12-17-2014

Phenotypic Effects of Predicted SigI on Virulence in Bacillus anthracis

Jenny Gi Yae Kim
Georgia State University

Adam Christopher Wilson
Georgia State University

Recommended Citation

Kim, Jenny Gi Yae and Wilson, Adam Christopher, "Phenotypic Effects of Predicted SigI on Virulence in Bacillus anthracis." Thesis, Georgia State University, 2014.
doi: https://doi.org/10.57709/6381709

PHENOTYPIC EFFECTS OF PREDICTED σ^I ON VIRULENCE IN *BACILLUS ANTHRACIS*

by

JENNY KIM

Under the Direction of Adam C. Wilson, PhD

ABSTRACT

Alternative sigma factors play a key role in the physiology of *Bacillus anthracis* by regulating the transcription of the appropriate genes required for adaptation and survival. Under specific conditions, alternative sigma factors activate transcription by binding to the promoter of the genes relevant to the condition and initiate synthesis of RNA. Here we report that the transcription of predicted *sigI* gene in *B. anthracis*, BAS3231, is induced by elevated temperatures and involved in the regulation of virulence gene expression. We show that BAS3231 is required for cell viability at elevated temperatures. We have also demonstrated that mutation in the BAS3231 gene results in a decrease in virulence gene expression. Our study provides new insight into the role of alternative sigma factors in *B. anthracis.*

INDEX WORDS: *Bacillus anthracis*, *Bacillus subtilis,* SigI, Heat stress, Virulence

PHENOTYPIC EFFECTS OF PREDICTED σ^I ON VIRULENCE IN *BACILLUS ANTHRACIS*

by

JENNY KIM

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2014

PHENOTYPIC EFFECTS OF PREDICTED σ^I ON VIRULENCE IN *BACILLUS ANTHRACIS*

by

JENNY KIM

Committee Chair: Adam C. Wilson

Committee: Zehava Eichenbaum

Parjit Kaur

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

December 2014

DEDICATION

I would like to dedicate this thesis to the love and support from all the family and friends who encouraged me in this study. I would like to especially thank my parents Steve and Mi Hyang Kim and my brother Andrew Kim for being such a huge support. They encouraged me to constantly keep my head up high and to remain the strong person they raised me to be during times when giving up was the easy way out. It is trite, but there are no words to describe the gratification I have for the constant support I received during this unforgettable learning experience from my family and without them I wouldn't be where I am today. I owe a gratitude for pursing my Masters in Science to my friends Paul Salazar, Lauren Adel, Sonia Im and Woori Koh for constantly staying by my side during difficult times, making me laugh and being the best support group I could have ever ask for.

To my lab members Sam Han and Mila Iakovenko who have generously given their time and expertise to provide invaluable advice, support and collaboration. My thanks must also go to my PI, Adam Wilson for giving me the opportunity to join his lab and mature into the scientist I have grown up to be. He has given me constant encouragement for improvement, the courage to challenge what is known about science and the strength to stand up for what I believe in.

Finally, I would like to dedicate this work to my late grandmother, Ok Chul Kim who very recently passed away from cervical cancer. She was the absolute paragon of compassion, strength, kindness and appreciation. She constantly pushed me to believe that the sky is the limit and that I will always make her proud regardless of where I end up in life. She consistently motivated me to live the life she sacrificed her life for and her strength is what kept me going when I wanted to give up. She was the woman I constantly strive to be and I hope to make the same impact on my community that she had on hers.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1   INTRODUCTION

Bacillus anthracis is a gram-positive, endo-spore forming bacterium known as the etiologic agent of anthrax (1,2). Spores of *B. anthracis* can result in systemic infection through three different modes of infection according to their method of entry: cutaneous, pulmonary and gastrointestinal (1). Upon infection, *B. anthracis* spores germinate into viable vegetative cells within the host and begin to spread to all tissues of the body. The high increase in vegetative cells result in the death of the host leading to the sporulation of the bacteria until another host becomes infected. Because *B. anthracis* is predominately found in soil, it is frequently exposed to variable living conditions. The bacteria's ability to withstand different environmental conditions such as thermal, oxidative and osmotic stress is imperative to its virulence and survival (20). As a common disease of livestock and occasionally humans, understanding the regulation of virulence is a prioritized topic of interest in bacterial pathogenesis of anthrax.

Virulence of *B. anthracis* is conferred by two virulence plasmids: pXO1 and pXO2. The pXO1 plasmid carries gene encoding the anthrax toxin subunits (1, 2, 3): *pagA*, encoding protective antigen, *lef*, encoding lethal factor, and *cya*, encoding edema factor. The master regulator of toxic gene expression is AtxA (4, 5, 6) which activates toxin gene expression in the presence of bicarbonate and in response to body temperature (7). The pXO2 virulence plasmid encodes a 5 gene operon that encodes the genes responsible for the synthesis of the poly-γ-D-glutamic acid (polyglutamate) capsule (8). Both virulence plasmids are necessary for the lethality of *B. anthracis* (4, 5, 6,7).

In order for *B. anthracis* to survive under different environmental conditions, certain strategies are used to transcribe essential virulence genes, such as the toxin genes, and

sporulation-related genes. One method of regulation of genes necessary for survival under

different conditions is through alternative sigma factors (14). Sigma factors are responsible for

promoter recognition and will bind to the RNA polymerase to initiate the transcription of RNA.

In doing so, the active core RNAP is able to transcribe genes regulated by its cognate sigma

factor such as the *Bacillus subtilis* sporulation genes that are regulated by its sporulation-

associated sigma factor (14, 9). Under conditions non-specific to the sigma factor associated

with environmental stress, the activity of some sigma factors is regulated by a specific inhibitor,

the anti-sigma factor. Anti-sigma factors act as antagonists by negatively regulating the activity

of its cognate sigma factor. The activity of the sigma factor is controlled by the anti-sigma factor

which binds to the sigma factor to inhibit the formation of the sigma factor-containing RNA

polymerase complex (13). One response to changes in environmental conditions is the

organism's ability to respond to a sudden elevation in the surrounding temperature. Response to

this increase in temperature is through the heat shock response (15). Heat shock response genes

are responsible for coding proteins appropriate for the survival of the bacteria at elevated

temperatures (15). The regulation of the heat shock genes are controlled by transcriptional

regulators such as alternative sigma factors. Sigma factors responsible for the regulation of heat

shock genes have been identified to be associated with cell viability under extreme

environmental conditions such as elevated temperatures (15). Because survival under various

conditions and utilization of complex systems to sense these changes are essential to the survival

of bacteria, it is hypothesized that sigma factors may be linked to the regulation of virulence in

Bacillus species (10, 14, 24).

The alternative sigma factor, σ^I, is a sigma factor found in *B. subtilis* to be associated

with heat survival with temperature-sensitive growth seen in *sigI* mutant strains at elevated

temperatures(10). In *B. subtilis,* the expression of the *sigI* is autoregulated by activated σ^I and is regulated by its anti-sigma factor, RsgI (16). Because of the high degree in homology between *B. subtilis* and *B. anthracis,* the predicted *sigI* gene in *B. anthracis*, BAS3231, is hypothesized to have a function similar to σ^I in *B. subtilis.* Although σ^I is required for adaptation to heat stress, the role of σ^I in virulence is not yet understood. σ^I in *B. subtilis* however, is not limited to the regulation of the heat shock genes and have been found to play a role in cell wall hydrolase. LytE, a peptidoglycan hydrolase, is involved in the synthesis and turnover of cell walls. An actin homolog gene, *mreBH,* responsible for the localization of autolysin LytE, belongs to the σ^I regulon in *B. subtilis.*   Because *mreBH* has been found to be a target of σ^I in *Bacillus subtilis*, it is hypothesized that not only does σ^I in *B. anthracis* transcriptionally regulate heat shock genes, but also target genes responsible for cell wall metabolism.

In this study, we investigate the role of σ^I on virulence and heat shock response. Our results suggest that σ^I affects toxin gene expression of *pagA, cya* and *lef*. We verified that a predicted anti-sigma factor, RsgI regulates the expression of σ^I at elevated temperatures. We also report that the σ^I in *B. anthracis* has a heat shock response function similar to that seen with *B. subtilis*.

2   MATERIALS AND METHODS

2.1   Strains and Growth

Relevant strains and plasmids used for this study are listed in Table 2.1.1. *B. anthracis* strain, 34F2(pXO1+ pXO2-) was grown in LB broth or BHI (brain heart infusion) broth supplemented with the appropriate antibiotics according to specific plasmids used with the appropriate concentrations: 7.5 µg/mL chloramphenicol, 7.5 µg/mL kanamycin. Transformation of plasmids were performed using competent cells of *B. anthracis* and were prepared using the method as described elsewhere (17) and electroporation was performed using a Bio-Rad Gene Pulse (2500V, 25µF, 400Ω, 4mm electroporation cuvette). *Escherichia coli* TG1, C600 and DH5α competent cells were prepared chemically as previously described (18) and clones were selected using appropriate antibiotics on LB agar: 7.5 µg/mL chloramphenicol, 30 µg/mL kanamycin. Plasmid construction and markerless deletion in *B. anthracis* was performed using methods previously described (19) Transformants were screened for incorporation of the mutation and plasmid retention of pXO1. LB-Bic was used to induce toxin expression by growing *B. anthracis* in LB containing 0.1M HEPES [pH 8.0] and 0.8% $NaHCO_2$ under 5% CO_2.

Table 1.1.1 Relevant Strains and Plasmids

Strain or plasmid	Relevant characteristic(s)
B. anthracis strains	
34F2	Parental
AW-A059	Insertional disruption of BAS3231
AW-A094	Markerless deletion of BAS3231
AW-A118	Markerless deletion of BAS3230
AW-A125	Markerless deletion of BAS3231-0
Plasmids	
pORI-Cm-I-SceI	pORI-Cm vector with I-SceI recognition site and (Cm^r)
pTCV-lac	Promoterless vector, transcriptional *lacZ* fusion (Kan^r)
pTCVlac-*pagA*	*pagA-lacZ* transcriptional fusion in pTCV-lac (Kan^r)
pTCVlac-*atxA12*	*atxA12-lacZ* transcriptional fusion in pTCV-lac (Kan^r)
pAW193	*cya-lacZ* transcriptional fusion in pTCV-lac (Kan^r)
pAW194	*lef-lacZ* transcriptional fusion in pTCV-lac (Kan^r)

2.2   β-Galactosidase Assay

B. anthracis lacZ fusion strains were constructed by fusion of predicted promoter on pTCV-lac replicative vector (21). Strains were grown in LB supplemented with 20 µg/mL kanamycin under inducing conditions at 37˚C. β-Galactosidase assays were performed as previously described in measure β-Galactosidase activity expressed in Miller Units (22, 23).

2.3   Heat Survival Assay

Survival of *B. anthracis* was assayed through a modification of the technique previously used (10) with the exception that survival temperature was 44˚C for 18 hours. Survival of strains was determined using serial dilution and colony counting after incubation at 37ºC for 15 hours.

3   RESULTS

3.1   Characterization of the *sigI* deletion strain

To investigate the role of *sigI* in virulence gene expression and cell growth, we made

markerless deletion strains in *B. anthracis* of the predicted *sigI* gene, BAS3231. When

characterizing growth of the deletion strain, the *sigI* mutant strain resulted in a decrease in

growth shown in Figure 3.1.1 compared to the parental strain, 34F2. As studied in *B. subtilis,* the

sigI mutant strains showed temperature-sensitive growth under elevated temperatures so a

growth deficiency under inducing conditions at 37˚C was not expected (10). However, with the

loss of *sigI* in *B. anthracis*, a deficiency in growth is observed at 37˚C. The growth deficiency

seen at non-elevated temperatures in the *sigI* mutant strain may be due to differential activities of

the *sigI* gene, for example, *sigI* in *B. anthracis* may function as another regulator other than heat

shock response
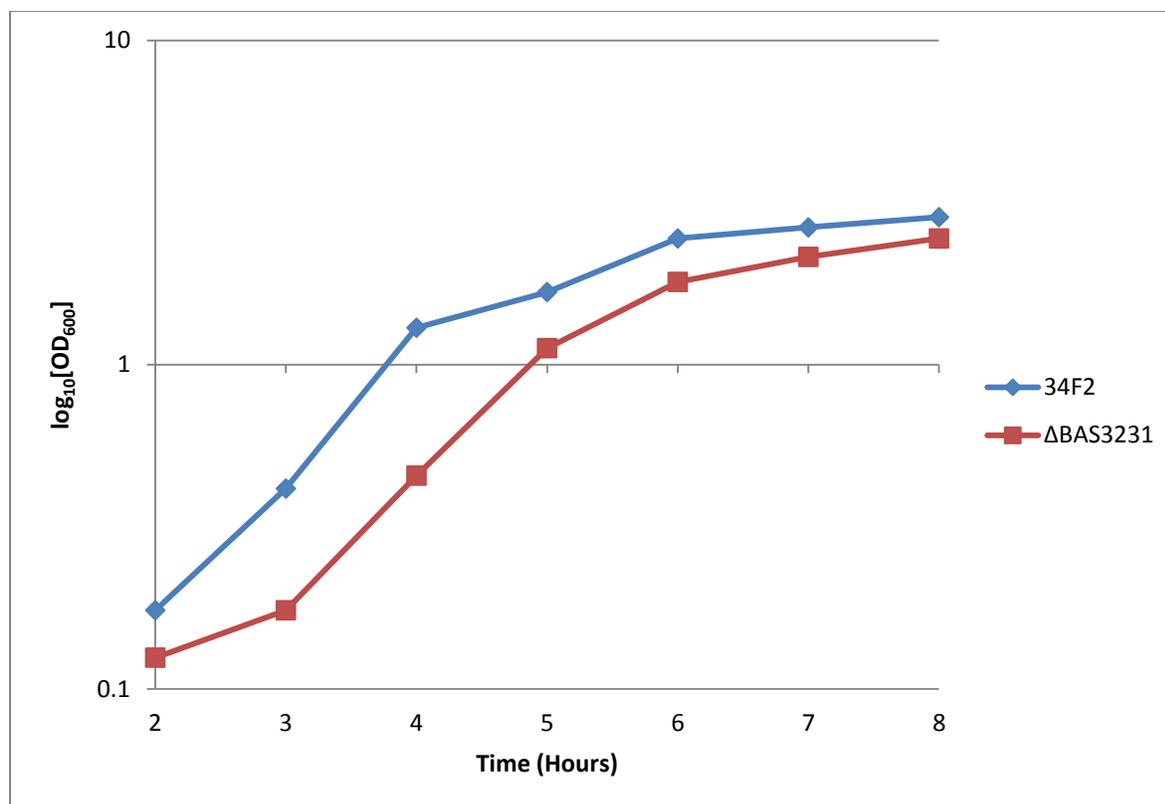
Figure 3.1.1 Cell growth of *sigI* mutant strain.
Cell growth of parental, 34F2, and the *sigI* mutant strain grown in LB at 37°C.

In addition to measuring growth, *lacZ* fusion plasmids were made with the promoter regions of virulence genes *atxA, pagA, cya* and *lef* to monitor virulence gene expression. As shown in Figure 3.1.2, the loss of the predicted *sigI* gene, BAS3231, resulted in a decrease of transcription of *pagA, cya* and *lef* during both exponential and stationary phase compared to the wild type strain. Unlike the change in expression of *pagA,* transcription of virulence regulatory gene *atxA* did not show a significant difference between the mutant and the wild type. This may suggest that the σ^I does not directly affect virulence gene expression through the master regulator, AtxA but through another mechanism to regulate *pagA, cya* and *lef.*
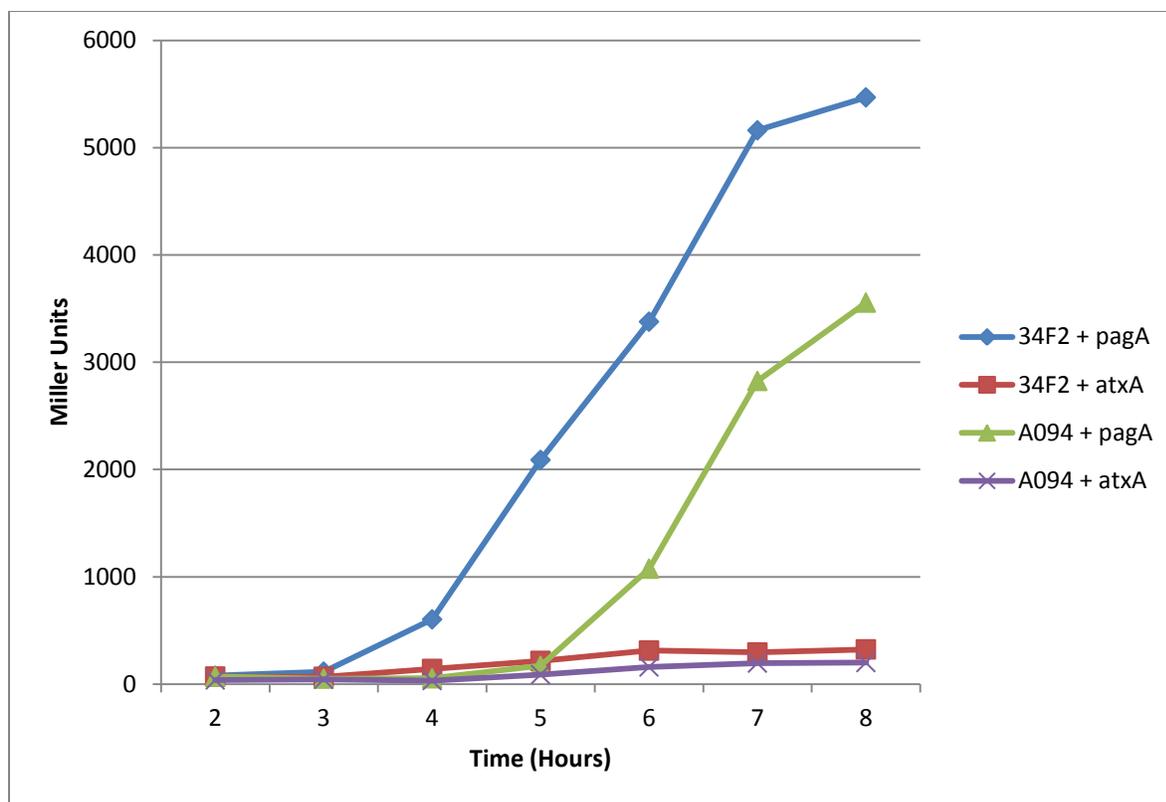
Figure 3.1.2 *pagA* **and** *atxA* **expression levels in** *sigI* **mutant strain.**
Virulence gene expression in the *sigI* **mutant strain. B-Galactosidase activity in** *pagA* **and**
atxA **reporter strains grown in LB-Bic supplemented with 7.5 µg/mL kanamycin under**
inducing conditions at 37°C.

Because change in virulence gene expression in late stationary phase showed the most

evident differences, we focused more carefully at the 8 hour mark into the stationary phase.

When measuring expression of all 4 virulence genes at 8 hours, the *sigI* mutant consistently

showed a significant decrease in *pagA, cya* and *lef* expression; however, there was no change in

atxA expression as seen in Figure 3.1.3. These results suggest that the loss of *sigI* is required for

the transcription of toxin genes *pagA, cya* and *lef* but does not affect expression of the master

regulator *atxA* which indicates that σ[I] either directly or indirectly affects *pagA, cya* and *lef*

without affecting transcription of the master virulence regulator.

Figure 3.1.3 *pagA*, *atxA, cya* and *lef* expression levels in *sigI* mutant strain. Virulence gene expression in *sigI* mutant strain at 8hrs. B-Galactosidase activity in *pagA* and *atxA, cya* and *lef* reporter strains grown in LB-Bic supplemented with 7.5 μg/mL kanamycin under inducing conditions at 37°C. ***, p<0.0001

3.2   Characterization of *rsgI* deletion strain

Although the interaction between σ^I and its anti-sigma factor, RsgI, in *B. subtilis* has already been established, interaction between the two proteins in *B. anthracis* has yet been characterized (13). The primary function of an anti-sigma factor is to bind to the sigma factor and hinder its ability to form an RNAP holoenzyme. Because σ^I is a known transcription factor activated by heat, low expression levels of virulence genes at inducing conditions were unexpected. To determine whether the predicted anti-sigma factor gene, BAS3230, could be affecting virulence gene expression, deletion strains of BAS3230 were constructed and characterization of growth and virulence gene expression were done. If the primary role of the predicted anti-sigma factor is to solely regulate the expression of *sigI,* deletion of the BAS3230

(*rsgI*) gene would show no change in both growth and virulence gene expression compared to the parental strain.

Under the same conditions used to perform the previous phenotype characterization, the same growth curve was done to determine whether loss of BAS3230 alone would affect cell viability over time as shown in Figure 3.2.1 These results suggest that loss of *rsgI* has no effect on cell growth at both exponential and stationary phase, indicating that *rsgI* has no role in cell growth as was shown with the *sigI* mutant strain.

Figure 3.2.1. Cell growth of *rsgI* mutant strain.
Cell growth of parental, 34F2, and the *rsgI* mutant strain grown in LB at 37°C.

To determine whether the predicted anti-sigma factor plays a role in virulence gene expression, β-Galactosidase assays were done using all 4 reporters: *pagA, atxA, cya,* and *lef.* As

seen in Figure 3.2.2, deletion of BAS3230 had no effect on virulence gene expression of all 4

reporter genes. This was anticipated as anti-sigma factors do not function in gene regulation

directly. This may also confirm that σ^I alone is responsible for virulence gene expression and
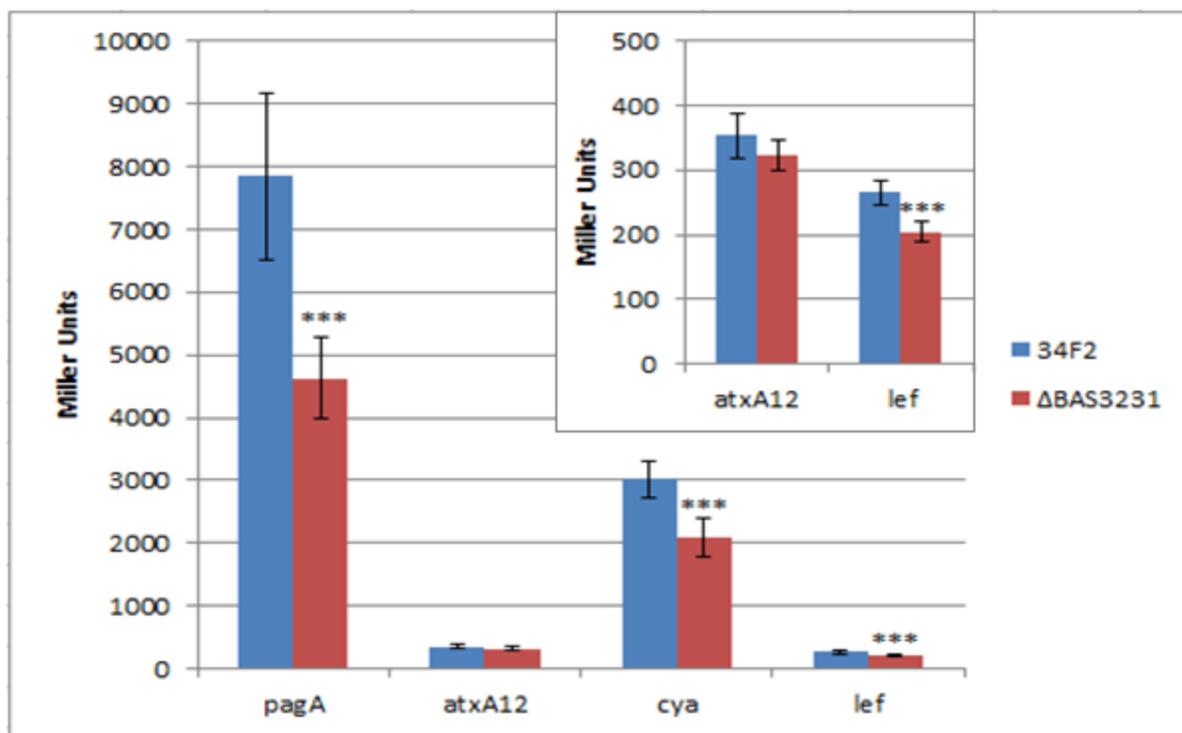
growth deficiency.
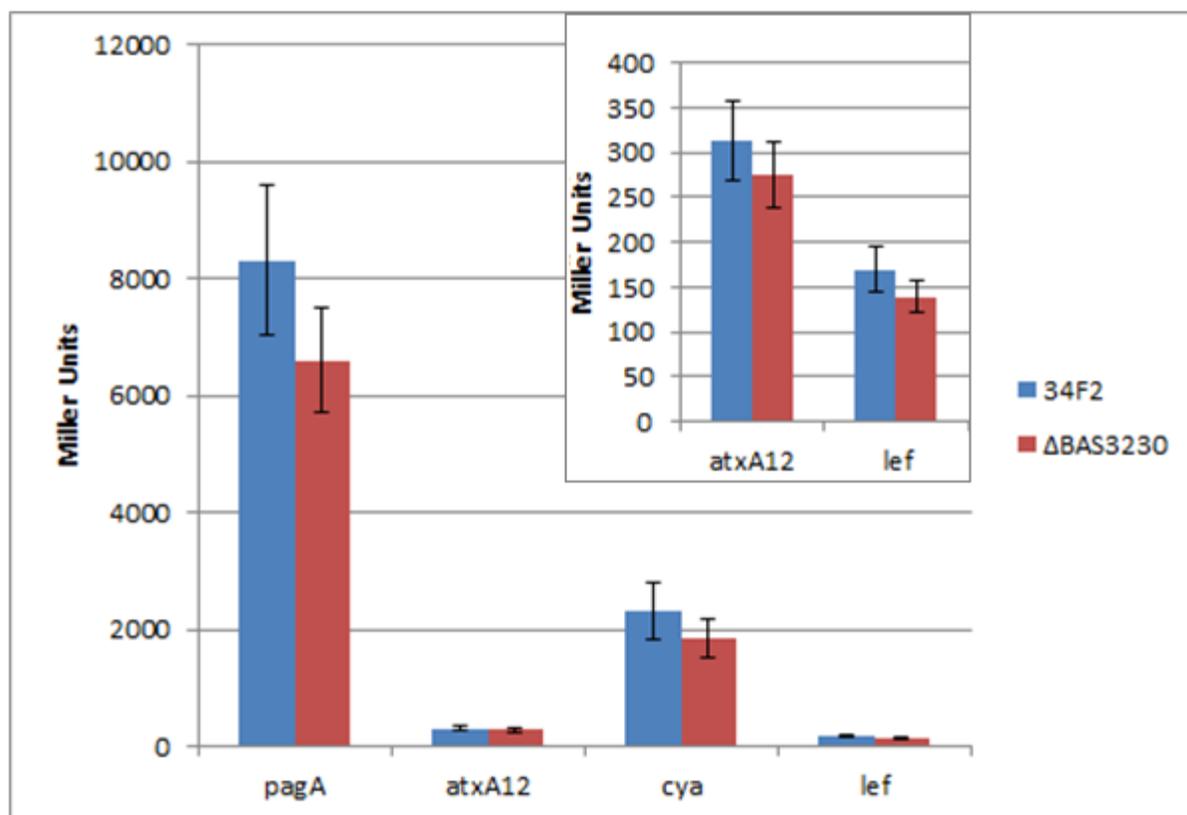
Figure 3.2.2 *pagA, atxA, cya* and *lef* **expression levels in** *rsgI* **mutant strain.**
Virulence gene expression in the rsgI mutant strain at 8hrs. β-Galactosidase activity in
pagA **and** *atxA***,** *cya* **and** *lef* **reporter strains grown in LB-Bic supplemented with 7.5 µg/mL**
kanamycin under inducing conditions at 37˚C.

3.3   Characterization of *sigI-rsgI* double deletion strain

Our earlier analysis of the *sigI* and *rsgI* mutant strains shows that the growth deficiency and

lowered virulence gene expression were seen with the single deletion of the predicted *sigI* gene,

BAS3231. To eliminate the possibility that another factor such as a neighboring gene or the

presence of the anti-sigma factor may be causing this change in phenotype, a double gene deletion of both the sigma factor and its anti-sigma factor was constructed. The double gene deletion strain was analyzed using the same conditions as the single gene deletion analysis for both growth and virulence gene expression. The double gene deletion of BAS3231-0 had a growth deficiency similar to the single gene deletion of BAS3231 under the same conditions as seen in Figure 3.3.1. This suggests that with the absence of both the sigma factor and its regulating anti-sigma factor, the phenotype is similar to the single deletion of the sigma factor alone. This observation was anticipated due to the understandings that without the anti-sigma factor present to regulate its cognate sigma factor; the sigma factor will consistently be active. However, with the sigma factor deleted in lieu of the anti-sigma factor, both the transcription protein and its regulator are no longer present to initiation any type of transcription.
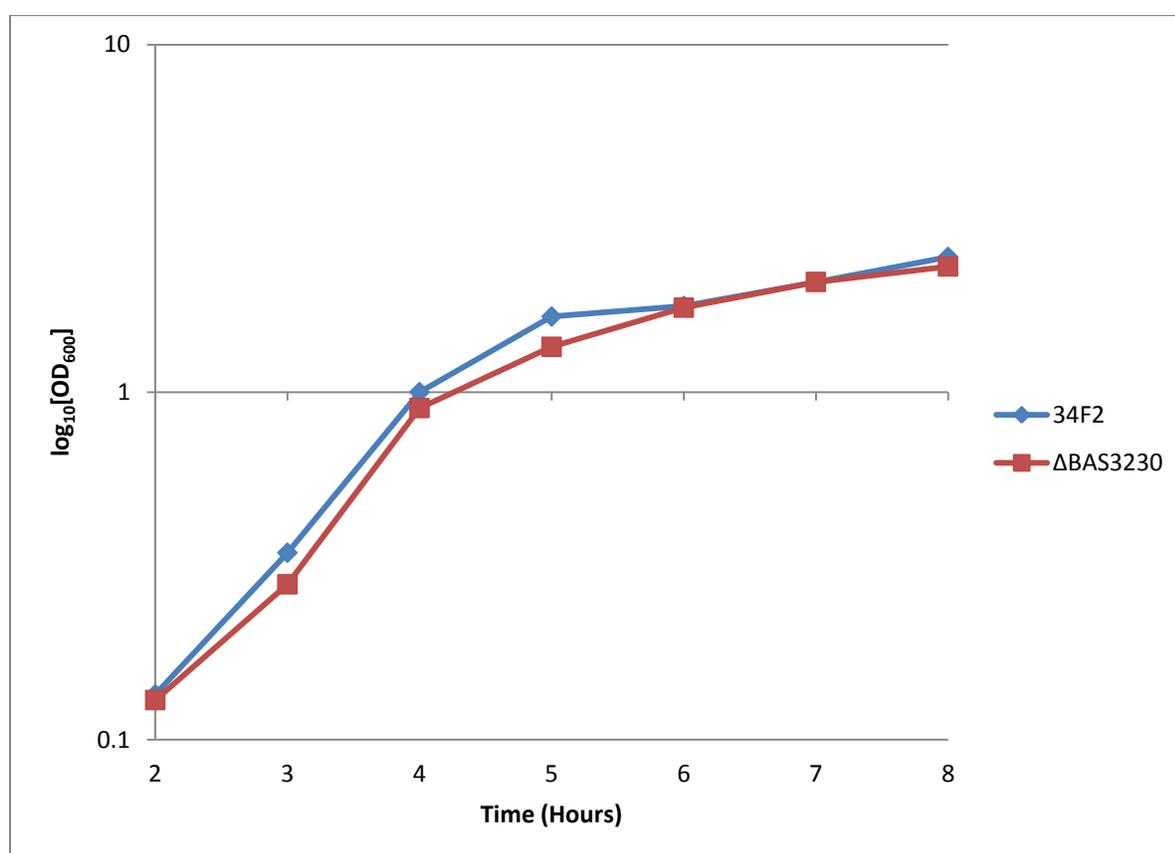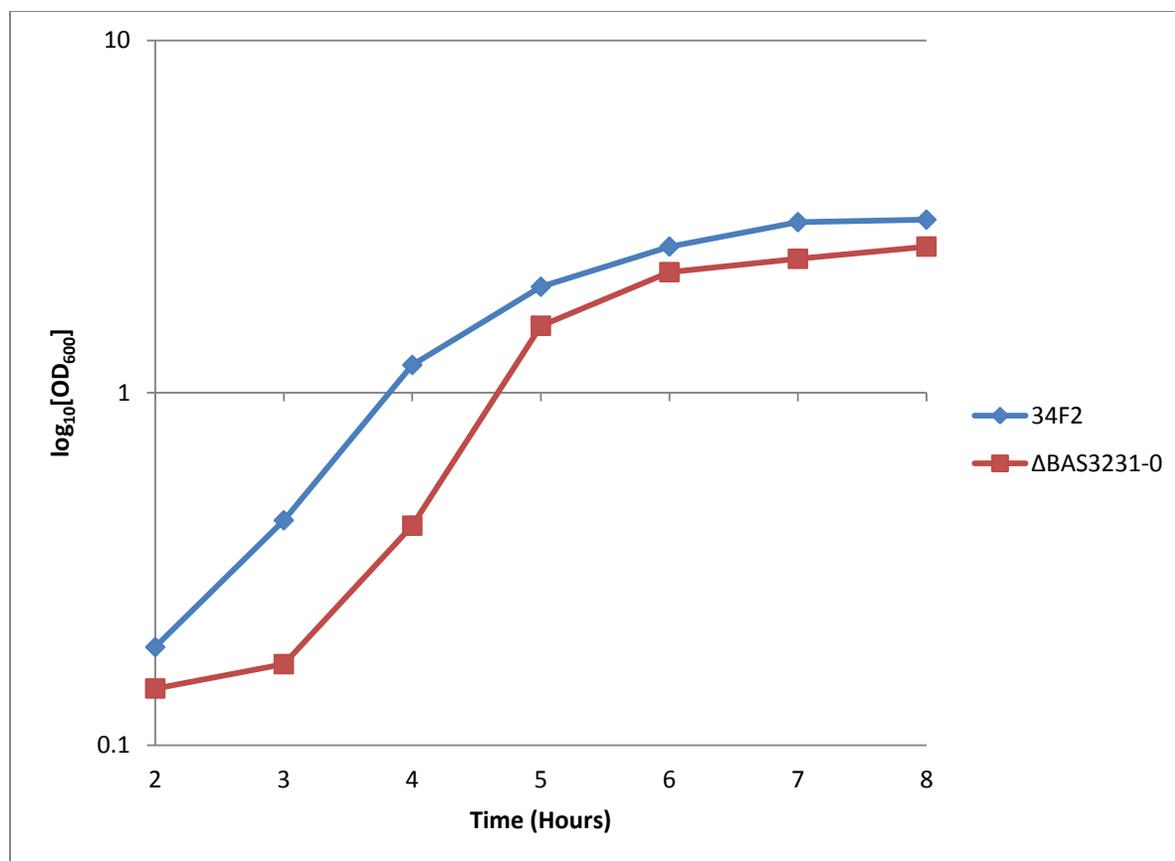
Figure 3.3.1 Cell growth *sigI-rsgI* mutant strain.
Cell growth of parental, 34F2, and the *sigI-rsgI* mutant strain grown in LB at 37˚C.

To further characterize the double mutant strain and to verify that the predicted *sigI* gene is responsible for the decrease in virulence gene expression of *pagA, cya* and *lef,* we performed a β-Galactosidase assay using the same conditions as mentioned before for the single gene deletions. When virulence gene expression was analyzed in the double mutant, we observed decrease in virulence gene expression similar to the phenotype seen with the single *sigI* deletion mutant as shown in Figure 3.3.2. The double gene deletion of both predicted *sigI* and *rsgI* had a decrease in *pagA, cya* and *lef*. When the same assay was performed at late stationary phase, gene expression of *pagA, cya* and *lef* were lower compared to the parental strain, 34F2.
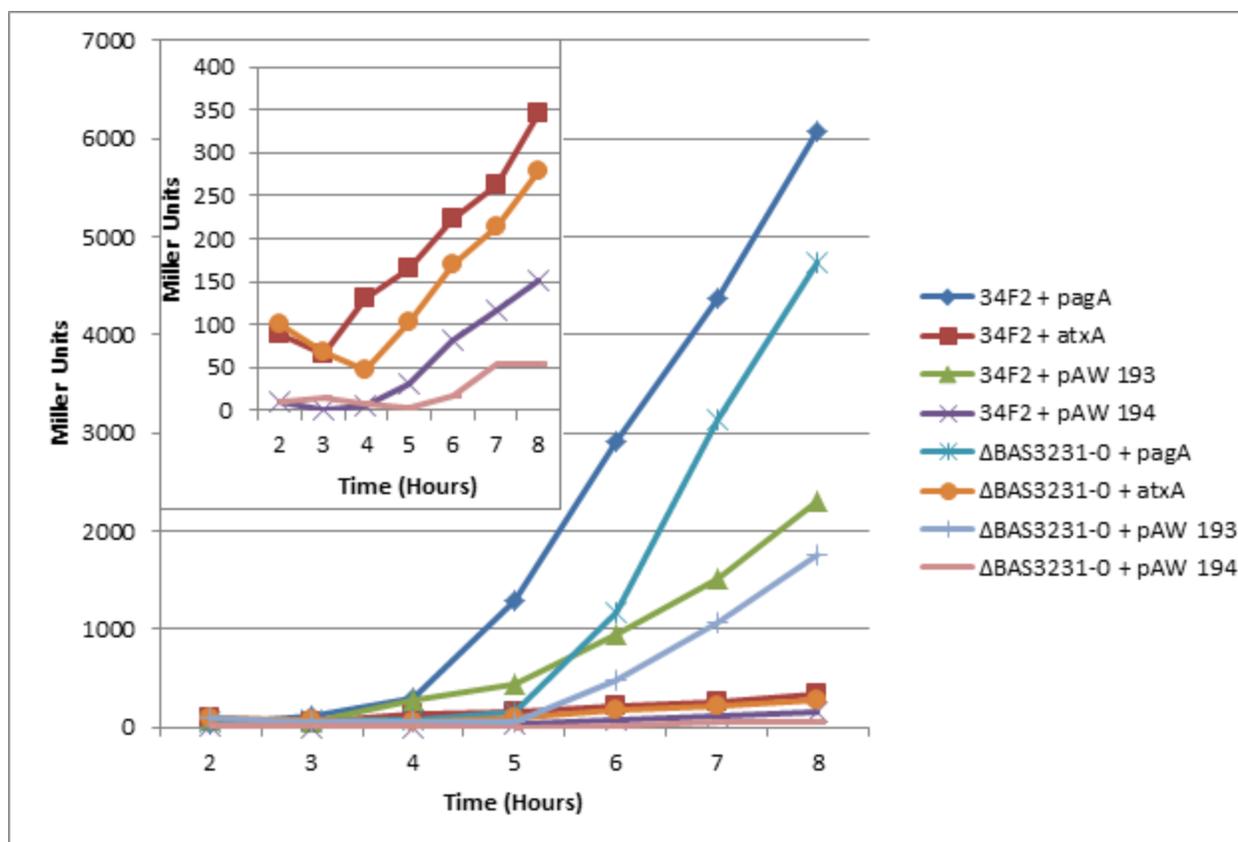
Figure 3.3.2 *pagA, atxA, cya* and *lef* **expression levels in** *sigI-rsgI* **mutant strain. Virulence gene expression in the** *sigI-rsgI* **mutant strain. β-Galactosidase activity in** *pagA* **and** *atxA, cya* **and** *lef* **reporter strains grown in LB-Bic supplemented with 7.5 μg/mL kanamycin under inducing conditions at 37˚C.**

This decrease in virulence gene expression seen with the double gene deletion of both *sigI* and *rsgI* was also observed in the *sigI* mutant strain. The decrease in *pagA, cya* and *lef* in the *sigI-rsgI* mutant strain were anticipated considering that the primary role of the anti-sigma factor is to regulate its cognate sigma-factor and is expected to have no effect on virulence gene expression. The data suggests that the role of the predicted anti-sigma factor does not play a role in virulence gene expression or growth and acts purely as the regulator of its sigma factor.

3.4 Predicted σ^I is induced by heat shock

In *B. subtilis*, the putative sigma factor σ^I has been found to be heat-inducible [10]. Because of the high similarity in homology between *B. subtilis* and *B. anthracis*, a heat shock survival assay was done with the *sigI* mutant strain in *B. anthracis*. The *sigI* deletion strain and the parental strain were grown at an elevated temperature of 44˚C and survival was determined. Survival rate was quantified by counting individual colonies after the plating of heat shock cultures followed by growth at 37˚C. Similar to the results seen with the *sigI* mutant strains in *B. subtilis* [10], *sigI* mutant strains in *B. anthracis* were unable to survive at elevated temperatures as seen in Table 3.4.1. The deletion mutant strains had similar growth as the parental at 37˚C; however, at an elevated temperature the mutant strains had a survival rate of almost 2% compared to the survival rate of 36% seen with the parental strain. These results suggest that just like σ^I in *B. subtilis,* the predicted σ^I in *B. anthracis* is a heat shock sigma factor that is necessary for the cell's ability to grow at elevated temperatures.

Table 2.4.1. Heat shock survival of *sigI* mutant strains at 44°C.
Survival of parental strain and *sigI* mutant strain after incubation in LB at 44˚C and 37˚C for 20 hours.

	CFU after incubation at:		
Strain	37˚C	44˚C	Survival rate (%)
34F2	$2.21\text{x}10^7$	$1.11\text{x}10^6$	35.67 ± 13.77
A094	$6.31\text{x}10^7$	$1.64\text{x}10^4$	1.744 ± 2.89

3.5   Transcription of *sigI* is induced by heat shock

sigI appears to be a part of an operon containing both the *sigI* predicted gene BAS3231 and the predicted anti-sigma factor, BAS3231. Upstream of the BAS3231 gene is a large intergenic region of 778bp between the genes BAS3231 and BAS3232 as seen in Figure 3.5.1. Although the neighboring gene BAS3232 lies in the same direction as the predicted *sigI* and *rsgI* gene, this intergenic region is a fairly large space between two neighboring genes. This allowed us to predict the presence of a possible *sigI* promoter within the intergenic space. Hypothesizing the presence of a promoter, we created a plasmid to measure promoter activity of BAS3231 during elevated temperatures.

Figure 3.5.1 Predicted BAS3231 (*sigI*) operon structure.

To measure promoter activity, a *lacZ* fusion plasmid was constructed using the entire 778bp intergenic region between the genes of BAS3231 and BAS3232. Promoter activity was measured using β-Galactosidase assay of both the parental strain and the *sigI* mutant strains at both 37°C and at an elevated temperature of 44°C as seen in Figure 3.5.2. At normal physiological conditions, promoter activity of both the parental strain and the *sigI* mutant strain falls to very low levels during late exponential phase, however, at approximately 5 hours of growth, promoter activity for both strains increases to levels up to 6 times higher. Interestingly, the mutant strain at 37°C showed a higher level of promoter activity at hour 8 compared to the

parental strain. When both strains were grown at an elevated temperature of 44˚C, promoter activity increased at an earlier time around 4 hours of growth, but activity in the mutant strain was significantly lower than the parental. These results not only provide evidence that σ^I is involved in heat shock but also that because promoter activity of *sigI* still increases at lower levels in the mutant strain during elevated temperature, regulation of the heat shock sigma factor could be induced by another promoter.

Figure 3.5.2 *sigI* promoter activity in *sigI* mutant strain.
BAS3231 promoter activity in the *sigI* mutant strain. β-Galactosidase activity in parental and *sigI* mutant strains grown in LB supplemented with 7.5 μg/mL kanamycin at 37˚C and 44˚C.

Promoter activity of the predicted *sigI* promoter was also measured in *rsgI* mutant strains using the same β-Galactosidase assay mentioned. Activity was measured in both the parental

strain and the *rsgI* mutant strains at 37˚C seen in Figure 3.5.3. As expected, when the cognate

regulator of the sigma factor is removed, transcription of the regulated gene will exceed normal

levels as seen in the parental strain. Activity of the promoter was measured in both LB and

inducing LB media containing bicarbonate to determine whether the promoter is inducible.

Miller units for BAS3231 promoter activity in the *rsgI* mutant strain remained the same

regardless of whether cultures were assayed in non-inducing LB or inducing LB. Interestingly,

promoter activity in the *rsgI* mutants were significantly higher than the parental strain. As

expected, an increase in promoter activity of *sigI* in the *rsgI* mutant strain was observed due to

the absence of its regulator with a 30-fold increase promoter activity. These results suggest that

without the presence of the anti-sigma factor to regulate levels of its cognate sigma factor,

promoter activity of the predicted *sigI* levels increase to extremely high levels even without

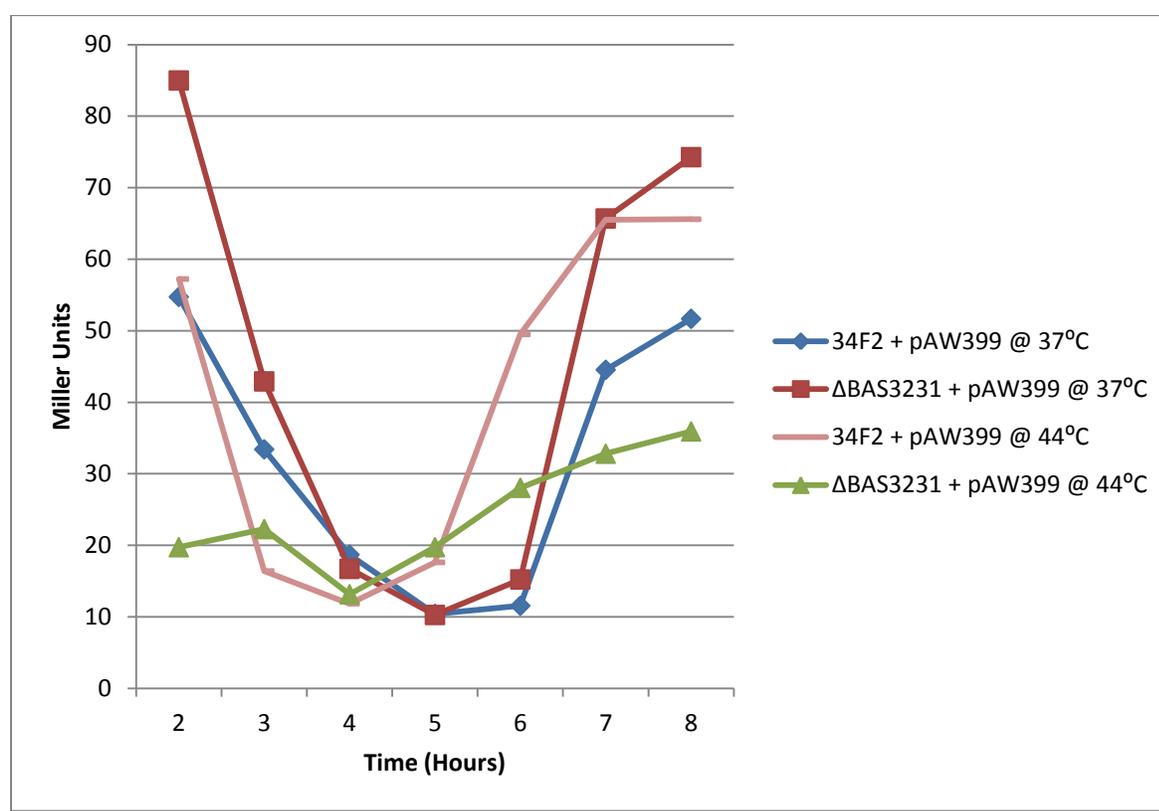induction or the presence of elevated temperatures.
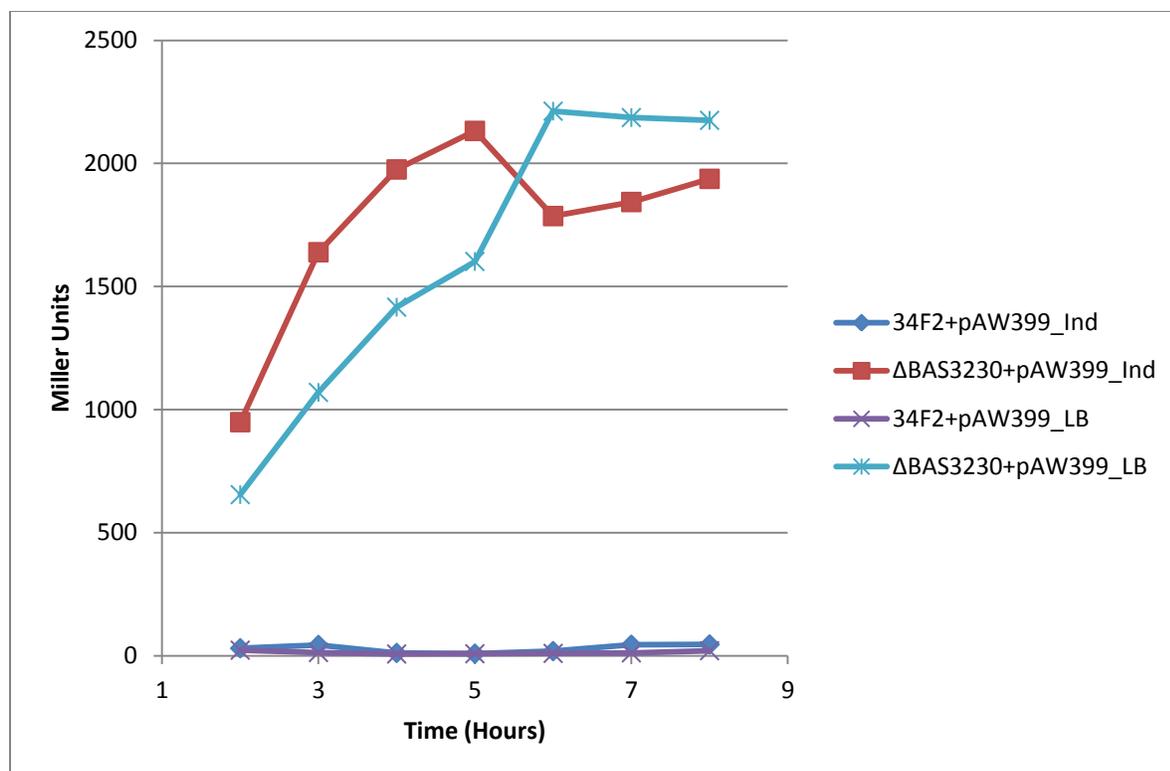
Figure 3.5.3 *sigI* **promoter activity in** *rsgI* **mutant strain.**
BAS3231 promoter activity in the *rsgI* **mutant strain β-Galactosidase activity of predicted**
in parental and *rsgI* **mutant strains grown in LB and LB-Bic supplemented with 7.5 μg/mL**
kanamycin under inducing conditions at 37˚C.

4   DISCUSSION

Heat shock sigma factors contribute to cell viability at elevated temperatures by initiating

the transcription of appropriate heat shock genes by activating the inactive RNAP. Due to the

homology between the two species *B. subtilis* and *B. anthracis*, the effect of deleting *sigI* in *B.*

anthracis was done to test whether the function of σ^I is conserved between the two similar

species. In *B. subtilis*, σ^I is induced by heat shock and mutant strains of the predicted *sigI* gene

are unable to grow at elevated temperatures. Very similar to *B. subtilis*, the σ^I in *B. anthracis* is

also induced by elevated temperatures. In addition to induction of σ^I by elevated temperatures,

sigI mutants in *B. anthracis* have decreased toxin gene expression and a deficiency in growth in both non-inducing and inducing conditions supplemented with CO_2/bicarbonate.

Mutant *sigI* strains in *B. anthracis* show a decrease in *pagA, cya* and *lef* toxin gene expression; however, levels of *atxA* remain the same compared to the parental 34F2 strain. Although σ^I is a heat shock sigma factor, virulence gene regulation mechanisms have not been found for the heat shock sigma factor and its correlation to virulence gene expression. *sigI* in *B. subtilis* has been linked to the regulation of cell wall synthesis pathways such as the WalRK two component system in growing and stressed cells (24, 25, 26). In *B. subtilis,* the alternative sigma factor, σ^I , is a target of the WalRK two component system and is targeted by the WalR regulon under heat stress (25). With the regulation of *sigI* and other genes associated with cell morphogenesis and cell wall hydrolysis by the WalR regulon, transcription of *sigI* has been found to be heat-inducible(25, 26). Whether the regulation of virulence gene expression by *sigI* in *B. anthracis* is direct or indirect, toxin gene expression appears to require the presence of *sigI*.

This report suggests that σ^I is involved in heat shock. In the taxonomically similar species, *B. subtilis*, *sigI* mutant strains showed temperature-sensitive growth at elevated temperatures resulting in the conclusion that transcription of sigma factor σ^I in *B. subtilis* is induced by heat shock (10). When the mutation strain of the putative *sigI* gene in *B. anthracis* was grown at elevated temperatures, temperature-sensitive growth was also seen. This provides evidence that, without the *sigI* gene, the strain is unable to withstand elevated temperatures. Mutant *sigI* strains in *B. anthracis* also had a decreased level of *sigI* promoter activity at elevated temperature. However, at 37°C, promoter activity of the *sigI* mutant strain was higher than the parental at not only early exponential phase but more importantly the late stationary phase. Our anticipated result was that at normal temperatures, the mutant strain would either have similar promoter

activity as the parental strain or slightly lower due to the previous data suggesting that *sigI* is induced by heat shock. However, the elevated promoter activity in the mutant strain at normal conditions may suggest the presence of a secondary transcriptional regulator. This suggests that, similar to the *sigI* operon seen in *B. subtilis*, a σ^A promoter may be involved in the regulation of *sigI*(26). If a second promoter is located in proximity to the *sigI* promoter, deletion of the *sigI* gene may increase the activity of this promoter. We hypothesize that the lower promoter activity seen in the parental strain at normal conditions is low due to the idea that the presence of both σ^I and σ^A could act as an interference with one another resulting in a lower level of promoter activity. If this is true, in the mutant strain, *sigI* is deleted which results in a decrease in interference between σ^I and σ^A which may cause the increase in promoter activity of *sigI*.

We also showed that the transcription of *sigI* is tightly regulated by its cognate anti-sigma factor, RsgI. When promoter activity was measured in strains missing the anti-sigma factor gene *rsgI*, *sigI* promoter activity increased 100 fold. The function of the anti-sigma factor is known to act as an antagonist by negatively regulating its sigma factor-dependent transcription by inhibiting the sigma factor. We found that with the *rsgI* mutant strain, promoter activity of *sigI* was increased significantly which was expected. Given the results of the no change in toxin gene expression with the *rsgI* mutant strain, it was hypothesized that the anti-sigma factor had no effect on virulence phenotypes other than to regulate the sigma factor. However, our findings of increased promoter activity of *sigI* in the *rsgI* mutant strain provides evidence that regulation by *rsgI* is incredibly controlled under elevated temperatures and without it , *sigI* would be strongly produced.

Although our results suggest that σ^I is a heat induced sigma factor, we are uncertain about the relationship between the role of this heat shock sigma factor and toxin gene expression. It is

possible that *pagA* is indirectly regulated by σ^I and that additional mechanisms are used for the regulation of toxin gene expression. While these observations provide evidentiary support of the homology found between the two species of *B. subtilis* and *B. anthracis*, the understanding of virulence regulation is still unknown. Additional work is required to identify the regulation mechanism and relationship between σ^I and *pagA, cya* and *lef* to define their relationship and the role of a heat shock sigma factor in virulence gene regulation.

5   FUTURE DIRECTIONS

5.1   Indirect or direct regulation of virulence gene expression by σ^I

Using methods such as *in vitro* transcription, microarray analysis and β-Galactosidase reporter assays, we hope to verify our hypothesis that the putative σ^I of *B. anthracis* either indirectly or directly regulates virulence gene expression. To determine whether there is a direct correlation between *pagA, cya* and *lef* expression and the presence of σ^I, *in vitro* transcription will be used. Using core RNA polymerase, recombinant σ^I proteins and templates for the toxin genes *pagA, cya* and *lef,* we will be able to determine whether σ^I alone is able to initiate the transcription of the toxin genes by regulating transcription from the virulence gene templates.

To determine whether there is an indirect regulation of toxin gene expression, microarray analysis will be used to measure fold change of genes affected by the deletion of *sigI* under normal physiological conditions and at elevated temperatures. Fold changes of genes expressed in the parental strain compared to the mutant strains at different conditions will provide information on the possible genes involved in a mechanistic pathway in virulence gene expression. Microarray analysis will not only provide additional evidence of changes in

virulence gene expression in the mutant strains but also shed light on other affected genes to make inferences on the possible mechanistic pathway of virulence gene regulation.

5.2 BAS3228 and its relationship in cell wall synthesis

Cell wall hydrolases are critical for bacterial virulence in its involvement with cell wall turnover and synthesis. In the taxonomically similar species *B. subtilis*, autolysin LytE is a peptidoglycan hydrolase that has been found to be driven by a σ^A promoter (11) and a two component system, YycFG (WalRK) (11). It has been found that an actin homolog gene *mreBH* belongs to the σ^I regulon and is responsible for the localization of LytE in *B. subtilis* (11). Because it has been found that the *mreBH* gene is a target of σ^I in *B. subtilis*, we have investigated neighboring genes upstream and downstream of the *sigI* gene. Downstream of the *sigI* gene, BAS3231, in *B. anthracis* lies the gene BAS3228 that has been annotated as a hydroxyl transferase. Because hydroxyl transferases are involved in the synthesis of polysaccharides that compose the peptidoglycan of gram-positive bacteria, we are hypothesizing that the transferase gene, BAS3228 plays an active role in not only cell wall synthesis but also an indirect or direct role in antibiotic resistance to β-lactam antibiotics such as Oxacillin. In our preliminary study of *B. anthracis sigI* mutant strains, it has been observed that some *sigI* mutant strains are susceptible to β-lactam class antibiotics, specifically Oxacillin. Given our hypothesis and observations, we hope to identify the role of the predicted transferase gene BAS3228 in the species' ability to withstand β-lactam antibiotics.

REFERENCES

1. Dixon, T. C., Meselson, M., Guillemin, J., & Hanna, P. C. (1999). Anthrax. *N Engl J Med, 341*(11), 815-826.

2. Koehler, T. M. (2009). Bacillus anthracis physiology and genetics. *Mol Aspects Med, 30*(6), 386-396.

3. Little, S. F., & Ivins, B. E. (1999). Molecular pathogenesis of Bacillus anthracis infection. *Microbes Infect, 1*(2), 131-139.

4. Bongiorni, C., Fukushima, T., Wilson, A. C., Chiang, C., Mansilla, M. C., Hoch, J. A., & Perego, M. (2008). Dual promoters control expression of the Bacillus anthracis virulence factor AtxA. *J Bacteriol, 190*(19), 6483-6492.

5. Fouet, A. (2010). AtxA, a Bacillus anthracis global virulence regulator. *Res Microbiol, 161*(9), 735-742.

6. Hammerstrom, T. G., Roh, J. H., Nikonowicz, E. P., & Koehler, T. M. (2011). Bacillus anthracis virulence regulator AtxA: oligomeric state, function and CO(2) -signalling. *Mol Microbiol, 82*(3), 634-647.

7. Bertin, M., Chateau, A., & Fouet, A. (2010). Full expression of Bacillus anthracis toxin gene in the presence of bicarbonate requires a 2.7-kb-long atxA mRNA that contains a terminator structure. *Res Microbiol, 161*(4), 249-259.

8. Candela, T., Mock, M., & Fouet, A. (2005). CapE, a 47-amino-acid peptide, is necessary for Bacillus anthracis polyglutamate capsule synthesis. *J Bacteriol, 187*(22), 7765-7772.

9. Piggot, P. J., & Hilbert, D. W. (2004). Sporulation of Bacillus subtilis. *Curr Opin Microbiol, 7*(6), 579-586.

10. Zuber, U., Drzewiecki, K., & Hecker, M. (2001). Putative sigma factor SigI (YkoZ) of Bacillus subtilis is induced by heat shock. *J Bacteriol, 183*(4), 1472-1475.

11. Margot, P., Wahlen, M., Gholamhoseinian, A., Piggot, P., & Karamata, D. (1998). The lytE gene of Bacillus subtilis 168 encodes a cell wall hydrolase. *J Bacteriol, 180*(3), 749-752.

12. Tseng, C. L., & Shaw, G. C. (2008). Genetic evidence for the actin homolog gene mreBH and the bacitracin resistance gene bcrC as targets of the alternative sigma factor SigI of Bacillus subtilis. *J Bacteriol, 190*(5), 1561-1567.

13. Dufour, A., & Haldenwang, W. G. (1994). Interactions between a Bacillus subtilis anti-sigma factor (RsbW) and its antagonist (RsbV). *J Bacteriol, 176*(7), 1813-1820.

14. Kazmierczak, M. J., Wiedmann, M., & Boor, K. J. (2005). Alternative sigma factors and their roles in bacterial virulence. *Microbiol Mol Biol Rev, 69*(4), 527-543.

15. Schumann, W. (2003). The Bacillus subtilis heat shock stimulon. *Cell Stress Chaperones, 8*(3), 207-217.

16. Asai, K., Ootsuji, T., Obata, K., Matsumoto, T., Fujita, Y., & Sadaie, Y. (2007). Regulatory role of RsgI in sigI expression in Bacillus subtilis. *Microbiology, 153*(Pt 1), 92-101.

17. Koehler, T. M., Dai, Z., & Kaufman-Yarbray, M. (1994). Regulation of the Bacillus anthracis protective antigen gene: CO2 and a trans-acting element activate transcription from one of two promoters. *J Bacteriol, 176*(3), 586-595.

18. Sambrook J., Russell D. W., (2001) Molecular cloning: a laboratory manual, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

19. Han, H., Wilson, A. C. (2013). The two CcdA proteins of Bacillus anthracis differentially affect virulence gene expression. *J Bacteriol, 195(23),* 5242-5249.

20. Cybulski, R. J., Jr., Sanz, P., Alem, F., Stibitz, S., Bull, R. L., & O'Brien, A. D. (2009). Four superoxide dismutases contribute to Bacillus anthracis virulence and provide spores with redundant protection from oxidative stress. *Infect Immun, 77*(1), 274-285.

21. Poyart, C., & Trieu-Cuot, P. (1997). A broad-host-range mobilizable shuttle vector for the construction of transcriptional fusions to beta-galactosidase in gram-positive bacteria. *FEMS Microbiol Lett, 156*(2), 193-198.

22. Miller J. H., (1972) Experiments in molecular genetics, p 352-355. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

23. Wilson, A. C., Hoch, J. A., & Perego, M. (2008). Virulence gene expression is independent of ResDE-regulated respiration control in Bacillus anthracis. *J Bacteriol, 190*(15), 5522-5525.

24. Hadjifrangiskou, M., Chen, Y., & Koehler, T. M. (2007). The alternative sigma factor sigmaH is required for toxin gene expression by Bacillus anthracis. *J Bacteriol, 189*(5), 1874-1883.

25. Huang, W. Z., Wang, J. J., Chen, H. J., Chen, J. T., & Shaw, G. C. (2013). The heat-inducible essential response regulator WalR positively regulates transcription of sigI, mreBH and lytE in Bacillus subtilis under heat stress. *Res Microbiol, 164*(10), 998-1008.

26. Salzberg, L. I., Powell, L., Hokamp, K., Botella, E., Noone, D., & Devine, K. M. (2013). The WalRK (YycFG) and sigma(I) RsgI regulators cooperate to control CwlO and LytE expression in exponentially growing and stressed Bacillus subtilis cells. *Mol Microbiol, 87*(1), 180-195.