg^{2+}-limitation, whereas Will et. al harvested the cells at mid-log phase in LB broth, washed them in N-minimal medium supplemented with high or low Mg^{2+} concentrations, and resuspended and incubated the cells in N-minimal medium with the corresponding Mg^{2+}

concentration for 30 minutes. Kong et. al also harvested the cells at mid-log phase in N-minimal medium supplemented with either high or low Mg^{2+} concentrations. The status of intracellular activity and mRNA transcript levels differ between mid-log phase and stationary phase and could affect how the PhoP regulon genes are expressed. Further qRT-PCR studies will need to be performed with cells grown to mid-log phase to determine if the phase of growth makes a difference.

Constitutive expression of the *mgtCBRU-cigR* operon and *mgtA* due to mutations in the regulatory ORFs that normally suppress expression in non-inducing conditions confers enhanced heat tolerance in *Salmonella* (Gall et al., 2016, 2018; O'Connor et al., 2009). H-NS behaving as another level of suppression informs us that it could influence the level of heat tolerance *Salmonella* typically exhibits in mesothermic temperatures. It would be interesting to determine a method of investigating how H-NS influences heat tolerance while accounting for the pleiotropic effects the mutation causes. Furthermore, PhoP-independent activation of the Mg^{2+} transporters with the unaltered regulatory ORFs should be investigated to determine if it also allows enhanced heat tolerance. Elucidating the mechanisms by which Mg^{2+} transporters are regulated will permit us to discover the connection between Mg^{2+} homeostasis and enhanced heat tolerance.

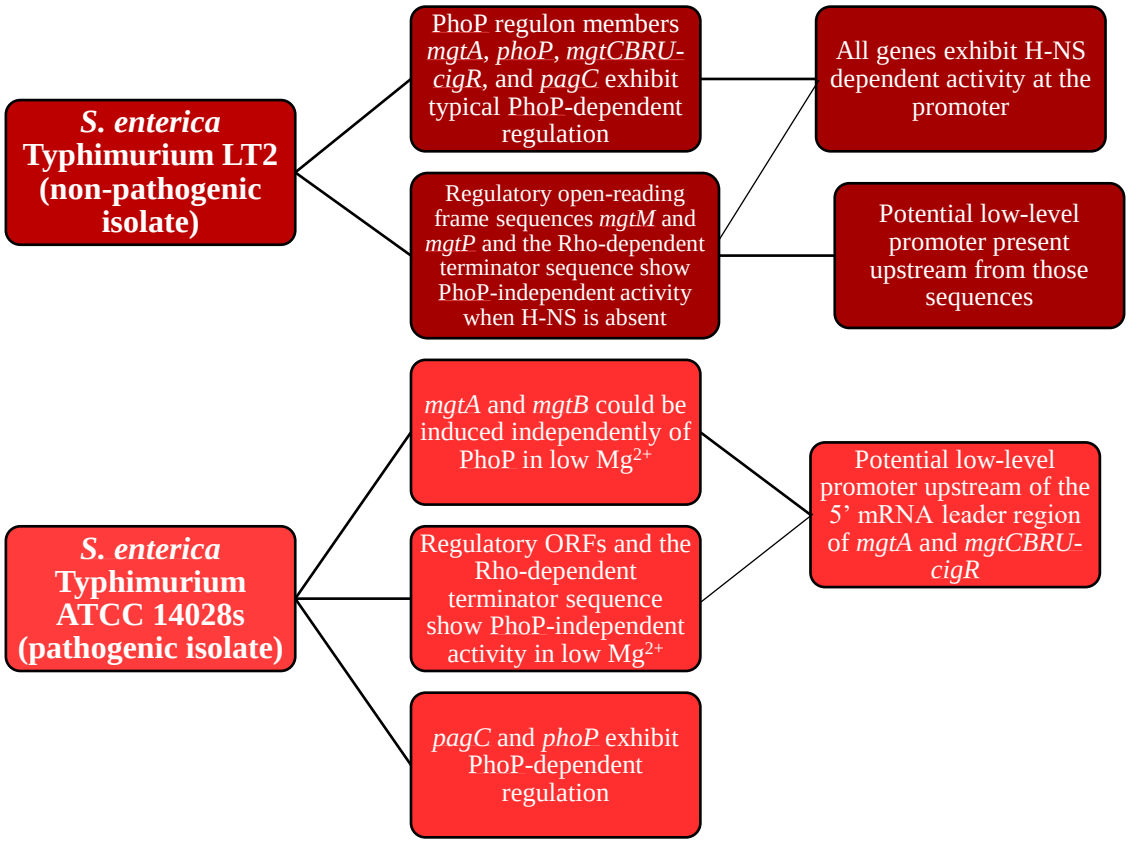


Figure 4.1: **Summary of findings regarding PhoP's and H-NS's activity at the promoters of the PhoP regulon genes studied in *S. enterica* Typhimurium LT2 and ATCC 14028s.**

APPENDIX

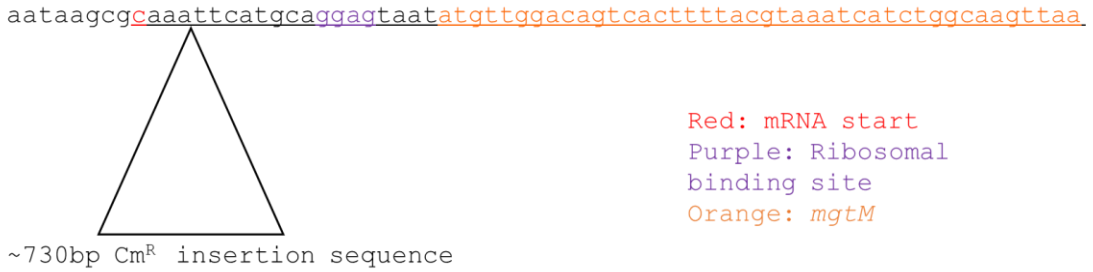
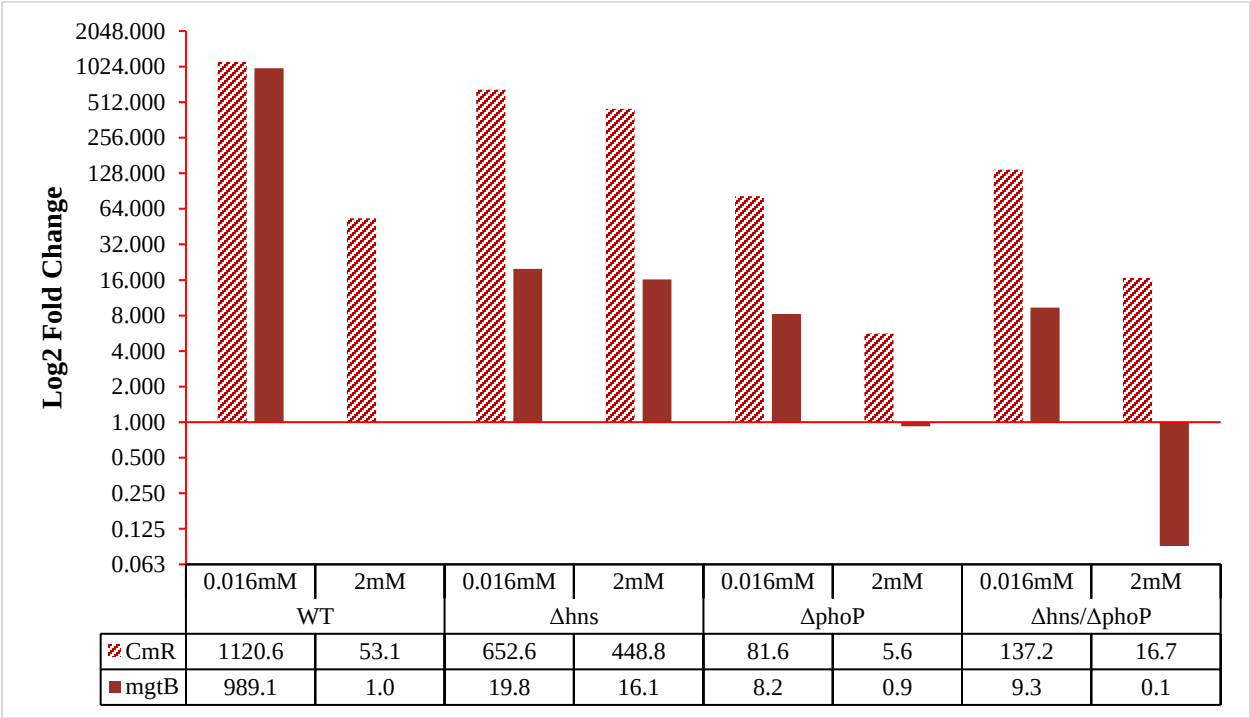


Figure 4.2: **Location of the chloramphenicol insertion sequence**. The figure depicts a segment of the 5'mRNA leader region including *mgtM*. A promoter-less chloramphenicol sequence was inserted between nucleotides 4A and 5T downstream from the mRNA start site.



	0.016mM	2mM	0.016mM	2mM	0.016mM	2mM	0.016mM	2mM
	WT		Δhns		ΔphoP		Δhns/ΔphoP	
CmR	1120.6	53.1	652.6	448.8	81.6	5.6	137.2	16.7
mgtB	989.1	1.0	19.8	16.1	8.2	0.9	9.3	0.1

Figure 4.3: **Fold-change expression levels of the chloramphenicol resistance gene (Cm^R) and *mgtB* genes in *hns*::IS*10*, *phoP*, and *hns*:: IS*10 phoP* strains in the ATCC 14028s background grown to mid-log phase at low (0.016 mM) and high (2 mM) Mg^{2+} concentrations.**

We attempted to separate the effects of regulation of transcription initiation of the *mgtCBRU-cigR* promoter by PhoP from the regulation of transcription termination by the regulatory ORFs and the Rho-dependent terminator sequence. To observe expression behavior upstream of the regulatory ORFs, a chloramphenicol resistance gene (Cm^R) was inserted directly

behind the promoter and upstream of *mgtM*. It was necessary to do so because the distance between the promoter and *mgtM* was too short to construct primers for qRT-PCR. Subsequently, we performed qRT-PCR to compare expression between Cm^R and *mgtB* in the wild type, the *hns* and *phoP* single mutants, and the *hns phoP* double mutant of the pathogen. Furthermore, studying expression in the *hns* mutants allows us to observe where H-NS may bind. Analyzing the qRT-PCR data had to be done differently than what was done before (see methods section). Instead, all normalized expression values were calibrated against *mgtB* in the wild type at high Mg^{2+} conditions to observe a fold change difference between Cm^R and *mgtB*. These results are presented as an appendix, because unfortunately, this experiment was done only once without replicates, and therefore, the data can be interpreted tentatively.

Expression of Cm^R was greater than expression of *mgtB*, further confirming the significant role the regulatory ORFs plays in suppressing expression. In the WT, the Cm^R gene was induced 21-fold (=1120.6 / 53.1) when comparing low and high Mg^{2+} conditions (Figure 5.2). This is the effects of regulation of transcription initiation by phosphorylated PhoP in response to Mg^{2+} limitation. *mgtB* is induced much more: 989-fold by low Mg^{2+} conditions, and this is due to the combination of increased transcription initiation by phosphorylated PhoP and the suppression of termination by the short ORFs. The regulation of expression of *mgtB* is very similar in the WT and in the *hns*, *phoP*, and *hns phoP* mutants in the strain with the Cm^R (Figure 5.2) and in the strain without Cm^R (Figure 3.5), therefore the Cm^R insertion does not interfere with the expression of *mgtB*.

At first glance it seems that the regulatory ORFs and the terminator sequence have a greater impact on repression of *mgtB*, however, it may be more insightful to observe absolute transcript levels upstream and downstream the attenuating sequences. Further directions for this

investigation should be to determine reproducibility of the results and inactivate the regulatory ORFs after the insertion of the chloramphenicol resistance gene to observe differences in expression. Additionally, performing RNA sequencing to measure differences in expression before and after the regulatory ORFs and terminator would be optimal for our purposes. This method would permit us to quantify absolute amounts of transcript rather than observing only fold-change differences.

REFERENCES

Abrahams, G. L., & Hensel, M. (2006). Manipulating cellular transport and immune responses: Dynamic interactions between intracellular Salmonella enterica and its host cells. *Cellular Microbiology*, *8*(5), 728–737. https://doi.org/10.1111/j.1462-5822.2006.00706.x

Bijlsma, J. J. E., & Groisman, E. A. (2003). Making informed decisions: Regulatory interactions between two-component systems. *Trends in Microbiology*, *11*(8), 359–366. https://doi.org/10.1016/S0966-842X(03)00176-8

Boyle, E. C., Bishop, J. L., Grassl, G. A., & Finlay, B. B. (2007). Salmonella: From Pathogenesis to Therapeutics. *Journal of Bacteriology*, *189*(5), 1489–1495. https://doi.org/10.1128/JB.01730-06

Castang, S., McManus, H. R., Turner, K. H., & Dove, S. L. (2008). H-NS family members function coordinately in an opportunistic pathogen. *Proceedings of the National Academy of Sciences*, *105*(48), 18947–18952. https://doi.org/10.1073/pnas.0808215105

Chen, J., Lau, Y. F., Lamirande, E. W., Paddock, C. D., Bartlett, J. H., Zaki, S. R., & Subbarao, K. (2010). Cellular Immune Responses to Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) Infection in Senescent BALB/c Mice: CD4+ T Cells Are Important in Control of SARS-CoV Infection. *Journal of Virology*, *84*(3), 1289–1301. https://doi.org/10.1128/JVI.01281-09

Choi, E., Lee, K.-Y., & Shin, D. (2012). The MgtR regulatory peptide negatively controls expression of the MgtA Mg2+ transporter in Salmonella enterica serovar Typhimurium. *Biochemical and Biophysical Research Communications*, *417*(1), 318–323. https://doi.org/10.1016/j.bbrc.2011.11.107

Datsenko, K. A., & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proceedings of the National Academy of Sciences*, *97*(12), 6640–6645. https://doi.org/10.1073/pnas.120163297

Davis, R. W., Botstein, D., Roth, J. R., & Cold Spring Harbor Laboratory. (1980). *Advanced bacterial genetics*. Cold Spring Harbor Laboratory.

Falkow, S. (1996). The Evolution of Pathogenesis in Escherichia, Shigella, and Salmonella. In Neidhardt, F. C., Curtis III, Roy, Ingrahm, John L., C. Lin, Edmund C., Low, K. Brooks, Magasanik, Boris, Reznikoff, William S., Riley, Monica, Schaechter, Moselio, &

Umbarger, H. Edwin, *Escherichia coli and Salmonella: Cellular and Molecular Biology* (2nd ed., Vol. 2, pp. 2723–2739). ASM Press.

Gall, A. R., Datsenko, K. A., Figueroa-Bossi, N., Bossi, L., Masuda, I., Hou, Y.-M., & Csonka, L. N. (2016). Mg2+ regulates transcription of mgtA in Salmonella Typhimurium via translation of proline codons during synthesis of the MgtL peptide. *Proceedings of the National Academy of Sciences*, *113*(52), 15096–15101. https://doi.org/10.1073/pnas.1612268113

Gall, A. R., Hegarty, A. E., Datsenko, K. A., Westerman, R. P., SanMiguel, P., & Csonka, L. N. (2018). High-level, constitutive expression of the *mgtC* gene confers increased thermotolerance on *Salmonella enterica* serovar Typhimurium: Constitutive expression of the *mgtC* gene. *Molecular Microbiology*, *109*(3), 327–344. https://doi.org/10.1111/mmi.13988

Gerlach, R., & Hensel, M. (2007). Salmonella Pathogenicity Islands in host specificity, host pathogen-interactions and antibiotics resistance of Salmonella enterica. *Berliner Und Münchener Tierärztliche Wochenschrift*, *120*, 317–327. https://doi.org/10.2376/0005-9366-120-317

Gordon, B. R. G., Li, Y., Wang, L., Sintsova, A., van Bakel, H., Tian, S., Navarre, W. W., Xia, B., & Liu, J. (2010). Lsr2 is a nucleoid-associated protein that targets AT-rich sequences and virulence genes in Mycobacterium tuberculosis. *Proceedings of the National Academy of Sciences*, *107*(11), 5154–5159. https://doi.org/10.1073/pnas.0913551107

Groisman, E. A. (1998). The ins and outs of virulence gene expression: Mg2+ as a regulatory signal. *BioEssays*, *20*(1), 96–101. https://doi.org/10.1002/(SICI)1521-1878(199801)20:1<96::AID-BIES13>3.0.CO;2-3

Groisman, E. A. (2001). The Pleiotropic Two-Component Regulatory System PhoP-PhoQ. *Journal of Bacteriology*, *183*(6), 1835–1842. https://doi.org/10.1128/JB.183.6.1835-1842.2001

Heithoff, D. M., Conner, C. P., Hentschel, U., Govantes, F., Hanna, P. C., & Mahan, M. J. (1999). Coordinate Intracellular Expression of Salmonella Genes Induced during Infection. *Journal of Bacteriology*, *181*(3), 799–807.

Higgins, C. F., Dorman, C. J., Stirling, D. A., Waddell, L., Booth, I. R., May, G., & Bremer, E. (1988). A physiological role for DNA supercoiling in the osmotic regulation of gene expression in S. typhimurium and E. coli. *Cell*, *52*(4), 569–584. https://doi.org/10.1016/0092-8674(88)90470-9

Hollands, K., Proshkin, S., Sklyarova, S., Epshtein, V., Mironov, A., Nudler, E., & Groisman, E. A. (2012). Riboswitch control of Rho-dependent transcription termination. *Proceedings of the National Academy of Sciences*, *109*(14), 5376–5381. https://doi.org/10.1073/pnas.1112211109

Isturiz, T., & Wolf, R. E. (1975). In vitro synthesis of a constitutive enzyme of Escherichia coli, 6-phosphogluconate dehydrogenase. *Proceedings of the National Academy of Sciences*, *72*(11), 4381–4384. https://doi.org/10.1073/pnas.72.11.4381

Kehres, D. G., & Maguire, M. E. (2002). Structure, properties and regulation of magnesium transport proteins. *Biometals*, *15*(3), 261–270. https://doi.org/10.1023/A:1016078832697

Kolter, R., & Yanofsky, C. (1982). Attenuation in Amino Acid Biosynthetic Operons. *Annual Review of Genetics*, *16*(1), 113–134. https://doi.org/10.1146/annurev.ge.16.120182.000553

Lee, E.-J., & Groisman, E. A. (2010). An antisense RNA that governs the expression kinetics of a multifunctional virulence gene. *Molecular Microbiology*, *76*(4), 1020–1033. https://doi.org/10.1111/j.1365-2958.2010.07161.x

Lee, J.-W., & Lee, E.-J. (2015). Regulation and function of the Salmonella MgtC virulence protein. *Journal of Microbiology*, *53*(10), 667–672. https://doi.org/10.1007/s12275-015-5283-1

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Miller, S. I., Kukral, A. M., & Mekalanos, J. J. (1989). A two-component regulatory system (phoP phoQ) controls Salmonella typhimurium virulence. *Proceedings of the National Academy of Sciences*, *86*(13), 5054–5058. https://doi.org/10.1073/pnas.86.13.5054

Navarre, W. W. (2006). Selective Silencing of Foreign DNA with Low GC Content by the H-NS Protein in Salmonella. *Science*, *313*(5784), 236–238. https://doi.org/10.1126/science.1128794

Navarre, W. W., McClelland, M., Libby, S. J., & Fang, F. C. (2007). Silencing of xenogeneic DNA by H-NS--facilitation of lateral gene transfer in bacteria by a defense system that recognizes foreign DNA. *Genes & Development*, *21*(12), 1456–1471. https://doi.org/10.1101/gad.1543107

Neidhardt, F. C., Bloch, P. L., & Smith, D. F. (1974). Culture Medium for Enterobacteria. *Journal of Bacteriology*, *119*(3), 736–747. https://doi.org/10.1128/jb.119.3.736-747.1974

Nielsen, M.-B., Knudsen, G. M., Danino-Appleton, V., Olsen, J. E., & Thomsen, L. E. (2013). Comparison of heat stress responses of immobilized and planktonic Salmonella enterica serovar Typhimurium. *Food Microbiology*, *33*(2), 221–227. https://doi.org/10.1016/j.fm.2012.09.020

O'Connor, K., Fletcher, S. A., & Csonka, L. N. (2009). Increased expression of Mg2+ transport proteins enhances the survival of Salmonella enterica at high temperature. *Proceedings of the National Academy of Sciences*, *106*(41), 17522–17527. https://doi.org/10.1073/pnas.0906160106

Papp-Wallace, K. M., & Maguire, M. E. (2008). Magnesium Transport and Magnesium Homeostasis. *EcoSal Plus*, *3*(1). https://doi.org/10.1128/ecosalplus.5.4.4.2

Perez, J. C., Latifi, T., & Groisman, E. A. (2008). Overcoming H-NS-mediated Transcriptional Silencing of Horizontally Acquired Genes by the PhoP and SlyA Proteins in Salmonella enterica. *The Journal of Biological Chemistry*, *283*(16), 10773–10783. https://doi.org/10.1074/jbc.M709843200

Rychlik, I., & Barrow, P. A. (2005). Salmonella stress management and its relevance to behaviour during intestinal colonisation and infection. *FEMS Microbiology Reviews*, *29*(5), 1021–1040. https://doi.org/10.1016/j.femsre.2005.03.005

Sambrook, J., Fritsch, E., & Maniatis, T. (1982). *Molecular cloning: A laboratory manual* (2nd ed.). Cold Spring Harbor Laboratory Press.

Snavely, M. D., Gravina, S. A., Cheung, T. T., Miller, C. G., & Maguire, M. E. (1991). Magnesium transport in Salmonella typhimurium. Regulation of mgtA and mgtB expression. *Journal of Biological Chemistry*, *266*(2), 824–829. https://doi.org/10.1016/S0021-9258(17)35247-X

Véscovi, E. G., Soncini, F. C., & Groisman, E. A. (1996). Mg2+ as an Extracellular Signal: Environmental Regulation of Salmonella Virulence. *Cell*, *84*(1), 165–174. https://doi.org/10.1016/S0092-8674(00)81003-X

Walthers, D., Li, Y., Liu, Y., Anand, G., Yan, J., & Kenney, L. J. (2011). Salmonella enterica Response Regulator SsrB Relieves H-NS Silencing by Displacing H-NS Bound in Polymerization Mode and Directly Activates Transcription *. *Journal of Biological Chemistry*, *286*(3), 1895–1902. https://doi.org/10.1074/jbc.M110.164962

Will, W. R., Bale, D. H., Reid, P. J., Libby, S. J., & Fang, F. C. (2014). Evolutionary expansion of a regulatory network by counter-silencing. *Nature Communications*, *5*(1), 5270. https://doi.org/10.1038/ncomms6270

Yeom, J., Pontes, M. H., Choi, J., & Groisman, E. (2018). A protein that controls the onset of a Salmonella virulence program. *The EMBO Journal*, *37*(14), e96977. https://doi.org/10.15252/embj.201796977

Yeom, J., Shao, Y., & Groisman, E. A. (2020). Small proteins regulate Salmonella survival inside macrophages by controlling degradation of a magnesium transporter. *Proceedings of the National Academy of Sciences*, *117*(33), 20235–20243. https://doi.org/10.1073/pnas.2006116117

Zwir, I., Latifi, T., Perez, J. C., Huang, H., & Groisman, E. A. (2012). The promoter architectural landscape of the Salmonella PhoP regulon. *Molecular Microbiology*, *84*(3), 463–485. https://doi.org/10.1111/j.1365-2958.2012.08036.x