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The study of sigma factors o5816 and c7615 in Bacillus cereus

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ABSTRACT

The Gram-positive and ubiquitous bacterium *Bacillus cereus* is an emerging human and animal pathogen, and is capable of adapting to numerous extracellular stresses such as the low pH environment of the gastrointestinal tract. One mechanism *B. cereus* might utilize to regulate the acid-tolerance response system is through alternative sigma factors: proteins that bind and direct RNA polymerase to promoter-specific genes. A previous study found that alternative sigma factors σ5816 and σ7615 were induced dramatically at low pH, but it was unclear whether these sigma factors mediated an acid-tolerance response. Here, molecular tools were developed to further study these σ factors, including a regulatable expression system and sigma-specific polyclonal antibodies. We were unable to identify a growth-deficient or growth-enhanced phenotype in strains that had these sigma factors deleted or could over-express these sigma factors, respectively. Through transcriptome analysis and qRT-PCR, we attempted to define the σ5816 regulon. The proteolytic subunit of a Clp protease, ClpA, was found to potentially be part of the σ5816 regulon. Understanding how *B. cereus* uses sigma factors to adapt to environmental stresses may give insight on how this emerging pathogen interacts and survives in its host.

KEY WORDS: *Bacillus cereus*, extracytoplasmic function, alternative sigma factor, stress response, acid tolerance response

DEDICATION

I dedicate this thesis to my mother, Julie, and my step-father, Terry. Without your love, guidance, and support, I would not have accomplished nearly as much as I have. I owe my successes to the two of you. Thank you.

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List of Abbreviations

ATCC	The American Type Culture Collection™
ATR	Acid Tolerance Response
β-MCE	Beta-Mercaptoethanol
Da	Dalton
ECF	Extra-Cytoplasmic Function
EDTA	Ethylenediaminetetraacetic Acid
FDR	False Discovery Rate
FPLC	Fast Protein Liquid Chromatography
HTH	Helix-Turn-Helix
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
KU	Klett Units
LB	Luria Broth
MCS	Multiple Cloning Site
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
MW	Molecular Weight
NGS	Next Generation Sequencing
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PMF	Proton Motive Force
PSI	Pounds per Square Inch
OD	Optical Density
OFA	Oxalate/Formate Antiporter
O/N	Over Night
ONPG	Ortho-Nitrophenyl-β-Galactoside
ORI	Origin of Replication
qRT-PCR	Quantitative Reverse-Transcriptase Polymerase Chain Reaction
RE	Restriction Enzyme
RIP	Regulated Intramembrane Proteolysis
RNAP	RNA Polymerase
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
SOD	Superoxide Dismutase
SOLiD	Support Oligonucleotide Ligation Detection™
TBS	Tris-Buffered Saline
TCS	Two-Component System
TE	Tris-EDTA
WT	Wild-Type

PART 1: INTRODUCTION

1.1 An overview of *Bacillus cereus*

Bacillus cereus is a Gram-positive, facultative anaerobic, rod-shaped bacterium and, like other species of *Bacillus*, capable of forming endospores (Kotiranta *et al.*, 2000). Here, however, the major physiological similarities end among different *B. cereus* strains. With a bacterial genome of 5.5 Mbp, *B. cereus* exhibits a high degree of genetic heterogeneity; correspondingly, the different strains of *B. cereus* display a diverse range of phenotypes (Kotiranta *et al.*, 2000; Rasko *et al.*, 2005). For example, a particular *B. cereus* isolate may exhibit motility, secrete a paracrystalline surface protein layer (S-layer), and form biofilms, while a different isolate lacks these capabilities altogether (Kotiranta *et al.*, 2000). While remarkable phenotypic and genetic differences exist between various strains of *B. cereus*, striking similarities arise when one compares *B. cereus* as a species to other members of the genus *Bacillus* (Rasko *et al.*, 2005). Ultimately, these conflicting findings have complicated the classification of *B. cereus* and closely related organisms (Rasko *et al.*, 2005; Vilas-Bôas *et al.*, 2007).

1.2 Taxonomy

The genomic sequence of *B. cereus* is highly similar to a few other members of *Bacillus* (Ivanova *et al.*, 2003). For example, strains of *B. cereus, Bacillus anthracis,* and *Bacillus thuringiensis* differ by less than one percent in their 16S rRNA gene sequences (Ash *et al.*, 1992; Chen *et al.*, 2002), and share greater than 80% sequence similarity according to DNA-DNA hybridization experiments (Kaneko *et al.*, 1973). These two

criteria, in conjunction, are generally sufficient for assigning strains to the same species (Schleifer, 2009). Moreover, modern molecular techniques such as multilocus sequence typing (MLST) and multilocus enzyme electrophoresis (MLEE) cannot consistently discriminate between strains of these three *Bacillus* species (Helgason *et al.*, 1998; Helgason *et al.*, 2004; Tourasse *et al.*, 2011). Yet, each of these *Bacillus* members has earned a different reputation in bacteriology: *B. anthracis* is the etiological agent of the notorious and lethal disease anthrax; *B. thuringiensis* is important in biotechnology for the production of insecticidal compounds; and *B. cereus* has traditionally been thought of as a ubiquitous, though generally innocuous, bacterium that has garnered attention as an emerging human and animal pathogen (Vilas-Bôas *et al.*, 2007).

The designation of closely related *Bacillus* strains to one of these three aforementioned species was based on the presence of certain characteristic features, such as the lethal toxin produced by *B. anthracis*, the insecticidal, parasporal inclusions produced by *B. thuringiensis*, and the emetic toxin, cereulide, produced by *B. cereus*. However, these distinguishing features are known to be largely plasmid-encoded rather than chromosomal in origin (Ezzell *et al.*, 2009; Vilas-Bôas *et al.*, 2007). An increasing amount of evidence indicates that these plasmids can, in many situations, be transferred between closely related species (Vilas-Bôas *et al.*, 2007). In short, the taxonomy of these bacteria is disputed, with some researchers proposing that strains of *B. anthracis* and *B. thuringiensis* should be reclassified as strains of *B. cereus* (Helgason *et al.*, 2004). Nevertheless, a non-taxonomic ranking at the level of subgenus, known as the *Bacillus cereus* group and of which *B. cereus* is the archetypical and eponymous member, was created to group six highly related *Bacillus* species. This group includes not only *B.*

cereus, B. anthracis, and *B. thuringiensis,* but also *Bacillus weihenstephanensis*, *Bacillus mycoides,* and *Bacillus pseudomycoides* (Tourasse *et al.*, 2011).

1.3 Habitat

While primarily thought of as a soil-dwelling bacterium, *B. cereus* is ubiquitous and can be found in many other environments, including both fresh and marine waters, decaying organic matter, and as part of the microflora in the guts of many invertebrates and vertebrates (Bottone, 2010; Jenson *et al.*, 2003). *B. cereus* is generally mesophilic, growing optimally at temperatures between 30°C and 37°C (Kotiranta *et al.*, 2000). However, various strains have been found to be psychrotolerant as well as psychrotrophic, growing at temperatures between 4°C and 7°C (Larsen and Jørgensen, 1999; TeGiffel *et al.*, 1997). Other isolates have been found to be moderately thermophilic, growing at temperatures above 43°C (Kotiranta *et al.*, 2000). The diversity in the range of potential habitats of *B. cereus* is explained by the fact that the bacterium, relative to many other microbes, has a large genome that encodes numerous metabolic pathways. Consequently, *B. cereus* tolerates many environmental conditions and has simple nutritional requirements (Kotiranta *et al.*, 2000).

B. cereus and other *Bacillus* species have traditionally been thought endemic to the soil, where they are abundant: as many as 10^6 *Bacillus* spores can be found per gram of soil (Nicholson *et al.*, 2002; Hong *et al.*, 2009). Recently, more attention has been given to examining the roles of *B. cereus* and *Bacillus* spp. as part of gut microflora in both animals and humans. While *B. cereus* is known to be part of the natural gut microflora of many invertebrates, *Bacillus* spp. in general are not considered permanent

members of the human gut microflora. Results from recent studies, however, have shown that healthy individuals excrete on average 10^4 *Bacillus* spores per gram of feces: a number too great to be explained by the ingestion of soil-derived foods alone (Hong *et al.*, 2009). Conjectures have since been put forth proposing *Bacillus* spp., including *B. cereus*, have acquired the ability to inhabit also the gastrointestinal tracts of humans. While no solid evidence has yet shown that *B. cereus* can be a long-term colonizer of the human gastrointestinal tract, the bacterium has long since been recognized as a potential transient member of the human gut microflora: *B. cereus* is well-known for causing self-limiting food borne-illness (Kotiranta *et al.*, 2000; Jenson *et al.*, 2003; Turnbull and Kramer, 1985).

1.4 Pathogenesis

By far the most common manifestation of *B. cereus* infection is food-borne illness. Both the dormant spores and vegetative cells of *B. cereus* possess features that contribute to the wide dissemination and overall ubiquity of these bacteria in the food industry (Bottone, 2010). Spores are particularly difficult to kill as they are resistant to acidity, freezing, desiccation, irradiation, high temperature, and other stresses (Bottone, 2010). Thus, these spores can survive pasteurization, gamma-ray irradiation, organic acid treatment, and many other procedures commonly employed to preserve or sterilize food (Kamat *et al.*, 1989; Larsen *et al.*, 1999). Moreover, the great hydrophobicity of the spore allows for the tight adhesion to a variety of substrates, such as food processing machinery and equipment (Arnesen *et al.*, 2008). Vegetative cells may secrete S-layers

or form biofilms, features that have been both shown to increase *B. cereus* adhesion to fomites, foods, and human intestinal epithelial cells (Kotiranta *et al.*, 1998).

Virulent strains can cause both emetic- and diarrheal-type food poisoning. The emetic form has an incubation period of between one and five hours, and is usually associated with the consumption of contaminated wheat and grains; comparatively, the diarrheal form has a longer incubation time of eight to sixteen hours, and can arise from the consumption of contaminated meat, vegetables, and dairy products (Granum *et al.*, 1997). The prevalence of each type varies geographically, with the diarrheal-type predominating in countries of Scandinavia and eastern Europe and the emetic-type predominating in the United Kingdom and Japan (Kramer *et al.*, 1989).

B. cereus strains are capable of producing a variety of virulence factors. The emetic-type food poisoning is caused by cereulide, a heat-stable toxin that is pre-formed in food and exerts its emetic effect even if the food-contaminating bacteria have been thoroughly killed through prior cooking (Granum *et al.*, 1994). Other *B. cereus* strains can produce a variety of diarrheal toxins, with the most important being hemolysin (HBL) and non-hemolytic enterotoxin (NheI), both of which are tripartite toxins (Lund *et al.*, 1997). Several other virulence factors are capable of being produced, including cytolysin, phospholipase C, and a variety of proteases and collegenases (Arnesen *et al.*, 2008).

Previous studies found *B. cereus* strains were responsible for 17.8% of food-borne disease in Finland, 11.5% in the Netherlands, and 2.2% in Canada (Kotiranta *et al.*, 2000). In 1990, *B. cereus* was found to be the most common agent responsible for food-

borne disease in Norway (Kotiranta *et al.*, 2010). Although *B. cereus* is currently thought responsible for only a minority of food-borne illnesses in many countries, the exact incidence of food-borne illness caused by *B. cereus* in populations is not known. Cases of food poisoning are often unreported, incorrectly attributed to other pathogens, such as *Salmonella enterica* and *Clostridium perfringens*, or are undiagnosed altogether (Kotiranta *et al.*, 2000). Thus, *B. cereus* likely plays a larger role in food-borne illness than is currently perceived. While food-borne disease attributed to *B. cereus* is usually self-limiting, numerous cases have resulted in hospitalization and even death (Bottone, 2010).

Aside from food poisoning, *B. cereus* infections can give rise to more severe disease, especially among neonates, the elderly, and the immunocompromised. Such diseases include bacterial meningitis, sepsis, and ocular infections that may cause permanent blindness (Arnesen *et al.*, 2008; Bottone, 2010; Lund *et al.*, 1997). Due to its ubiquity, *B. cereus* has been traditionally dismissed as a contaminant in the clinical setting as opposed to being recognized as a potential etiological agent of disease (Bottone, 2010). However, an increasing amount of severe, non-gastrointestinal infections are being attributed to *B. cereus*, and the bacterium is considered an emerging pathogen (Bottone, 2010).

1.5 Stress response and signal transduction systems

All microbes must maintain homeostasis in order to survive. A problem arises, however, in that environments are dynamic rather than static: the fluctuation of environmental conditions is non-conducive to cellular homeostasis (Chalancon and

Madan Babu., 2011). A bacterium in its natural setting has to therefore adapt to the presence or change in magnitude of abiotic stresses, such as pH and temperature shifts. Similarly, during the development of an infection, pathogens must adapt to the hostile environments of their host. For example, in order to colonize the gastrointestinal tract, *B. cereus* must survive oxidative, thermal, and especially acidic stresses imparted by the host's innate immune system (van Schaik *et al.*, 2004). Microbes must therefore utilize signal transduction systems to first detect and subsequently respond to environmental stresses.

A bacterium's stress response involves quickly activating specific sets of genes that encode proteins or enzymes able to relieve the given stress. Since the vast majority of genetic expression in bacteria is regulated at the level of transcriptional initiation, it is not surprising that many stress-response systems regulate the potential for RNA polymerase (RNAP) to bind gene-specific promoters (Hogel *et al.*, 2001; Browning *et al.*, 2004). Perhaps the most prevalent players that control RNAP binding are transcription factors (TF). TFs are DNA-binding proteins and may be classified as either an activator or a repressor, depending if the TF enhances or inhibits RNAP binding to the promoter of a given gene, respectively (Latchman, 1997).

TFs are critical components of two particular signal transduction pathways: one- and two-component systems. Both systems are prevalent in eubacteria and play important roles in sensing and responding to environmental and intracellular stresses. The simplest signal transduction system is the one-component system, as it consists solely of a regulatable TF. This TF has both an input and output domain; in the presence of an appropriate stimulus, the input domain activates the output domain, allowing the TF to

bind to gene-specific DNA sequences and thus modulating gene expression (Ulrich *et al.*, 2005). In the two-component system, the TF, called a response regulator, is activated through an intermediary protein rather than by the stimulus directly. This second component is generally a membrane-bound histidine kinase that acts as a sensor. Upon receiving a specific extracellular stimulus, the sensor kinase activates the appropriate response regulator through phosphorylation (Laub *et al.*, 2011; Stock *et al.*, 2000).

After one- and two-component systems, which are the most abundant signal transduction systems in prokaryotes, regulators called alternative sigma factors play the biggest role in dealing with stress (Helmann, 2002).

1.6 Overview of sigma factors

In vivo, RNAP may exist as either one of two forms: as a core enzyme, which consists of the assembled RNAP subunits $\alpha_2\beta\beta'\omega$; or as holoenzyme, which consists of the core enzyme bound to the prokaryotic transcription initiation factor sigma (σ) (Helmann and Chamberlin, 1988). Although catalytically active, the core enzyme alone is unable to bind DNA with any sequence specificity, and is thus unable to efficiently transcribe mRNA from specific genes (Helmann and Chamberlin, 1988). However, σ factor is able to recognize and bind specific promoter elements, including hexameric sequences located 35 (-35) and 10 (-10) bases upstream of the transcription start site (+1) (Vassylyev *et al.*, 2002). After binding to the β and β' subunits of core RNAP, σ factor can redirect the holoenzyme to specific promoters resulting in highly efficient, gene-selective transcription (Burgess *et al.*, 1969). As σ factors can recognize and bind to a

promoter that precedes hundreds of non-adjacent genes, a bacterium can quickly and efficiently modulate gene expression by regulating the activity of a single type of σ factor (Helmann and Chamberlin, 1988). In addition to promoter recognition, σ factor mediates DNA strand separation at the promoter and around the transcription start site, a process termed promoter melting (Helmann and Chamberlin, 1988). This strand separation is needed for the transition from the closed promoter complex to the open promoter complex so that initiation may occur (Von Hippel *et al.*, 1998).

The σ factor alone is nonfunctional, and does not bind DNA or initiate promoter melting. The association of σ factor with RNAP during the formation of holoenzyme, however, causes conformational changes that restore σ function (Helmann and Chamberlin, 1988). Previously, σ factor was believed to always dissociate from RNAP once elongation of RNA transcripts commenced. However, recent evidence suggests that, in some situations, σ factor can be retained by RNAP while elongation proceeds (Yang and Lewis, 2010).

1.6.1 Classification

Sigma factors are divided into two unrelated families: the large σ^{70} family and the significantly smaller σ^{54} (Helmann and Chamberlin, 1988). The σ^{70} family, so named for the molecular mass (in kDa) of the first sigma discovered, is further subdivided into four groups based on sequence similarity (Gross *et al.*, 1998).

Group 1 includes the prototypical σ^{70}, which was first discovered in *Escherichia coli,* as well as the closely related σ^{70} orthologs found in all bacterial species (Burgess *et*

al., 1969; Lonetto *et al.*, 1992). The σ factors of this group are known also as primary sigma factors, as one member is needed and present in every bacterium for the expression of house-keeping genes, especially genes needed during exponential growth. Primary σ factors are therefore essential for bacterial viability. The consensus sequence recognized by members of this group is TTGACA at the -35 region and TATAAT at the -10 region (Helmann, 2002).

Group 2 σ factors are closely related to group 1 in sequence, but are dispensable for growth. Due to this high degree of sequence similarity, the polypeptide regions determining promoter selectivity are nearly identical among σ factors of both group 1 and 2 (Helmann, 2002). Consequently, a majority of promoters can be recognized by both groups, though group 2 σ factors can regulate a small number of genes that group 1 cannot. The regulons of group 1 and group 2 σ factors thus overlap but are not identical, as group 2 σ factors can better tolerate specific base degeneracies at certain positions within the group 1 consensus promoter (Helmann, 2002).

Perhaps the best studied group 2 sigma is σ^S of *E. coli*, a sigma expressed upon a cell's entry into stationary phase as well as under conditions causing general cellular stress (Hengge-Aronis *et al.*, 1999; Helmann, 2002). The biological roles of many group 2 σ factors are unclear: in bacterial species with several group 2 σ factors, single and even multiple deletion mutants do not show an obvious phenotype (Buttner *et al.*, 1992). Group 2 σ factors most likely fine-tune the precise sets of genes being expressed by a cell under various conditions, as well as allow redundancy in the function of σ factors (Helmann, 2002).

Group 3 includes σ factors with a more divergent sequence relative to σ^{70} (Helmann, 2002). Through phylogenetics, group 3 σ factors have been clustered together based on evolutionary relatedness. Members within a cluster tend to have conserved or highly related functions, and control both stress responses and developmental processes (Gruber and Gross, 2003). Clearly defined clusters have been described for group 3 σ factors that control heat-shock response, sporulation, and flagellar biosynthesis (Gruber and Gross, 2003).

The last currently recognized group in the σ^{70} family is the group 4 σ factors, also called extracytoplasmic function (ECF) σ factors (Lonetto *et al.*, 1992). This group will be discussed in greater detail later on (see 1.8.1 Extracytoplasmic function sigma factors).

J. Helmann has proposed the establishment of a fifth group of σ factors. This group would include regulatory proteins with the most divergent sequence relative to σ^{70} (Helmann, 2002). Suggested members include regulators of toxins from low GC Gram-positive bacteria, including TxeR, which regulates two major toxin genes in *Clostridium difficile*, and BotR, which regulates botulinum neurotoxin in *Clostridium botulinum* (Helmann, 2002).

Sigma factors of the σ^{54} family exhibit little if any sequence similarity with the σ^{70} group, and also utilize a different mechanism for promoter melting (Gruber and Gross, 2003). To catalyze the formation of the open promoter complex, σ^{54} members must work in conjunction with an ATP-dependent activator (Rappas *et al.*, 2007; Buck *et al.*, 2000; Studholme *et al.*, 2000). Moreover, σ^{54} mediates promoter-specificity in a different fashion from σ^{70}, recognizing conserved bases at the -24 and -12 positions of the

promoter as opposed to -35 and -10 elements (Helmann, 2002). Bacterial species generally have at most one σ^{54} member encoded in their genome, although no conserved function has yet been identified among members of the family (Gruber and Gross, 2003). The best studied member, σ^{54} of *E. coli*, is involved in nitrogen metabolism. In other bacteria, σ^{54} has been found to regulate a diverse range of functions, but the family is believed to be less important than the σ^{70} family in regulating stress responses (Kazmierczak *et al.*, 2005). No members of this family have been found in high GC, Gram-positive bacteria or cyanobacteria (Studholme *et al.*, 2000).

1.6.2 Structure of group 1 σ factors of the σ^{70} family

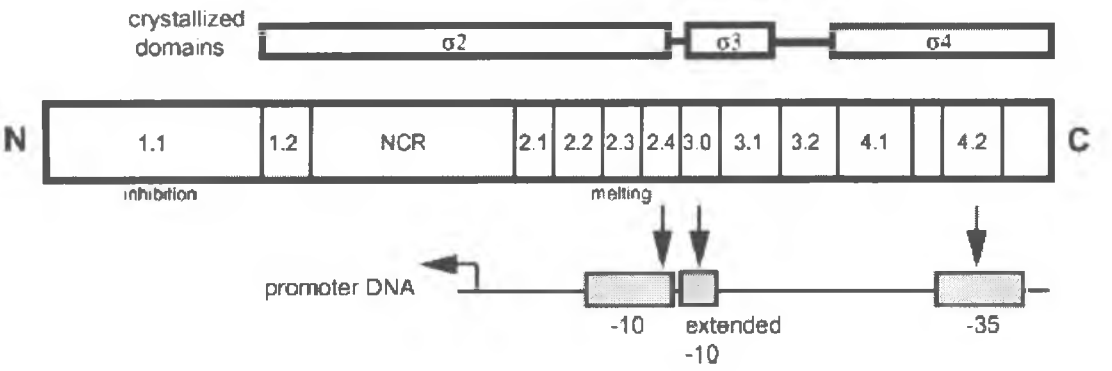
Due to experimental complexities associated with X-ray crystallography, a high-resolution structure has not been obtained for any σ factor alone; however, several high-resolution structures have been solved for individual domains of σ factors, as well as σ in association with RNAP in the context of holoenzyme (Vassylyev *et al.*, 2002). Malhotra *et al.* (1996) were the first to obtain a partial crystal structure of σ^{70} in *E. coli*, and σ factor domains from other bacterial species have since been visualized (Vassylyev *et al.*, 2002). This structural information, in conjunction with biochemical and genetic experiments, has helped elucidate the functions of the domains of σ factor.

The prototypical σ^{70} and its related orthologs are divided into four domains, also known as regions, numbered one through four (Fig. 1). The domains are interconnected via flexible linker peptides (Gribskov *et al.*, 1983; Lowe *et al.*, 1979; Severinova *et al.*, 1996). These domains are further divided into sub-regions, with each sub-region having a distinct role during transcription initiation, such as making protein-protein contacts with

RNAP or protein-DNA contacts with various elements of prokaryotic promoters (Helmann, 2002) (Fig. 1).

Region 1.1 is an auto-inhibitory domain that masks the DNA-binding determinants of unbound σ factor; upon the formation of holoenzyme, the conformation of σ changes such that region 1.1 is rendered inactive (Helmann and Chamberlin, 1988) (Fig. 1). Region 2.4 consists of five α-helices and contains the strongest DNA binding determinant of σ factor, recognizing the -10 region of promoters (Gruber and Gross, 2003) (Fig. 1). Like other DNA-binding proteins, the α-helices are arranged in a helix-turn-helix (HTH) motif (Murakami *et al.*, 2002). Region 2.3 contains numerous aromatic and basic residues that are involved in DNA melting during the formation of the open promoter complex (Gruber and Gross, 2003) (Fig. 1). Region 3 recognizes the extended -10 element located only in certain promoters (Barne *et al.*, 1997; Murakami *et al.*, 2002) (Fig. 1). Region 4.2 has the strongest RNAP-binding determinant and consists of four helices which recognize the -35 consensus promoter sequence (Campbell *et al.*, 2002) (Fig. 1).

Figure 1. Domains and sub-regions of σ^{70}. σ^{70} and its orthologs are classified as group 1 σ factors and consist of four main domains (numbered 1 through 4), three of which have been visualized by crystallography (σ2-σ4). Domains are further divided into discrete sub-regions, which play various roles during transcription initiation. Region 1.1 is an auto-inhibitory domain which renders σ inactive until it binds RNAP during holoenzyme formation. The non-conserved region (NCR) is found in only certain group 1 σ factors, and an associated function has yet to be discovered. Region 2.4 makes contacts with both RNAP and the -10 promoter element. Region 3.0 Recognizes the extended -10 sequence found only in certain promoters. Region 4.2 makes contacts with both RNAP and the -35 promoter element (Helmann, 2011).


crystallized domains
σ2
σ3
σ4
N
1.1
1.2
NCR
2.1
2.2
2.3
2.4
3.0
3.1
3.2
4.1
4.2
C
inhibition
melting
promoter DNA
-10
extended -10
-35

1.6.3 Structure of non-group 1 σ factors of the σ^{70} family

Group 2 σ factors are closely related to primary σ factors in sequence, and thus have conserved regions 1 through 4 (Lonetto *et al.*, 1992). Sigma factors of group 3 and 4, however, lack regions 1 and 3. As a result, σ factors of these groups are significantly smaller than members of group 1 and 2, generally having a molecular weight of between 25 and 35 kDa (Gruber and Gross, 2003; Helmann, 2002). The conservation of domains 2 and 4 among all groups in the σ^{70} family highlights the importance of these domains in binding core RNAP subunits as well as promoter elements (Lonetto *et al.*, 1992).

1.7 Sigma factor regulation

Sigma factors provide a powerful mechanism by which cells dramatically effect changes in global gene expression with alacrity. Understandably, a cell must tightly regulate the activity of σ factors, else undesirable or even lethal changes in gene expression arise (Helmann, 2002). The regulation of σ factor activity can occur at the level of transcription, translation, post-translational modification, and protein stabilization and degradation (Helmann, 2002). In addition, the affinity for which σ factor binds core RNAP can be altered by accessory proteins, adding yet another mechanism for σ regulation (Helmann, 2002). Sigma factors that play critical roles in numerous regulatory pathways can often be controlled at all of these levels. Thus, σ factor regulation is multi-layered and involves numerous mediators (Helmann, 2002).

1.8 Alternative sigma factors

While primary σ factors of group 1 in the σ^{70} family are needed to maintain the constitutive expression of house-keeping genes, a bacterium must be able to fine-tune expression by transcribing from specific, situational genes in response to changing environmental conditions (Helmann, 2002; Gruber and Gross, 2003). Although this can be achieved through previously mentioned one- and two- component systems, many bacteria depend also on non-primary σ factors of groups 2 through 4 from the σ^{70} family, also known as alternative σ factors, to modulate gene expression. These alternative σ factors are most often expressed or activated in response to specific environmental or cellular cues, and subsequently compete with primary σ factor for a fraction of free core RNAP to allow transcription from alternative promoter sites (Gruber and Gross, 2003).

1.8.1 Extracytoplasmic function sigma factors

ECF σ factors of group 4 in the σ^{70} family are perhaps the most important of all alternative σ factors in regulating stress responses. The ECF group of σ factors was first proposed by Lonetto *et al.* (1994) to classify a group of transcriptional regulatory molecules that were predicted to function in the same fashion but bare little sequence similarity to *E. coli* σ^{70}. The founding members of this group were initially found to regulate cell-surface molecules, transport systems, and other processes relating to the cell-envelope physiology; thus, these newly identified σ factors were said to have extracytoplasmic function (ECF), giving rise to the name of this group of σ factors (Lonetto *et al.*, 1992; Helmann, 2002). Today, ECF σ factors make up the fourth and largest group in the σ^{70} family, and these σ factors are the most divergent in sequence

from σ^{70} relative to other groups, exhibiting extraordinary variability even between one another (Helmann, 2002). Perhaps as a consequence of their sequence diversity, ECF σ factors have been increasingly found to mediate not only extracytoplasmic functions, but also a diverse range of other cellular responses, especially those associated with stress and virulence (Helmann, 2002).

1.8.2 ECF sigma factors in stress responses and virulence

Bacteria that are able to inhabit numerous different habitats, and are thus able to tolerate a wider range of potential environmental stresses, generally have more ECF σ factors encoded in their genomes (Helmann, 2002). In these bacteria, the number of ECF σ factors often outnumbers σ factors of all other groups combined. One extreme example includes the soil-dwelling, ubiquitous bacterium *Streptomyces coelicolor*, which encodes over 50 putative ECF σ factors (Paget *et al.*, 2002). Comparatively, an average of only six ECF σ factors are encoded in the bacterial genomes sequenced to date (Staroń *et al.*, 2009). The two model organisms in which σ factors have been best studied, *E. coli* and *B. subtilis*, have two and seven ECF σ factors respectively (Helmann, 2002). In stark contrast to *S. coelicolor,* bacteria that have evolved highly specific life styles in response to relatively constant environmental conditions, including obligate intracellular parasites, may encode not a single ECF σ factor (Helmann, 2002).

In many situations, pathogenesis is dependent on a bacterium's ability to sense changes in its local environment and adapt to specific stresses (Kazmierczak *et al.*, 2005). ECF σ factors have since been found to regulate virulence and virulence-associated genes in a number of bacteria: in *Salmonella enterica* serovar Typhimurium, the *rpoE* gene

encodes σ^E which regulates genes involved in oxidative stress, thus aiding the survival of these bacteria in host macrophages (Cano *et al.*, 2001; Humphreys *et al.*, 1999); *Pseudomonas aeruginosa* secretes siderophores in response to iron-limiting conditions through the ECF σ factor encoded by *pvdS* (Miyazaki *et al.*, 1995; Ochsner *et al.*, 2002; Stintzi *et al.*, 1999); and *Mycobacterium tuberculosis* has at least four ECF σ factors which contribute to virulence, although the exact mechanisms through which this is achieved is currently being investigated (Kazmierczak *et al.*, 2005).

1.8.3 ECF sigma factor regulation

The fitness of a bacterium depends on having highly efficient bioenergetics. With regards to environmental stress, an appropriate cellular response must be invoked only when the stress is present. Additionally, the stress response must be rapid and robust once triggered. ECF σ factors, for example, must be inactive in the absence of the stress to which they respond; however, when the stress is present, ECF σ factors must immediately gain activity, as the transcriptional changes they bring forth will take time to translate at the level of protein. To achieve this, prokaryotes have evolved highly efficient regulatory systems to control ECF σ factor activity (Helmann, 2002).

Many of the ECF σ factors that have been studied in detail are co-transcribed with negative regulators in an operon. The most common negative regulator is called an anti-σ factor (Helmann, 2002). The σ factor and anti-σ factor interaction operates analogously to the two-component system: in the absence of the appropriate stress or stimulus, the anti-σ factor sequesters σ often at the cell membrane, disallowing σ to bind cytoplasmic core RNAP and thus inhibiting σ activity; in the presence of the appropriate stimulus, the

anti-σ factor is degraded by a specific protease and may release the ECF σ factor where it is free to compete for binding to core RNAP (Helmann, 2002). Numerous mediators and even other regulatory pathways, including two-component systems, can interact with anti-σ factor, resulting in additional layers of ECF σ factor regulation (Helmann, 2002).

ECF σ factors often auto-regulate the very operon from which they were transcribed. Thus, a stress can elicit a quick and robust cellular response: not only is there already sequestered σ factor ready to impart changes in genetic expression, but additional uninhibited σ factor is then rapidly transcribed (Helmann, 2002).

1.9 Sigma factors in *Bacillus cereus*

The genome of the type strain *B. cereus* ATCC 14579, henceforth referred to as strain 14579, was sequenced by Ivanova *et al.* in 2003, and genomic analysis found twenty putative σ factors. A number of these σ factors are orthologous to those studied in *B. subtilis* and other bacterial species: the primary σ factor in strain 14579 is σ^A, orthologous to *E. coli* σ^{70}; σ^B is orthologous to *B. subtilis* σ^B; sigma factors σ^E,σ^F, σ^G, σ^H, and σ^K are othologous to the five similarly named σ factors found in *B. subtilis* that play critical roles during sporulation; and σ^L is the only σ^{54} representative found.

To date, the majority of the work conducted on σ factors in *B. cereus* involves σ^B encoded by the *sigB* gene. Strain 14579 σ^B is conserved in sequence and function to the well-studied *B. subtilis* σ^B, a group 2 σ factor of the σ^{70} family expressed upon entry into stationary phase. *B. subtilis* σ^B is induced by a wide variety of stresses and regulates an estimated 150 genes (Hecker *et al.*, 2007). As a regulator of the general stress response,

σ^B is similar in function to *E. coli* σ^S. Regulation of σ^B is complex, involving at least 12 different regulatory proteins that function in a branched signaling cascade (Hecker *et al.*, 2007; Helmann, 2011). Eventually, these signals converge on and regulate the activity of RsbW, the anti-σ factor of σ^B (Helmann, 2011). Like in *B. subtilis*, studies involving *B. cereus* σ^B have found that the σ factor regulates a general stress response, being induced by heat-shock, oxidative, ethanol, acidic, and osmotic stresses (van Schaik *et al.*, 2004).

In addition to the aforementioned σ factors, strain 14579 encodes ten ECF σ factors. The functions of these ECF σ factors are largely unknown, as ECF σ factors in general have not been well studied in *B. cereus* relative to model organisms such as *E. coli* and *B. subtilis*. ECF σ factor σ^W in strain 14579 has orthologs that have been studied to a limited extent in other bacterial species; however, the functions of this σ factor are not entirely understood (Ades *et al.*, 2003; Huang, 1999). Aside from σ^B, how exactly *B. cereus* uses other alternative σ factors, and in particular ECF σ factors, to survive in a wide range of environments is not known.

1.10 Strategies in determining ECF σ factor function

With the advent of cheaper, quicker, and more efficient high-throughput sequencing technologies, the number of sequenced bacterial genomes is increasing exponentially. Sequenced genomes provide a wealth of information, including the presence and location of putative genes. However, identifying the functions of genes, such as novel ECF σ factors, often proves challenging. A variety of strategies have been used successfully to elucidate σ factor function.

1.10.1 Finding conditions which induce ECF σ factor expression

ECF σ factors often auto-regulate their own expression when activated by the stress to which they respond. Thus, the main function of a particular ECF σ factor may be gleamed by identifying a condition or stress which dramatically induces ECF σ factor expression, and this induction may be detected by quantitative reverse transcription PCR (qRT-PCR) or Western blotting. Additional experiments, however, are needed to confirm that these σ factors are regulating genes important in responding to the stress.

1.10.2 Finding a phenotype in a ECF σ factor mutant

One simple and direct way to understand the function of an ECF σ factor is to take a genetic approach: make a deletion mutant and look for a phenotype under specific conditions of stress. If a lethal or growth-defective phenotype is found in the mutant relative to wild-type (WT) under a particular condition, the ECF σ factor of study is likely playing an important role in coping with the tested stress. This approach may be ineffective if no phenotype associated with the mutant is found, either due to a lack of insight as to the appropriate conditions to test, or due to other stress-response systems providing functional redundancy.

1.10.3 Determining genes regulated by ECF σ factor

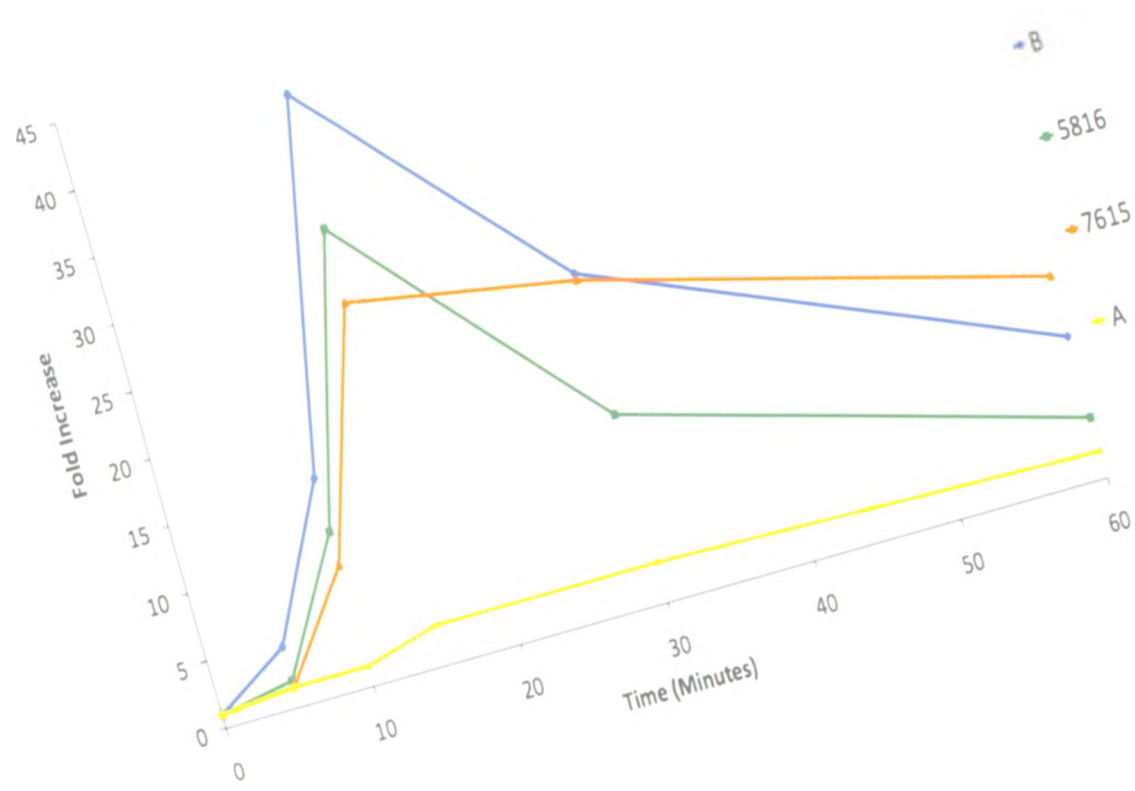
Identifying the specific set of genes that an ECF σ factor regulates (regulon) may directly give insight as to ECF σ factor function if these genes have been previously annotated (Haldenwang, 1995). The regulon of a putative ECF σ factor can be

determined by a variety of methods. One method involves transcriptome analysis. If the condition(s) which induces the expression of said ECF σ factor is identified, the set of genes being induced by said condition in WT may be compared to the set of genes being induced in a mutant with the σ factor in question deleted. A decrease or even absence in expression of select genes in the mutant relative to WT would make these genes potential candidates for the σ factor regulon. Alternatively, if a strain can be made with the activity of the ECF σ factor substantially bolstered, either by over-expressing the σ factor itself or deleting any known negative regulators, one can identify regulon candidates by looking for up-regulated genes relative to the transcriptome of WT.

1.11 *B. cereus* ATCC 14579 ECF σ factors σ5816 and σ7615

The Linn laboratory has been interested in putative, novel ECF σ factors that may influence pathogenesis in *B. cereus*. Previous RT-PCR experiments conducted by T. Linn (unpublished) have identified a condition which dramatically up-regulates the expression of σ^B along with two putative ECF σ factors. When strain 14579 is shifted from a pH of 7.2 to a pH 5.4, gene expression of σ5816 and σ7615 increases over 25 fold and 30 fold, respectively (Fig. 2). Both σ5816 and σ7615 are annotated as ECF σ factors in the GenBank® (NCBI) database, as each CDS has both conserved domain Sigma70 region 2 and conserved domain Sigma70 region 4, as determined by sequence alignments to *E. coli* σ^{70} (Conserved Domain Database).

Figure 2. Expression of σ factors in *B. cereus* ATCC 14579 following acid treatment. The expression levels of primary σ factor σ^A, group 2 σ factor σ^B, and two ECF σ factors, σ5816 and σ7615, are tracked following a pH shift from 7.2 to 5.4 by qRT-PCR. Expression was normalized to *rpoB*, the gene encoding the β subunit of RNAP (T. Linn, unpublished data).


B
5816
7615
A
Fold Increase
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
Time (Minutes)

As σ5816 and σ7615 are up-regulated seemingly in response to acid stress, it was hypothesized that these two ECF σ factors promote the expression of genes involved in an acid-tolerance response. Surprisingly, subsequent experiments did not find a dramatic growth- or survival-defective phenotype in a triple mutant that was deleted for σ^B, σ5816, and σ7615 (BcL8) relative to strain 14579 (WT) when these strains were shifted from a neutral pH to an acidic pH (J. Leung, unpublished). Two possibilities exist that may explain the absence of a dramatic phenotype in BcL8. First, σ5816 and σ7615 may indeed regulate the acid stress stimulon, but *B. cereus* possesses an additional regulatory system(s) that can compensate for the loss of these ECF σ factors and thus rescue the expression of these genes. Second, σ5816 and σ7615 are not being induced by (and responding to) acid stress directly, but rather by a secondary effect(s) of low pH; accordingly, BcL8 may show an obvious phenotype if directly stressed by a currently unknown condition. One of the possible candidates for this unknown condition is cell envelope stress, as *B. cereus* cells are known to undergo lysis as the pH drops slightly past the values that have induced the expression of these σ factors (lysis at below pH 5). To explore these possibilities and ultimately determine the function(s) of ECF σ factors σ5816 and σ7615, this study will use some of the aforementioned strategies.

1.12 Hypothesis and research objectives

We hypothesize that σ5816 and σ7615 are important for a stress-specific response(s) in strain 14579. To identify the nature of this stress, and thus the overall function of these σ factors, we have three major objectives:

1. Develop molecular tools to study σ5816 and σ7615:

a. an expression system to create strains that can over-express these σ factors in a regulated fashion

b. raise antibodies specific towards these two σ factors so that σ factor protein expression can be quantified by Western blot

2. Look for phenotypes associated with over-expression or deletion of these σ factors:

   a. examine whether σ7615 or σ5816 over-expression can ameliorate growth in acid stress

   b. examine whether σ7615 or σ5816 deletion results in a growth-defective phenotype under acid-related stresses, including different types of cell-envelope stress

3. Attempt to identify the regulon of σ5816 through transcriptome analysis through two approaches:

   a. identify genes that are substantially up-regulated in a σ5816 over-expressing strain relative to WT at neutral pH

   b. identify genes that are not as up-regulated in an acid-stressed σ5816 deletion mutant (BcL1) as they are in WT.

CHAPTER 2: MATERIALS AND METHODS

2.1 Strains, cultures, and growing conditions

For a list of strains used during this project, see Table 1. Strain 14579 and mutant strains were grown at 30°C or 37°C in Luria broth (LB) (Bertani, 1951) at 200 rotations per minute (RPM) unless otherwise indicated; over-expression strains were grown similarly, but supplemented with chloramphenicol (2 μg/mL; Sigma). *E. coli* strains were grown at 37°C in LB at 200 RPM supplemented with either ampicillin (100 μg/mL; Sigma) or kanamycin (30 μg/mL; Sigma) as required for the selection of various plasmids.

2.2 Transformation protocols

2.2.1 Transformation of *E. coli* strains

Competent *E. coli* DH5α and *E. coli* XL1-Blue cells were transformed via heat-shock. Specifically, 10 μL of plasmid DNA was added to a 200 μL aliquot of competent cells, and placed on ice for 30 min. The resulting mixture was transferred to a 42°C water bath for 90 seconds with gentle agitation and then transferred back onto ice for another 30 min. Eight hundred μL of SOC medium (Hanahan, 1983) were added, and the transformation mixture was incubated at 37°C for one h with moderate agitation. Ten μL and 100 μL volumes of the transformation mixture were plated onto LB supplemented with either 100 μg/mL ampicillin or 30 μg/mL kanamycin. Plates were incubated at 37°C overnight (O/N). Single transformants colonies were streaked twice prior to their use in subsequent experiments.

Table 1: Bacterial strains used in this study

Strain	Description	Source or reference
B. cereus		
ATCC 14579	*B. cereus* type strain, WT	ATCC
BcL1	*B. cereus* ATCC 14579 Δσ5816	T. Linn
BcL8	*B. cereus* ATCC 14579 $\Delta\sigma^B$, Δσ5816, Δσ7615	T. Linn
BcL23	*B. cereus* ATCC 14579::p5-σ7615	This study
BcL25	*B. cereus* ATCC 14579::p5-σ5816	This study
BcL31	*B. cereus* ATCC 14579::pCCEXP5	This study
BcCClacZ	*B. cereus* ATCC 14579::pCClacZ	This study
BcCClacZ-H	*B. cereus* ATCC 14579::pCClacZ-H	This study
E. coli		
BL21	F- *ompT hsdSB(rB-,mB-) gal dcm*	Invitrogen
DH5α	*supE Δlac U169 (Φ80lacZΔM15)hsdR recA endA gyrA hri relA*	(Hanahan, 1985)
MG4	F- lambda- *ilvG- rfb*-50 *rph*-1 *ΔlacIZY*	T. Linn
NEB Express	*fhuA2 [lon]ompT gal sulA11 R(mcr-73::miniTn10—Tet^S)2 [dcm] R(zgb-210::Tn10—Tet^S) endA1 Δ(mcrC-mrr)114::IS10*	New England BioLabs
SCS110	*rpsL (Strr) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44D (lac-proA)* [F′ *traD36 proAB lacI^{q}ZΔM15*]	Stratagene
XL1-Blue	F′::Tn*10 proA$^+$B$^+$ lacI^q Δ(lacZ)M15/recA1 endA1 gyrA96* (Nal^r) *thi hsdR17* ($r_k^- m_k^+$) *glnV44 relA1 lac*	New England BioLabs

E. coli MG4 cells were made competent on the same day of each transformation. Cultures were grown to 70 klett units (KU) and the cells were harvested by centrifugation at 6000 g for 5 min at 4°C. Pellets were washed with 10 mL 0.1M $MgCl_2$ and resuspended in 0.1 M $CaCl_2$. Cells were incubated on ice for 30 min. Subsequently, 10 µL of plasmid DNA and 90 µL of saline-sodium citrate: 0.1M $CaCl_2$ (3:4 v/v) were added to 200 µL of competent *E. coli* MG4 cells. This mixture was incubated on ice for another 30 min, then heat-shocked at 42°C for 2 min. Cells were mixed with 1 mL LB and allowed to incubate at 37°C for 60 min. 10 µL and 100 µL aliquots of the transformation mixture were plated on LB agar supplemented with 100 µg/mL ampicillin at 37°C O/N.

Competent *E. coli* SCS110 cells (Stratagene) were transformed via electroporation. A 40 µL aliquot of cells was mixed with 2 µL of plasmid DNA, put into a 0.2 cm GenePulser® Cuvette (Bio Rad) and pulsed at 2.2 kV with a capacitance of 25 µFD and resistance of 200 Ω using a Gene Pulser™ (Bio Rad).

2.2.2 Transformation of *B. cereus* ATCC 14579

Strain 14579 has a restriction system that cleaves methylated adenine in the sequence GATC; therefore, plasmids derived from *E. coli* strains had to first be passaged through the *dam*⁻ strain *E. coli* SCS110 prior to strain 14579 transformation.

Strain 14579 cells were made competent on the same day of each transformation. An 80 mL culture was grown to 20 KU and cells were harvested by centrifugation at 10 000 g for 8 min at 4°C. Pellets were washed twice in 18 mL EBX buffer (10% sucrose;

15% glycerol; 0.5 mM $MgCl_2$; 1 mM KPO_4 pH 7.8). Cells were resuspended in 200 µL ice-cold EBX.

For each transformation, 100 µL of electrocompetent cells were mixed with 1 µg of plasmid and incubated on ice for 10 min. The mixture was added to a GenePulser® cuvette and pulsed at 1.5 kV with a capacitance of 25 µFD and resistance of 600 Ω. The cells were added to 2 mL of LB supplemented with 1% glucose and incubated for 2 h at 30°C with moderate agitation. A 100 µL volume was plated onto LB agar with 5 µg/mL chloramphenicol. A 1 mL aliquot of the remaining cells was concentrated by being spun down at 9000 g for 3 min at RT and reducing the volume to 300 µL. The cells were resuspended and plated as described above. Plates were incubated at 30°C O/N. Single transformants colonies were streaked twice prior to use in experiments.

2.3 Creating an expression system in *B. cereus*

For a list of plasmids used in this study, see Table 2. The pAD123 plasmid was used as the backbone of our expression vector, and modifications were made. First, the *gfpmut3a* gene was excised from pAD123 using *Bam*HI (New England BioLabs) and *Sph*I (New England BioLabs) cut sites, and the 5' overhangs resulting from this restriction enzyme (RE) digest were filled in using T4 DNA polymerase to create blunt ends. The linear plasmid was then re-circularized by blunt-end ligation using T4 DNA ligase. Next, the P_{spank} and $P_{hyperspank}$ promoters, along with their downstream *lacI* genes, were excised from pDR110 and pDR111 respectively and cloned into the multiple cloning site (MCS) of pAD123 using the *Eco*RI and *Bam*HI cut sites. The resulting

P_{spank}- and $P_{hyperspank}$-based constructs were named pCCEXP4 and pCCEXP5 respectively.

Table 2: Plasmids used in this study

Plasmid	Description	Source or reference
Plasmid		
pET28a(+)	N-terminus 6x His-tagged fusions	Novagen®
pCCEXP4	Vector backbone with P_{spank}	This study
pCCEXP5	Vector backbone with $P_{hyperspank}$	This study
pUC-lacZ	*lacZ* reporter	R. Losick
pDR-110	Vector backbone with P_{spank}	D. Rudner
pDR-111	Vector backbone with $P_{hyperspank}$	D. Rudner
pAD123	*E. coli* to Gram-positive shuttle vector, used as pCCEXP4 and pCCEXP5 backbone	*Bacillus* Genetic Stock Center
Expression Plasmid		
pET-σ5816	6xHis-tagged σ5816	This study
pET-σ7616	6xHis-tagged σ7615	This study
pCClacZ	*lacZ* in pCCEXP4	This study
pCClacZ-H	*lacZ* in pCCEXP5	This study
p5-σ5816	σ5816 in pCCEXP5	This study
p5-σ7615	σ7615 in pCCEXP5	This study

2.4 Measuring β-Galactosidase expression from pCCEXP4 and pCCEXP5 systems

The *lacZ* reporter gene, encoding β-galactosidase, was excised from pUC18-*spo*VG(*rbs*)-*lacZ* at the *Hin*dIII and *Sph*I sites and cloned into the MCS of both pCCEXP4 and pCCEXP5. The resulting constructs were named pCClacZ and pCClacZ-H for P_{spank}- or $P_{hyperspank}$-driven *lacZ* expression, respectively. Constructs were transformed into strain 14579.

BcCClacZ and BcCClacZ-H cultures were grown in either the presence or absence of 1 mM IPTG to 40 KU and subsequently placed on ice for 20 min. Cells from 4 mL of each culture at each condition were harvested by centrifugation at 9000 g for 3 min at RT. Pellets were washed with 500 µL of ice-cold 25 mM Tris-HCl, pH 7.5 and subsequently frozen at -80°C until the day of the assay. Pellets were resuspended in 800 µL Z-Buffer (pH 7.0; 60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, and 50 mM β-mercaptoethanol) supplemented with lysozyme (2.5 mg/mL; Sigma) and incubated at 37°C for 5 min. Eight µL of 10% Triton X100 was then added to the samples. Assays were subsequently carried out as described by Miller (1972), with the exception that cell debris was pelleted via centrifugation prior to optical density (OD) measurement. Unit definition was calculated as $(1000 \times OD_{420}) / (\text{reaction time (min)} \times OD_{595})$.

2.5 Construction of ECF sigma factor over-expression *B. cereus* ATCC 14579 strains

DNA fragments containing the σ5816 (*BC2794*) and σ7615 (*BC2386*) genes were amplified from strain 14579 genomic DNA (gDNA) by PCR using the MyCycler™

thermal cycler (Bio-Rad) and the primers listed in Table 3. Genes were inserted into each of pCCEXP4 and pCCEXP5, and the constructs were recovered in *E. coli* XL1-Blue and eventually transformed into strain 14579. Plasmid DNA from the over-expression strains was sequenced to ensure that the σ factor gene sequences were identical to those of strain 14579.

Table 3: List of primers used in this study

Description	Name	Sequence[a]
σ5816 amplification, insertion into pET28a(+)	s5-14	5′-CCAT**CATATG**TGCACAAAAGTAACTCATGT-3′
	s5-12a	5′-TCTA**GTCGAC**ATTCCTCCAATACTTGTTATG-3′
σ7615 amplification, insertion into pET28a(+)	s7-14	5′-CAGT**GCTAGC**GAAACGGAGCAGTTATATGAAG-3′
	s7-12a	5′-GCCC**GTCGAC**TTAGAAAAATAATAAAAAAC-3′
Sequencing pET28a(+) insert	T7p	5′-TAATACGACTCACTATAGGG-3′
	T7t	5′-GCTAGTTATTGCTCAGCGG-3′
σ5816 amplification, insert into pCCEXP4/5	5816A1	5′-TTTG**GTCGAC**GAATCGTTTATTAAGTAAGCGC-3′
	5816A3	5′-CGTT**GCATGC**TTCCTCCAATACTTGTTATG-3′
σ7615 amplification, insert into pCCEXP4/5	s7A-3a	5′-GCTA**GTCGAC**GTGCAGCGTTACACATTTTA-3′
	s7A-5a	5′-CGTT**GCATGC**TGGCGAAAAAGATGTACCTC-3′
Sequencing pCCEXP4/5 inserts	CCEXP4-seq1	5′-AGGACAAATCCGCCGCTCTAG-3′
	pCC3a	5′-GAACAATCACGAAACAATAAT-3′
BC5438 for RT-PCR	BC5445A	5′-AATAACAATGCACCGAATGATG-3′
	BC5445B	5′-ACATCAATAGCCCAGGAGGTTC-3′
BC2300 for RT-PCR	BC2300A	5′-TGTTGGTGATTTCTTCGGTATG-3′
	BC2300B	5′-CCTAGAAGTGCACCGATAAAGG-3′
BC5445 for RT-PCR	BC5438A	5′-ACCAAATCAAGATACGCCTTTG-3′
	BC5438B	5′-CCAGTTTACTGTATGCCACCAG-3′
BC2793 for RT-PCR	BC2793A	5′-AATAACAATGCACCGAATGATG-3′
	BC2793B	5′-ACATCAATAGCCCAGGAGGTTC-3′

[a] Bold and underlined sequences denote RE cut sites

2.6 SDS-PAGE and Western Blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole-cell protein extract was conducted by growing the strain of interest to 40 KU. For *E. coli*, cells were harvested from 1 mL of culture by centrifugation at 9000 g for 3 min at room temperature (RT), resuspended in 50 µL SDS-PAGE sample buffer (50 mM Tris-Cl, pH 6.8; 2% SDS; 10% glycerol; 0.1% bromophenol blue; 200 mM β-mercaptoethanol), and immediately plunged into a 100°C water bath for 3 min and subsequently frozen until day of assay. For *B. cereus,* cells were harvested from 2 mL of culture by centrifugation at 9000 g for 3 min at RT. Pellets were subjected to a freeze-thaw cycle (-20°C) twice. Pellets were subsequently re-suspended in 50 µL Tris pH 7.4 with 2.5 mg/mL lysozyme (Sigma) and incubated at 37°C for 20 min. Fifty µL of SDS-PAGE sample buffer was added. Samples were immediately plunged into a 100°C water bath for 3 min and subsequently frozen until day of the assay. Frozen samples were again plunged into a 100°C water bath for 3 min prior to SDS-PAGE analysis.

Samples were loaded onto 10%, 12%, or 13% polyacrylamide gels and ran for 45 min at 180V using the Mini-PROTEAN® II Electrophoresis Cell (Bio-Rad). Gels were washed using Milli-Q water for 15 min. Protein bands were visualized by using GelCode® Blue Stain Reagent (Thermo Scientific) or by Western blot. Western blotting was carried out via typical protocol. Specifically, proteins separated on an SDS-PAGE gel were transferred onto a nitrocellulose membrane (Bio-Rad) using Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). Transfer proceeded for 45 min at 180 mA. Membrane was then blocked O/N in 2.5% non-fat milk dissolved in water (Bio-Rad) at 4°C. Membrane was washed with Tris-buffered saline + Tween/Triton (1 M Tris-Cl pH

7.5; 5M NaCl; 0.05% Tween 20; 0.2% Triton X-100). Primary incubation was for 1 hour at RT using anti-β RNAP mAb (NeoClone) and rabbit antiserum raised against σ5816 (ProSci Inc.); secondary incubation was for 1 hour at RT using goat anti-rabbit IgG conjugated to IRDye800 (Rockland) and goat anti-mouse conjugated to IRDye700DX (Rockland). Membrane was scanned using Li-Cor ODYSSEY® Imager on both the 700 and 800 channels.

2.7 Testing σ5816 and σ7615 protein solubility and optimal temperature for expression

To determine whether σ5816 and σ7615 were soluble when their expression was induced in *E. coli* BL21, we assayed if the proteins were found mostly in either the pellet or supernatant fractions of centrifuged whole-cell lysates via SDS-PAGE. The σ5816 and σ7615 genes were amplified from strain 14579 gDNA using the primers listed in Table 3 via PCR. These genes were each cloned into the pet28a(+) vector to create the pET-σ5816 and pET-σ7615 plasmids, which were recovered in *E. coli* XL1-Blue via kanamycin (30μg/mL) selection. Pure plasmid preparations were obtained from transformants using the High-Speed Plasmid Mini Kit (Geneaid); these preparations were then used to transform *E. coli* BL21 (Novagen).

Strains were grown to 40 KU after which IPTG was added to a final concentration of 1 mM. Cultures were allowed to keep growing under three different conditions: at 37°C for two hours; at 30°C for three hours; or at 18°C for ten hours. Cells grown under each condition were harvested by centrifugation at 6000 g for 10 min at 4°C. Pellets were resuspended in 5 mL of Talon Buffer (20 mM Tris-HCl, pH 8.0; 400 mM NaCl; 10%

glycerol; 0.1% NP-40) supplemented with 1 mM β-MCE, 150 µg/mL lysozyme, and 10 µg/mL DNaseI. Lysate was prepared by sonicating the samples using 10 pulses at 15 seconds each pulse and 35% of the maximum power of a Model 300 Sonic Dismembrator (Fisher). Lysate was then centrifuged at 10,000 g for 30 min at 4°C to obtain the supernatant and pellet fractions. The pellet was resuspended in 5 mL Talon Buffer. Pre-sonicate, sonicated supernatant, and sonicated pellet samples were loaded onto an acrylamide gel and analyzed via SDS-PAGE.

2.8 Purification of His-σ7615 fusion

2.8.1 Growth, induction, and harvest of cells

A one litre culture of *E. coli* BL21 transformed with pET-σ7615 was grown in LB with 30 µg/mL kanamycin at 37°C until an OD_{600} of 0.650, after which the culture was shifted to 18°C and IPTG was added to a final concentration of 0.5 mM. The culture was grown for an additional ten hours before the cells were harvested by centrifugation at 5000 g for 15 min at 4°C. Cell pellets were stored at -20°C prior to protein purification.

2.8.2 Lysate preparation, clarification, and protein purification via FPLC

Cell pellets were resuspended in 30 mL binding buffer (50 mM Tris-Cl pH 7.5, 500 mM NaCl, 10 mM imidazole), and pooled. An EDTA-free protease inhibitor cocktail tablet (Roche) was added. The cells were lysed by passing the suspension through a B Series cell disrupter (Constant Systems Ltd.) five times at 22 000 pounds per square inch (PSI). Unbroken cells and cellular debris were pelleted by spinning the

lysate at 5000 g for 15 min at 4°C. The supernatant was collected and further clarified by being spun at 50,000 g for one hour at 4°C in an ultracentrifuge (Beckman). Lastly, this supernatant was filtered through a 0.45 μM membrane prior to purification via Fast Protein Liquid Chromatography (FPLC). The FPLC (ÄKTAdesign) pumps and lines were connected to a 1 mL HisTrap™ High-performance Purification column (GE Healthcare). The lysate was passed through the system and protein was eluted off the column using elution buffer (50 mM Tris-Cl pH 7.5, 500 mM NaCl, 500 mM imidazole, 0.2 μM filtered).

2.8.3 Post-purification dialysis and concentration determination

Fractions containing His-σ7615 were pooled and dialyzed in 500 mL of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 2H_2O$, 1.76 mM KH_2PO_4) pH 7.40 using dialysis tubing with a 10 kDa cut-off. The buffer was changed twice, with each change occurring 8 h apart. The purified, dialyzed protein was checked via SDS-PAGE, and its concentration measured using the Bio-Rad Protein Assay, a modified assay based on the original method of Bradford (1976). Protein was aliquoted and stored at -80°C.

2.9 ECF sigma factor σ5816 purification

The pET-σ5816 plasmid was transformed into *E. coli* BL21. Growth, induction, harvest, and lysis of cells proceeded as described above. For the purification via FPLC, both the binding buffer and the elution buffer contained 6M guanidine HCl (Sigma).

2.10 Raising polyclonal anti-sera toward σ5816 and σ7615

Approximately 1 mg of purified His-σ5816 and His- σ7615 re-suspended in PBS pH 7.40 were sent to ProSci Inc. in Poway, California to raise rabbit, polyclonal antibodies towards these two sigma factors.

2.11 *B. cereus* growth curves for phenotype search

B. cereus strains were grown to 70 KU. Aliquots of the culture were diluted 15-fold into wells of a 100-well Honeycomb 2 microplate (Oy Growth Curves Ab Ltd.) containing LB plus various concentrations of bacitracin (Fluka Analytical) or vancomycin (Sigma). The wideband filter transmitting light at the 420_{nm} to 595_{nm} wavelengths was used to take OD readings every 15 min over the course of 12 h using the Bioscreen C™ (Oy Growth Curves Ab Ltd.). Growth curves were constructed by plotting the log OD versus time. Data were obtained in quadruplicate. *B. cereus* growth curves were also obtained when cells growing in LB were stressed with 0-0.022% bile salts (Sigma) or 0-4% NaCl.

For pH-shift experiments with BcL23 and BcL25, strains were grown to 80 KU in LB buffered to pH 7.40 with 10 mM MES and 10 mM MOPS plus either the absence or presence of 1mM IPTG. Aliquots of the culture were diluted 30-fold and shifted to pH values ranging from 5.10-7.40. Growth was assayed using the Bioscreen C as described above, with OD readings taken every 5 min over the course of 8 h. Growth curves were constructed as described above.

2.12 RNA extraction

A 30 mL culture of the appropriate strain was grown to 50 KU in LB (pH 7.40, buffered with 10mM MOPS and 10 mM MES) supplemented with 2 μg/mL of CAM, after which the first 1.6 mL sample of culture was taken for RNA extraction. 20 mL of the same culture was then transferred to a new flask containing IPTG at a final concentration of 1mM. Two additional 1.6 mL culture samples were taken for RNA extraction at 20 and 30 min post-induction. RNA extraction began as soon as each sample was taken.

Each 1.6 mL sample of culture was added to 1.6 mL of acid phenol-chloroform (Ambion) already equilibrated in a boiling water bath. This mixture was heated for 5 min with occasional vortexing, after which it was cooled on ice for 2 min. The 3.2 mL mixture was divided evenly into two 2.0 mL Eppendorf tubes and centrifuged at 14 000 g for 10 min at 4°C. The top, aqueous phase from each tube was taken and RNA was re-extracted with 800 μL of a phenol:chloroform:isoamyl alcohol mixture (25:24:1 ratio, pH 6.6; Ambion). The top phase was again taken and the nucleic acids were precipitated O/N at -20°C in the presence of 80 μL 3M sodium acetate and 630 μL of isopropanol. The precipitate was centrifuged at 14 000 g for 30 min at 4°C and the resulting pellet was washed with 500 μL of 70% ethanol. The sample was centrifuged again at 14 000 g for an additional 10 min at 4°C and subsequently allowed to air-dry for two min to remove any residual supernatant. The pellet was re-suspended in TE. DNA was degraded using the Turbo DNA-free™ kit (Ambion) and the remaining RNA was purified using RNeasy® Mini Kit (QIAGEN) according to the manufacturers' protocols. The RNA

concentration and purity were determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific); samples were frozen at -80°C.

RNA extracted from acid-induced strains proceeded as described above. Samples were shifted to acidic pH through the additi

on of 1N HCl.

2.13 qRT-PCR experiments

cDNA was created from previously prepared RNA samples by using 0.1 μg/μL RNA, 15 μg/μL random hexamers, 1 mM dNTPs (Roche), 10 U/μL reverse transcriptase (RT) (New England BioLabs), and 1x RT buffer (New England BioLabs). The RNA and hexamers alone were first incubated at 70°C for 3 min to remove any RNA secondary structure. The rest of the reagents were added, and this mixture was incubated at 42°C for 1 h, followed by a shift to 92°C for 10 min using a thermocycler. cDNA samples were diluted 16-fold and stored at -20°C until use.

The diluted cDNA, 5 mM of mixed primers of interest, and either Brilliant® II SYBR® Green qPCR Master Mix (Agilent) or SYBR® Green PCR Master Mix (Applied Biosystems) was used to set up 20 μL reactions in quadruplicate. qRT-PCR proceeded using the Rotor-Gene 6000 (Corbett Life Sciences). Ct values were calculated using a threshold of 0.015 fluorescent units. Changes in relative levels of gene expression were calculated by the ΔΔCT method first described by Pfaffl (2001). Data were normalized to the constitutively expressed *rpoB* gene, which encodes the β subunit of RNAP.

For the validation experiments of the RNA sequencing data, primer pairs were tested to ensure they had approximately equal amplification efficiency to *rpoB* (Table 3). Specifically, cDNA template dilutions of one-fold, two-fold, four-fold, 10-fold, 20-fold, and 40-fold were prepared. qRT-PCR was performed with each set of primers at each template dilution. Using a threshold of 0.015 fluorescent units, the Ct values of each primer set were subtracted from Ct value of *rpoB*. The differences were plotted, and a primer set was said to have equal amplification efficiency to *rpoB* if the slope of the plots was less than 0.01.

2.14 Whole-transcriptome sequencing of WT, BcL1, and BcL25

RNA samples for each condition and strain were prepared as previously described, with the exception that an additional mRNA enrichment step was performed. Specifically, rRNA in the newly extracted RNA samples was depleted using the MICROBExpress™ Bacterial mRNA enrichment kit (Ambion) according to the company's protocol. The depletion of rRNA and the high quality of the RNA samples were confirmed by the London Regional Genomics Centre through an Agilent 2100 Bioanalyzer. RNA samples were shipped on dry ice to The Centre for Applied Genomics (TCAG) at SickKids® in Toronto, Canada. cDNA library construction was completed by TCAG prior to RNA sequencing on the support oligonucleotide ligation detection (SOLiD) system developed by Applied Biosystems™. With assistance from G. Gloor, sequenced reads were aligned to the strain 14579 reference genome hosted on GenBank® (accession number AE016877.1) using Bowtie as described by Langmead *et al.* (2009). Only reads that mapped to gene-coding sequences were analyzed. In order to quantify

gene expression for analysis, the raw reads were normalized to i) the size of its mapped gene and ii) the number of reads mapped in each condition. This normalized value, called RPKM (Reads Per Kilobase of exon model per Million mapped reads), was used for gene expression comparison as first described by Mortazavi *et al.* (2008). RPKM was calculated for each gene using software developed by G. Gloor. In the analysis of the BcL25 transcriptome, differentially regulated genes relative to WT were called using the R package DESeq according to the statistical model presented by Anders and Huber (2010).

CHAPTER 3: RESULTS

3.1 Creating molecular tools to study σ5816 and σ7615

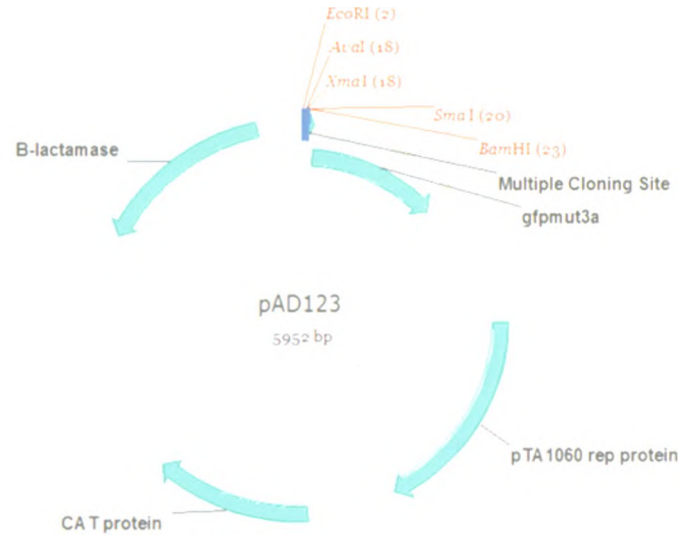
Two approaches taken in an attempt to identify the functions of σ5816 and σ7615 were to i) look for a phenotype(s) associated with the over-expression of these σ factors, and ii) identify the σ5816 regulon by looking for genes that were more highly expressed in a σ5816 over-expression strain relative to WT. To implement these approaches, a controllable expression vector effective in *B. cereus* had to first be created.

3.1.1 Creation of an expression system in *B. cereus*

The *E. coli*-Gram-positive shuttle vector pAD123 (Fig. 3A) was used with modifications as the backbone of the expression plasmid. First, the *gfpmut3a* gene was excised from pAD123, and the linear plasmid was then re-circularized by blunt-end ligation. The P_{spank} (Fig. 3B) and $P_{hyperspank}$ promoters, along with their downstream *lacI* genes, were excised from pDR110 and pDR111 (Table 2) plasmids respectively and inserted into the multiple cloning site (MCS) of pAD123. The only difference between P_{spank} and $P_{hyperspank}$ is that the latter promoter has a single base substitution (G to T) six bases downstream of the -10 element (Fig. 3B, asterisk). The resulting P_{spank}- and $P_{hyperspank}$-based constructs were named pCCEXP4 (Fig. 3C) and pCCEXP5 respectively. For details regarding the construction of pCCEXP4 and pCCEXP5, see chapter 2.3 in materials and methods.

Figure 3. Creation of a *B. cereus* expression system. Panel A. The plasmid pAD123 was used as a backbone for the construction of the pCCEXP4 and pCCEXP5 expression vectors. This high copy plasmid is a Gram-positive-*E. coli* shuttle vector, and encodes resistance to both chloramphenicol and ampicillin. An MCS is present, as are the ORIs for replication in both *B. cereus* and *E. coli*. **Panel B.** The P_{spank} promoter showing restriction sites for cloning, the flanking operators which bind the *lacI* repressor, and the -35 and -10 promoter sequences. Asterisk denotes position where a G to T base substitution results in $P_{hyperspank}$. **Panel C.** The P_{spank} promoter and *lacI* gene were excised from pDR110 and inserted into the MCS of pAD123 to create pCCEXP4. The pCCEXP5 vector has the $P_{hyperspank}$ promoter instead.

A


EcoRI (2)
AccI (18)
XmaI (18)
SmaI (20)
BamHI (23)
Multiple Cloning Site
gfpmut3a
B-lactamase
pAD123
5952 bp
pTA1060 rep protein
CAT protein

B


lacO
-35
-10
lacO
SalI
SphI
XhoI
HindIII
NheI

C

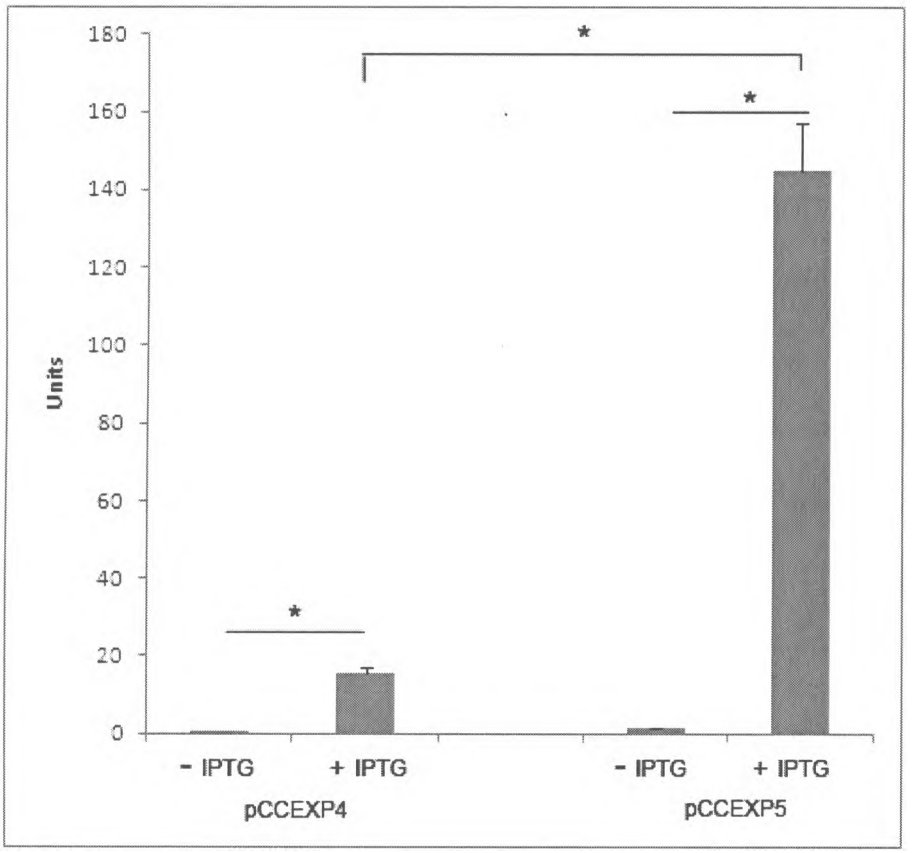

lacI
SphI (5260)
SalI (5242)
HindIII (5235)
lacO
lacP -10
lacP -35
lacO
Pspank
pCCEXP4
6614 bp
pTA1060 rep protein
CAT protein
B-lactamase

3.1.2 Testing the expression system in *B. cereus* ATCC 14579

The P_{spank} and $P_{hyperspank}$ promoters were designed to drive expression systems in *B. subtilis*. To our knowledge, the P_{spank} and $P_{hyperspank}$ promoters have not previously been used to drive transcription in *B. cereus*. In order to ensure the pCCEXP4 and pCCEXP5 expression vectors were working correctly for this study as well as potential future studies, the *lacZ* gene was inserted downstream of these promoters so expression could be easily assayed by measuring β-galactosidase activity. We found that expression of the *lacZ* reporter gene is induced by 1mM IPTG in both pCCEXP4 and pCCEXP5, and the absolute amount of induced *lacZ* activity is greater in pCCEXP5 (145.06 units) than pCCEXP4 (15.52 units) (Fig. 4A and Fig. 4B). However, the amount of uninduced *lacZ* activity is lower in pCCEXP4 relative to pCCEXP5; as a result, the induction ratio is dramatically higher in pCCEXP4 (402.59 fold induction) than pCCEXP5 (101.83 fold induction) (Fig. 4A and Fig. 4B).

Figure 4. β-galactosidase activity from pCCEXP4 and pCCEXP5 in *B.cereus* ATCC 14579. Panel A. β-galactosidase activity is dramatically up-regulated upon induction by 1mM IPTG. Induction of *lacZ* expression from pCCEXP5 and pCCEXP4 results in 145.06 units and 15.52 units respectively. Asterisk denotes $P < 0.001$ as calculated by unpaired, two-tailed Student's T-test. Three separate experiments conducted to obtain $n = 9$. Error bars show standard deviation. Unit definition calculated as $(1000 \times OD_{420}) / (\text{reaction time (min)} \times OD_{595})$. **Panel B.** Uninduced β-galactosidase activity is dramatically lower in pCCEXP4 than pCCEXP5. Consequently, the fold induction of β-galactosidase activity (induction ratio) is higher in pCCEXP4 at 402.59 relative to pCCEXP5 at 101.83. Three separate experiments conducted to obtain $n = 9$.

A



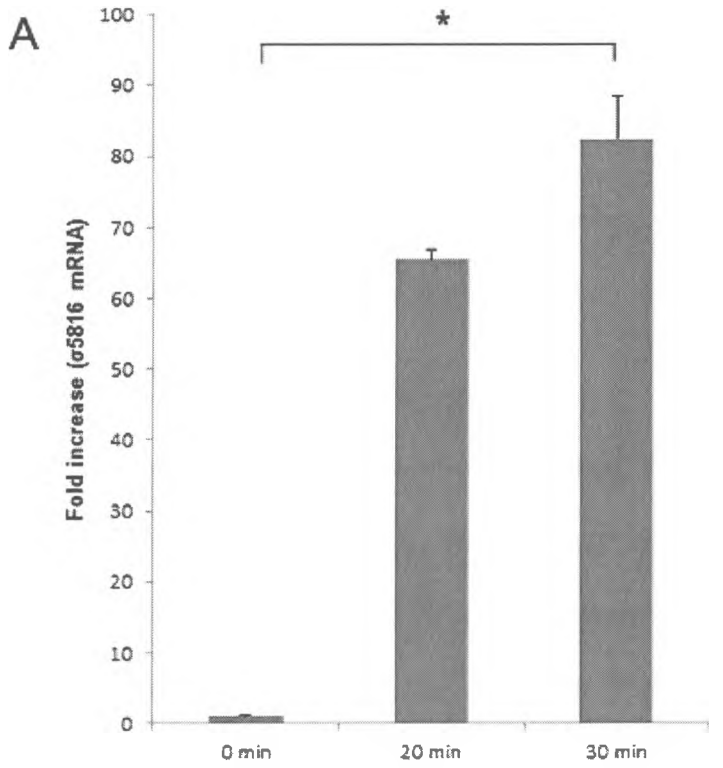
B

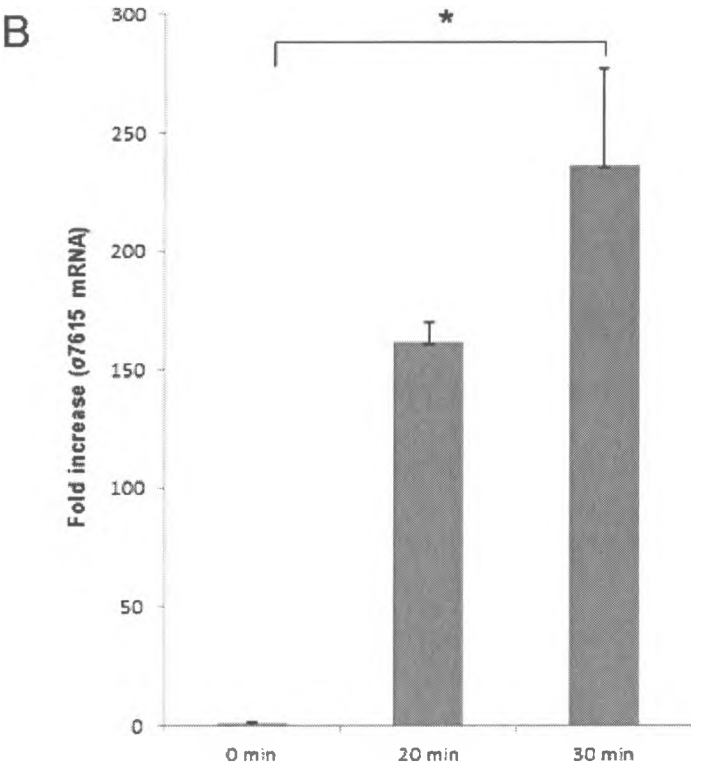
Plasmid	Avg. units of β-galactosidase activity		Induction Ratio
	Non-induced	Induced	
pCCEXP4	0.038	15.52	402.59
pCCEXP5	1.42	145.06	101.83

3.1.3 Quantifying σ5816 and σ7615 mRNA induced from pCCEXP5 via qRT-PCR

As the β-galactosidase assays indicate that the $P_{hyperspank}$ promoter is able to greatly drive expression from the pCCEXP5 vector when induced, we next tested if transcription of σ5816 and σ7615 could also be greatly up-regulated in this same system. The gene for either σ5816 or σ7615 was cloned into pCCEXP5 to create the plasmids p5-σ5816 or p5-σ7615 respectively (Table 2), and these plasmids were transformed into strain 14579. In a representative qRT-PCR experiment, we saw that 20 min after induction with 1 mM IPTG, the expression of σ5816 mRNA and σ7615 mRNA increased 66 fold and 162 fold respectively (Fig. 5A); after 30 min of induction, expression of σ5816 mRNA and σ7615 mRNA further increased to 114 fold and 236 respectively (Fig. 5B). The expression of these σ factors was normalized to the expression of the *rpoB* gene.

Figure 5. σ5816 and σ7615 expression from pCCEXP5 upon induction. Panel A. Induction of the $P_{hyperspank}$ promoter with 1mM IPTG can up-regulate the inserted σ5816 gene dramatically. Induction of gene expression was normalized to the *rpoB* gene. Asterisk denotes $P < 0.001$ as calculated by unpaired, two-tailed Student's T-test. $n = 3$. Error bars show standard deviation. **Panel B.** σ7615 is likewise up-regulated dramatically when inserted down-stream of the $P_{hyperspank}$ promoter. $n = 3$.


A
*
Fold increase (σ5816 mRNA)
100
90
80
70
60
50
40
30
20
10
0
0 min
20 min
30 min

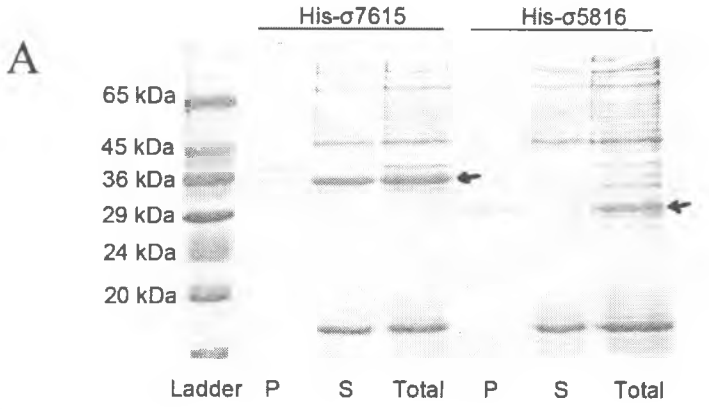

B
*
Fold increase (σ7615 mRNA)
300
250
200
150
100
50
0
0 min
20 min
30 min

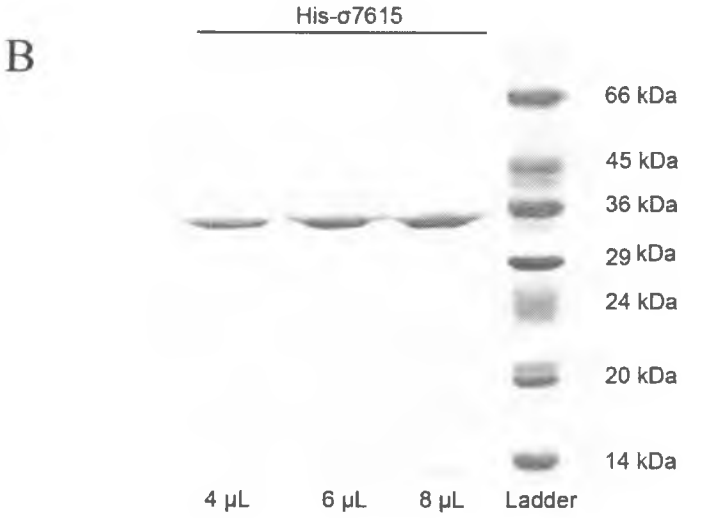
3.1.4 Purification of σ5816 and σ7615 in order to raise antisera

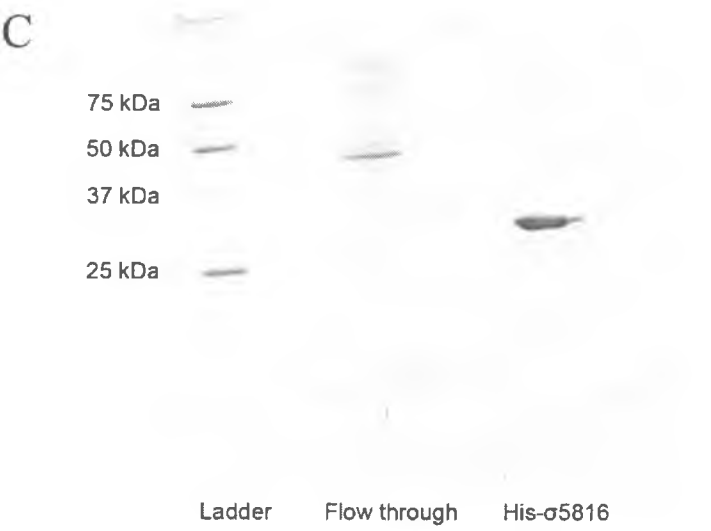
While qRT-PCR showed that induction of $P_{hyperspank}$ with IPTG could increase transcription of σ5816 and σ7615 mRNA, these experiments did not show that this increase in mRNA correlated to an increase in σ factor protein. Thus, antibodies specific for σ5816 and σ7615 were raised in order to show via Western blot that σ factor protein levels increased upon $P_{hyperspank}$ induction.

The pET system was first tried to create N-terminal 6x His-tag fusions, in order to purify these σ factors via FPLC using Ni^{2+} affinity columns. Through SDS-PAGE analysis of pellet and supernatant fractions of whole-cell lysate, His-σ7615 was found to be soluble while His-σ5816 was found to be insoluble (Fig. 6A). His-σ7615 was successfully purified, though degraded His-σ7615 or contaminating proteins may have been co-purified as indicated by the two faint bands migrating at the 20 kDa and 14 kDa mark (Fig. 6B). In an attempt to increase σ5816 solubility, a maltose-binding protein (MBP) fusion of σ5816 was created using the pMAL system (New England BioLabs). Upon induction, the MBP-fusion was still insoluble, even at multiple temperatures assayed: 37°C, 30°C, and 20°C (data not shown). Therefore, the pET system was used and the His-σ5816 was solubilized by re-suspending the whole-cell lysate in 6M guanidine hydrochloride. His-σ5816 was subsequently purified successfully (Fig. 6C). Both His-σ5816 and His-σ7615 were dialyzed in PBS pH 7.40 and sent to ProSci Inc. to raise rabbit anti-sera with polyclonal antibodies towards these proteins.

Figure 6. Purification of σ5816 and σ7615 via FPLC. Panel A. The solubility of His-σ5816 and His-σ7615 was determined by analyzing pellet and supernatant fractions from centrifuged whole-cell lysate of *E. coli* BL21. The vast majority of His-σ7615 was found in the supernatant and was considered soluble. However, His-σ5816 was found in the pellet fraction and was considered insoluble. Arrows show bands representing the induction of either His-σ7615 (33.1 kDa) or His-σ5816 (26.4 kDa). P, pellet; S, supernatant; Total, whole-cell lysate. **Panel B.** Increasing volumes of purified His-σ7615 was loaded onto an SDS-PAGE gel. Either degraded His-σ7615 or contaminating proteins appeared to be co-purified, as indicated by the migration of two faint bands alongside the 20 kDa and 14 kDa markers. **Panel C.** His-σ5816 was purified via FPLC using whole-cell lysate re-suspended in binding buffer and 6M guanidine hydrochloride. Guanidine hydrochloride was removed post-purification, with the protein sample dialyzed in PBS pH 7.40.


A
His-σ7615
His-σ5816
65 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
Ladder
P
S
Total
P
S
Total


B
His-σ7615
66 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
14 kDa
4 µL
6 µL
8 µL
Ladder


C
75 kDa
50 kDa
37 kDa
25 kDa
Ladder
Flow through
His-σ5816

3.1.5 IPTG induction of $P_{hyperspank}$ does not visibly increase σ5816 protein levels

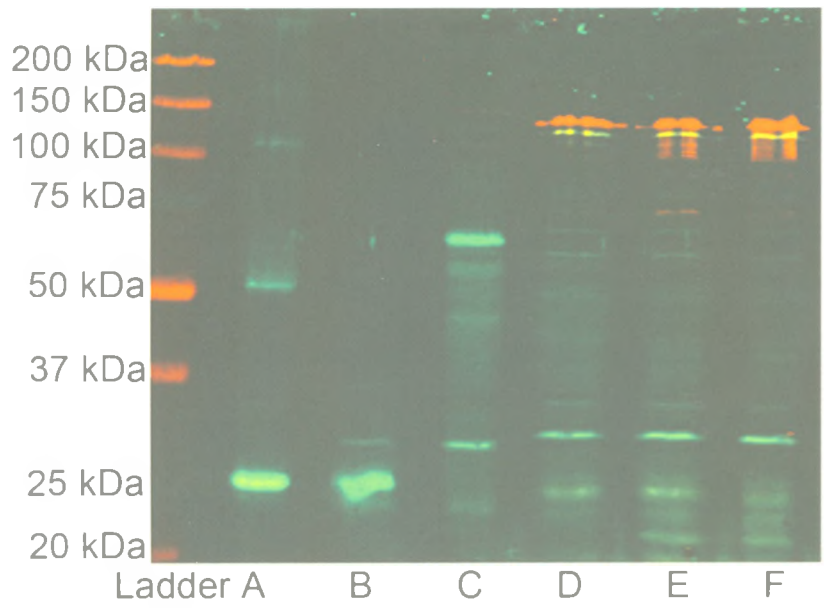
Purified His-σ5816 was obtained and sent to Pro-Sci Inc. to raise rabbit anti-sera. For the initial immunization, antigen was delivered in Freund's complete adjuvant. Every two weeks thereafter, over the course of eight weeks, antigen was delivered in Freund's incomplete adjuvant. Final bleeds were obtained from the rabbits at the end of the immunization project.

While qRT-PCR showed IPTG-induction of $P_{hyperspank}$ increased the transcription of σ5816 mRNA, it could not show whether IPTG-induction of $P_{hyperspank}$ increased σ5816 protein levels. Using rabbit anti-sera with polyclonal antibodies against σ5816, we looked for the expression of this protein following IPTG-induction. Bands were clearly seen that represented purified His-σ5816, as well as His-σ5816 and MBP-σ5816 in whole-cell lysates of *E. coli* expression strains (Fig. 7 lanes A, B and C). Bands likely representing σ5816 were seen in the whole-cell extracts of both strain 14579 and untreated BcL25 (strain 14579 with the plasmid p5-σ5816; σ5816 over-expression strain); these bands were of approximately equal signal intensity (Fig. 7 lanes D and E). Surprisingly, the lysate of BcL25 treated with IPTG for 30 min did not produce a more intense band relative to untreated BcL25, suggesting σ5816 proteins levels are not increasing upon $P_{hyperspank}$ induction with IPTG (Fig. 7 lane F).

The signal intensities of the bands representing σ5816 in strain 14579 and BcL25 were weaker than the bands representing His-σ5816 and MBP-σ5816 fusions found in the whole-cell lysates of *E. coli* expression strains (Fig. 7). The appearance of extra bands in all lanes suggests the polyclonal antibodies are cross-reacting with other proteins in both *E. coli* and *B. cereus* (Fig. 7). An extra band is seen at the 50 kDa mark of purified His-

σ5816; the same sample previously analyzed via SDS-PAGE did not exhibit this band on gels stained with Coomassie Blue (Fig. 6C).

Figure 7. IPTG induction of $P_{hyperspank}$ does not visibly increase σ5816 protein. Purified His-σ5816 and whole-cell lysates from *E. coli* and *B. cereus* strains were analyzed via Western blot. Goat anti-rabbit secondary antibody was used to detect polyclonal IgG antibodies in rabbit anti-serum raised against purified His-σ5816 on the green channel. Goat anti-mouse secondary antibody was used to detect anti-β monoclonal antibodies on the red channel. β, a subunit of core RNAP, was used as the loading control. Ladder (Bio-Rad) is pre-stained and fluoresced red. *A,* purified His-σ5816 (26.4 kDa); *B,* IPTG-treated TLE-9 (*E. coli* BL21 made to express His-σ5816 through pET system); *C,* IPTG-treated TLE-13 [*E. coli* 2523 NEB Express made to express MBP-σ5816 (71 kDa) through pMAL system]; *D,* whole-cell lysate of untreated strain 14579; *E,* whole-cell lysate of untreated BcL25; and *F,* whole-cell lysate of BcL25 treated with IPTG for 30 min. Extra bands, representing non-specific binding of polyclonal antibodies in the rabbit anti-sera, are seen in lanes A through F.


200 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
Ladder
A
B
C
D
E
F

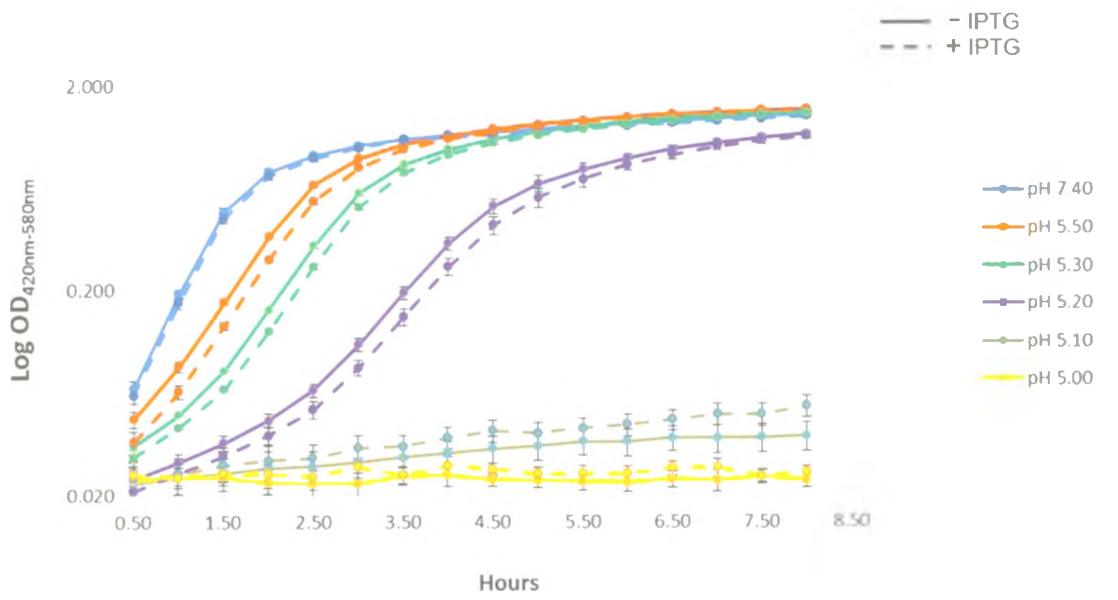
3.2 Phenotypes associated with either loss or gain of σ5816 and σ7615 expression are unable to be identified

3.2.1 Over-expression of σ5816 or σ7615 does not enhance *B. cereus* ATCC 14579 growth under acid treatment

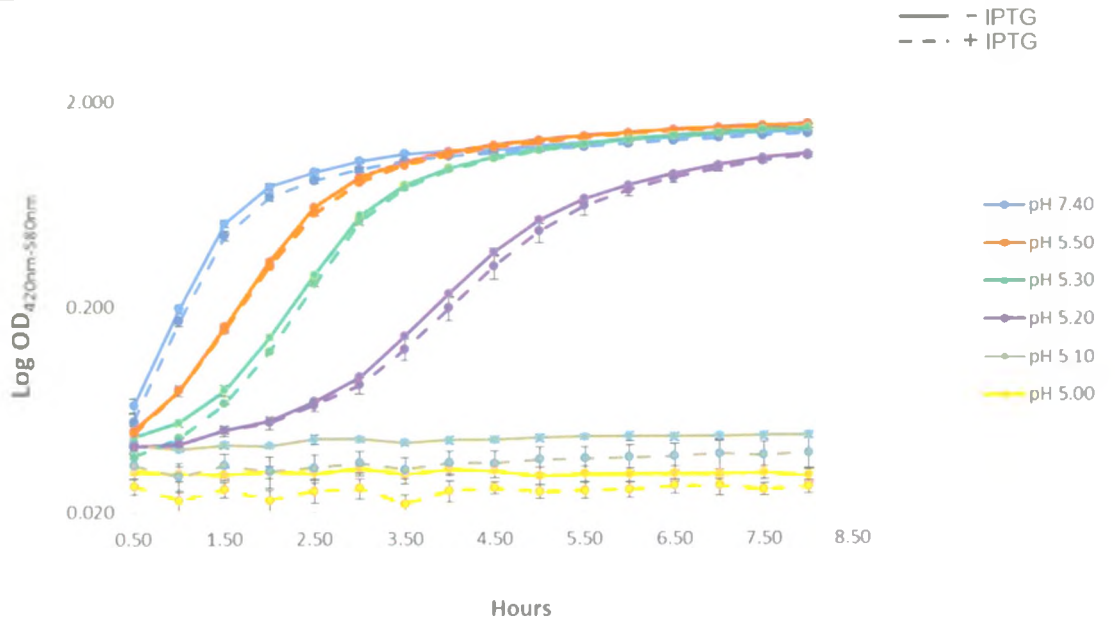
As previously mentioned, although acid treatment induced dramatically the transcription of σ^B, σ5816, and σ7615 (Fig. 2), the BcL8 mutant ($\Delta\sigma^B$, Δσ5816, Δσ7615) did not exhibit obvious growth or survival defects relative to WT when shifted to low pH. Using the new expression system described earlier, an alternative approach was taken by testing if the over-expression of either σ5816 or σ7615 could enhance growth at low pH. The growth of untreated and 1 mM IPTG-treated BcL25 (σ5816 over-expression), as well as untreated or 1 mM IPTG-treated BcL23 (σ7615 over-expression) was assayed in a range of acidic pH values. Cells in mid-exponential phase growing in buffered, neutral LB (pH 7.40) were taken for the acid-shift. Neither IPTG-treated BcL25 nor IPTG-treated BcL23 showed enhanced growth relative to their untreated conditions (Fig. 8A and 8B).

Figure 8. σ5816 or σ7615 over-expression does not affect *B. cereus* growth during acid treatment. Panel A. BcL25 treated with 1 mM IPTG (σ5816 over-expression, dashed line) had similar growth relative to untreated BcL25 (solid line) at 37°C in a range of pH values. Error bars show standard deviation of the mean of OD values obtained in quadruplicate. Growth curves were initially constructed using OD values taken every five minutes; however, for the sake of simplifying figure presentation, the following growth curves show readings taken at each half hour. The growth curves were unaffected by this modification. **Panel B.** BcL23 treated with 1 mM IPTG (σ7615 over-expression, dashed line) had similar growth relative to untreated BcL23 (solid line) at 37°C. Error bars show standard deviation.

A


- IPTG
+ IPTG
2.000
0.200
0.020
Log OD420nm-580nm
0.50
1.50
2.50
3.50
4.50
5.50
6.50
7.50
8.50
Hours
pH 7.40
pH 5.50
pH 5.30
pH 5.20
pH 5.10
pH 5.00

B


- IPTG
+ IPTG
2.000
0.200
0.020
Log OD420nm-580nm
0.50
1.50
2.50
3.50
4.50
5.50
6.50
7.50
8.50
Hours
pH 7.40
pH 5.50
pH 5.30
pH 5.20
pH 5.10
pH 5.00

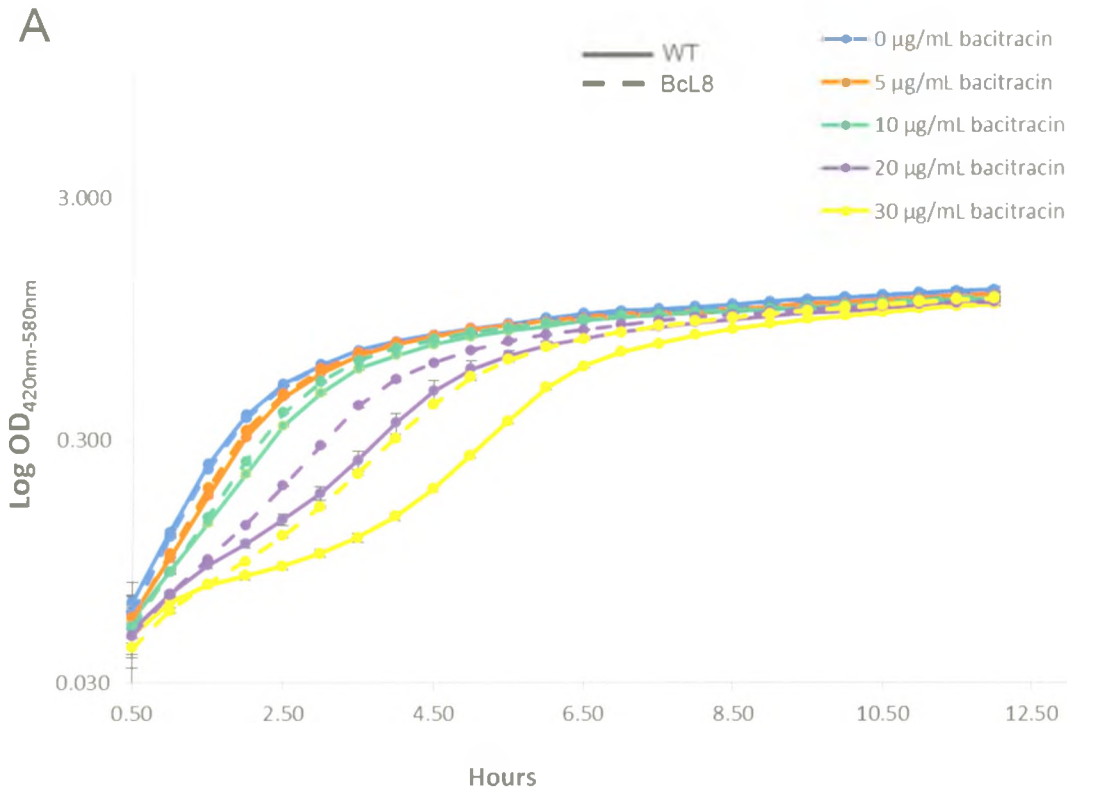
3.2.2 Loss of σB, σ7615, and σ5816 does not adversely affect growth of *B. cereus* under various conditions that cause cell-envelope stress

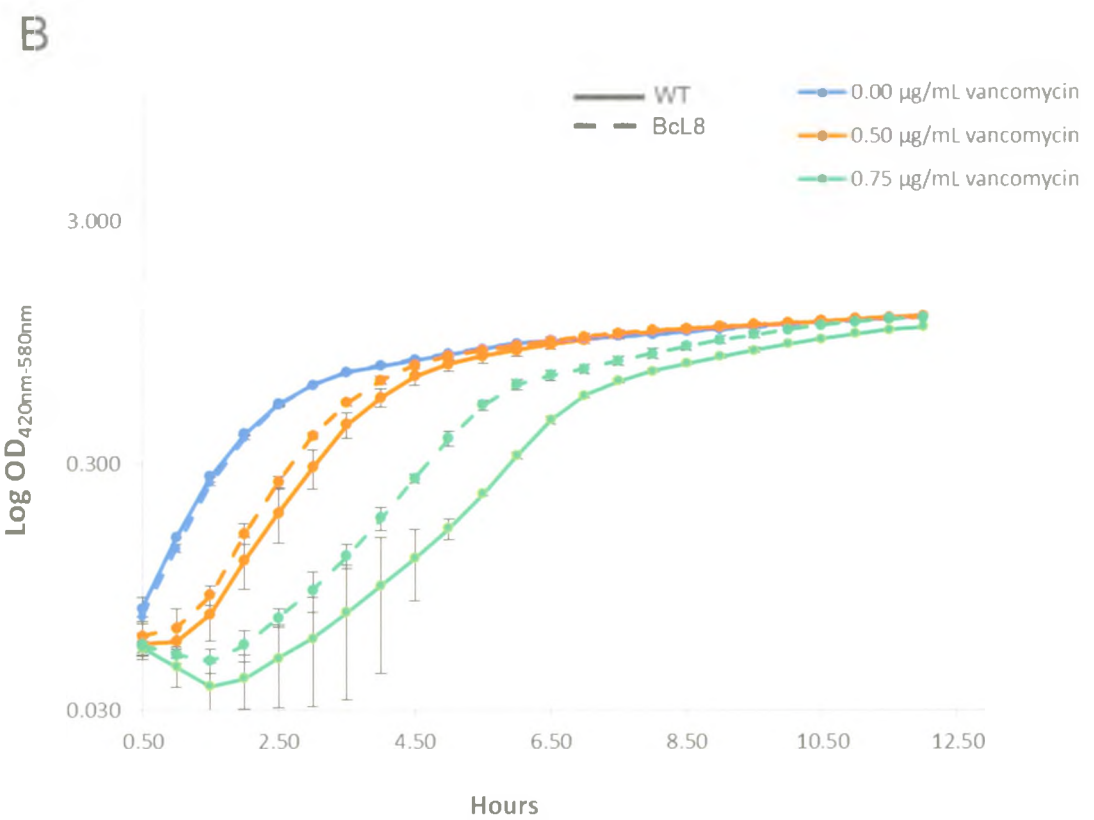
Despite the induction of σ5816 and σ7615 upon acid treatment in WT, this same acid treatment did not elicit an obvious phenotype in strains that lacked (BcL8) or could over-express these σ factors (BcL23 and BcL25). It is possible that these σ factors are not being induced by low pH directly (i.e. in response to an increase in H^+ concentration), but are rather sensing and responding to a different physiological cue that arises as a consequence of the low pH environment. Since cell lysis begins to occur in *B. cereus* at pH levels below 5.0, it is possible that these σ factors are responding to cell-envelope stress. To investigate whether any one or a combination of σ^B, σ5816, and σ7615 is important in mediating responses towards cell-envelope stress as opposed to acid stress, we looked for a more robust growth-defective phenotype in BcL8 relative to WT when cells were challenged with various agents that directly cause cell-envelope stress through different mechanisms.

BcL8 did not exhibit an obvious growth defect relative to WT when grown at 30°C in the presence of sub-lethal concentrations of either vancomycin or bacitracin, two antibiotics that interfere with cell wall biosynthesis (Fig. 9A and Fig. 9B). To see if a growth-defective phenotype is dependent on temperature, growth was assayed also at 37°C; however, still no growth defect was seen (data not shown). We also tested if growing the cultures initially at a lower concentration of 2 μg/mL bacitracin prior to shifting the cells to higher bacitracin concentrations would reveal a growth defect in BcL8, as this may have given the σ factors in WT more time to be transcribed and elicit a stress-response. Again, no growth defect was seen (data not shown). Interestingly,

BcL8 appeared to grow marginally better than WT at 20 μg/mL and 30 μg/mL bacitracin (Fig. 9A), as well as 0.75 μg/mL vancomycin (Fig. 9B).

Figure 9. Growth of BcL8 and WT in the presence of bacitracin or vancomycin. **Panel A.** BcL8 (dashed line) does not exhibit a growth deficiency relative to WT (solid line) at 30°C when challenged with bacitracin. BcL8 exhibits slightly better growth than WT at 20 μg/mL and 30 μg/mL bacitracin. Error bars show standard deviation of the mean of OD values taken in quadruplicate. **Panel B.** BcL8 (dashed line) does not exhibit a growth deficiency relative to WT (solid line) at 30°C when challenged with vancomycin. BcL8 exhibits slightly better growth than WT at 0.75 μg/mL vancomycin. Error bars show standard deviation.

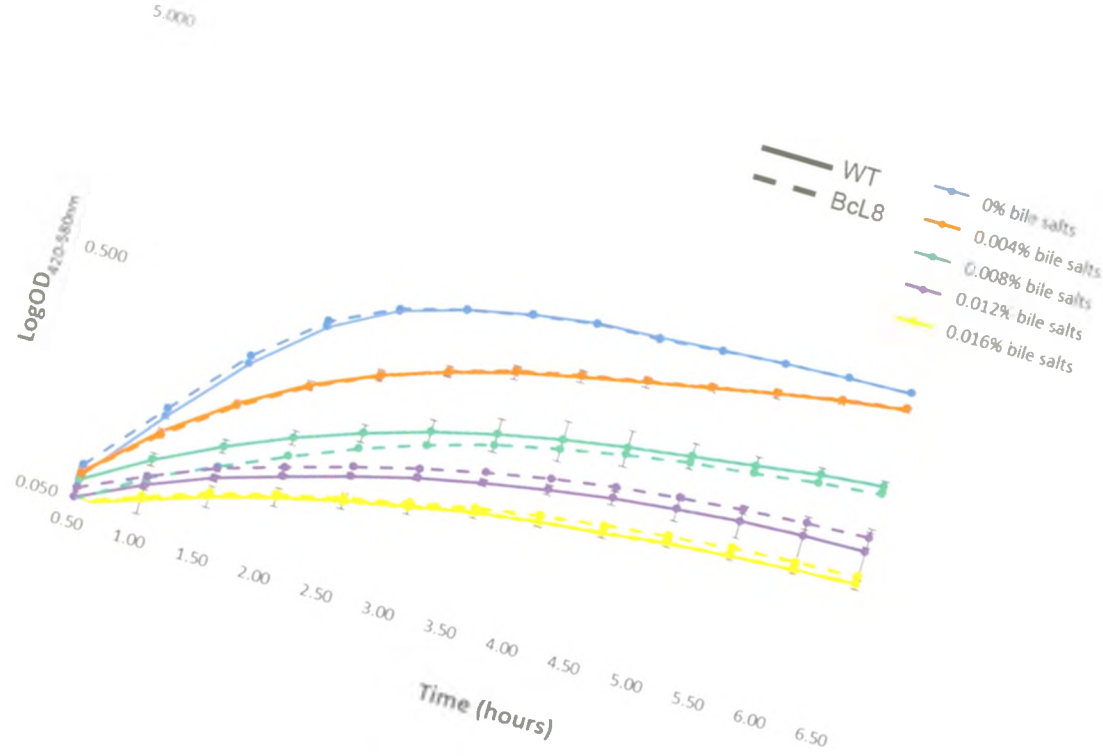

A
WT
BcL8
0 µg/mL bacitracin
5 µg/mL bacitracin
10 µg/mL bacitracin
20 µg/mL bacitracin
30 µg/mL bacitracin
3.000
0.300
0.030
Log OD420nm-580nm
0.50
2.50
4.50
6.50
8.50
10.50
12.50
Hours


B
WT
BcL8
0.00 µg/mL vancomycin
0.50 µg/mL vancomycin
0.75 µg/mL vancomycin
3.000
0.300
0.030
Log OD420nm-580nm
0.50
2.50
4.50
6.50
8.50
10.50
12.50
Hours

In addition to looking for a growth-defect phenotype in BcL8 relative to WT in the presence of cell-wall stress, we looked also for a growth-defect phenotype when BcL8 was subjected to either bile salt or osmotic stresses. BcL8 grew similar to WT under both conditions, and no phenotype was found (Fig. 10A and 10B).

Figure 10. Growth of BcL8 and WT in the presence of bile salts or NaCl. Panel A. BcL8 (dotted line) does not exhibit a growth defect relative to WT (solid line) when challenged with bile salts at 37°C. Error bars show standard deviation of the mean of OD values taken in quadruplicate. **Panel B.** BcL8 (dotted line) does not exhibit a growth defect relative to WT (solid line) when challenged with NaCl at 37°C. Error bars show standard deviation.

A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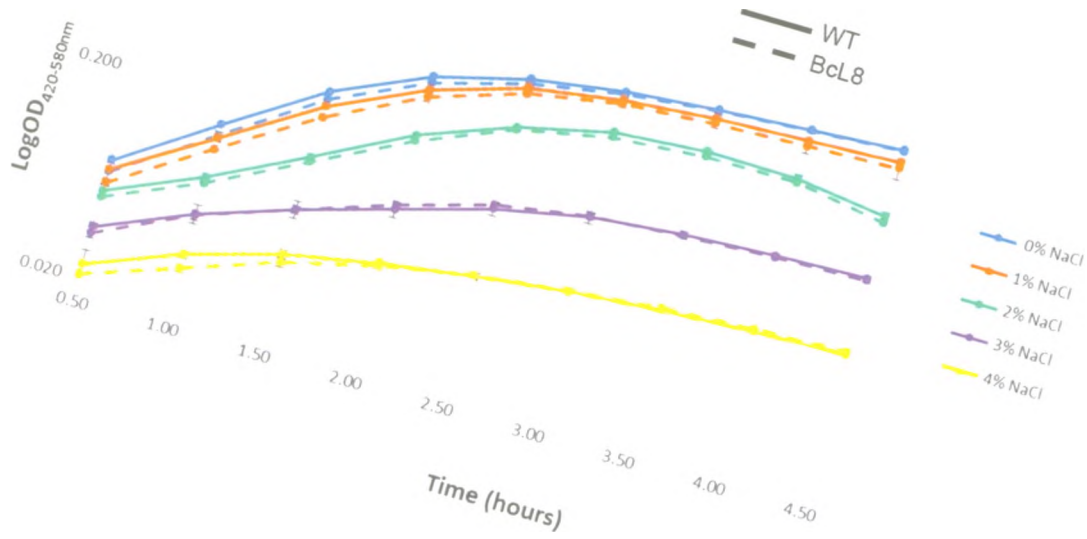
0.500
0.050
LogOD420-580nm
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
5.00
5.50
6.00
6.50
Time (hours)
WT
BcL8
0% bile salts
0.004% bile salts
0.008% bile salts
0.012% bile salts
0.016% bile salts

B


2.000
0.200
0.020
LogOD420-580nm
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00
4.50
Time (hours)
WT
BcL8
0% NaCl
1% NaCl
2% NaCl
3% NaCl
4% NaCl

3.3 Attempting to determine the regulon of σ5816 through transcriptomics

We attempted to identify the set of genes being specifically regulated by σ5816 in the hopes of gaining insight as to σ5816 function. Two approaches were taken in an attempt to achieve this. First, we wanted to identify genes induced by acid stress in both WT and a σ5816 single deletion mutant (BcL1). We then wanted to compare the expression levels of these acid-induced genes between these two strains, in hopes of identifying a core set of genes in BcL1 which was not as highly up-regulated by acid treatment as in WT. Second, we wanted to compare the expression levels of genes between IPTG-treated BcL25 (σ5816 over-expression) and untreated WT. This comparison should identify a core set of genes whose expression levels were substantially higher in IPTG-treated BcL25 relative to WT. The higher expression levels of this core set of genes in BcL25 relative to WT could potentially be due to heightened σ5816 activity.

To take both approaches, we needed to analyze five transcriptomes representing five different conditions: i) WT at neutral pH (pH 7.19), ii) WT under acid stress (pH 5.46), iii) BcL1 at neutral pH (pH 7.23), iv) BcL1 under acid stress (pH 5.45), and v) BcL25 at neutral pH and treated with 1 mM IPTG. Next generation sequencing (NGS) was used to analyze these five transcriptomes. For each condition, total RNA was extracted and then enriched for mRNA. These RNA samples were sent to The Centre for Applied Genomics (Toronto) for cDNA library construction and sequencing. Sequencing data was obtained using the SOLiD system. With assistance from G. Gloor, sequenced reads were aligned to the strain 14579 reference genome, and the number of reads

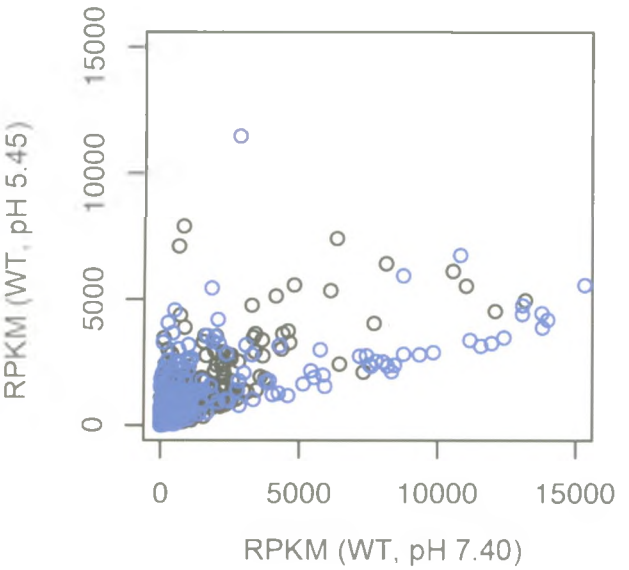
mapping to a gene coding sequence was used to calculate RPKM, a value used to quantify gene expression.

3.3.1 The effect of acid treatment on gene expression in WT *B. cereus* ATCC 14579

Following a pH-shift from 7.19 to 5.46, WT both up-regulates and down-regulates numerous genes, as indicated by substantial changes in RPKM values of genes between the two conditions. Thus, the transcriptional profile of WT changes drastically when challenged with acid stress (Fig. 11A). By calculating RPKM ratios (RPKM of a gene under the condition of acid-stress over RPKM of same gene under condition of neutral pH), the fold increase of many up-regulated genes in the acid-treated transcriptome was calculated. Many genes expected to play role in the acid-tolerance response (ATR) were up-regulated, including oxalate/formate antiporter (up 122 fold), α-acetolactate decarboxylase (up 119 fold), acetolactate synthase (up 115 fold) and superoxide dismutase [Mn+] (up 30 fold) (Fig. 11B). The 17 most up-regulated genes under the condition of acid treatment are shown in Fig. 10B.

Figure 11. WT up-regulates numerous genes upon acid stress. Panel A. RPKM plots of genes in WT at pH 7.40 versus RPKM plots of genes at pH 5.46 indicate the strain 14579 transcriptional profile changes dramatically upon acid stress. Blue and black dots represent genes located on the forward and reverse strands, respectively. **Panel B.** A list of the most highly up-regulated genes induced upon acid stress, as calculated by the RPKM ratio of each gene in the transcriptomes of strain 14579 under acid stress and at neutral pH.

A



B

Gene	Function	Induction Ratio
BC3419	long-chain-fatty-acid--CoA ligase	194.71
BC1436	Phage shock protein A	130.48
BC2300	oxalate/formate antiporter	122.89
BC0884	Alpha-acetolactate decarboxylase	118.95
BC0883	acetolactate synthase	115.32
BC2272	peptidylprolyl isomerase	88.46
BC0753	potassium-transporting ATPase subunit A	87.08
BC1435	hypothetical protein	85.38
BC1421	phosphoadenosine phosphosulfate reductase	72.50
BC1161	peptidylprolyl isomerase	72.18
BC2620	penicillin-binding protein transpeptidase	71.28
BC0785	hypothetical protein	59.13
BC0754	potassium-transporting ATPase subunit B	55.65
BC5438	antiholin-like protein LrgB	46.30
BC4341	GTP pyrophosphokinase	46.08
BC5439	murein hydrolase regulator LrgA	44.63
BC0755	potassium-transporting ATPase subunit C	42.36

3.3.2 Acid-induced gene expression profiles are highly similar in WT and BcL1

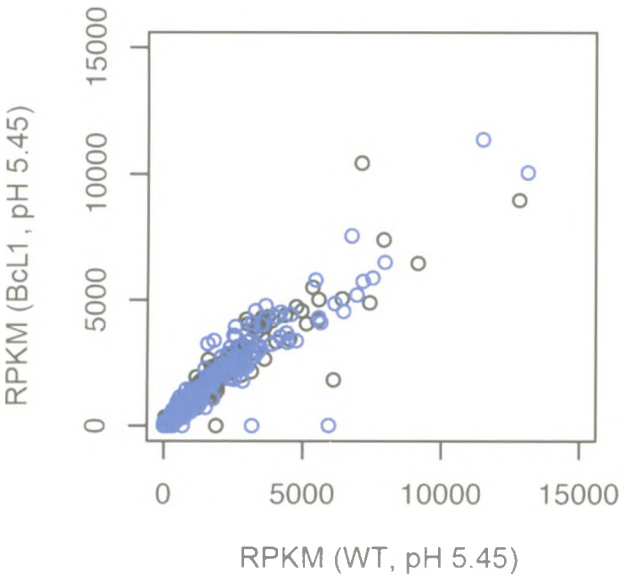
A plot of the RPKM values of genes in the transcriptome of acid-stressed BcL1 versus the RPKM values of the same genes in acid-stressed WT does not show dramatic transcriptional differences between the two strains (Fig. 12A). The vast majority of genes have a similar RPKM value in both transcriptomes, giving rise to linear distribution of plots along a 45° slope. However, a few plots were seen that lay close to the x-axis, representing genes that are strongly down-regulated in BcL1 (Fig. 12A).

To identify the set of genes that were not as strongly up-regulated upon acid treatment in BcL1 relative to WT, we calculated a new RPKM ratio (the RPKM ratio of a gene in acid-treated WT over the RPKM ratio of the same gene in acid-treated BcL1). This new ratio was used as the difference in gene expression (expression ratio) in WT relative to BcL1 upon acid treatment (Fig. 12B). Twenty-two genes located on the strain 14579 plasmid pClin15 were expressed at higher levels in WT relative to BcL1 (Fig. 12B, asterisk); however, the RPKM values of the vast majority of these plasmid-encoded genes were very low (< 1) in BcL1, strongly suggesting the plasmid was lost during the creation of the BcL1 strain. Aside from the plasmid-encoded genes, only the putative cytoplasmic protein encoded by *BC0917* is expressed substantially higher (tenfold change) in acid-induced WT relative to acid-induced BcL1 (Fig. 12B).

The transcriptomes of both WT and BcL1 under the condition of neutral pH were analyzed. The transcriptional profiles were highly similar between these two strains, although one hypothetical protein encoded by *BC3011* was expressed tenfold higher in WT relative to BcL1 (data not shown).

Figure 12. The transcriptomes of WT and BcL1 are highly similar upon acid-stress. **Panel A.** A scatter plot of the RPKM values of genes in both acid-induced WT and BcL1 shows the transcriptional profiles are highly similar, with only a few genes differing substantially in expression. Blue and black dots correspond to genes encoded on the forward and reverse strands, respectively. **Panel B.** A list showing 34 of the most highly expressed genes in acid-treated WT relative to acid-treated BcL1. Asterisk denotes plasmid-encoded genes. BcL1 has likely lost the pClin15 plasmid. Aside from the genes encoded by the plasmid, expression levels of none except *BC0917* differed by greater than tenfold in WT relative to BcL1.

A



B

Gene	Function	Expression Ratio
BCp0015	hypothetical protein	1044.50
BCp0013	putative cytoplasmic protein	711.00
BCp0020	hypothetical protein	704.85
BCp0001	hypothetical protein	680.49
BCp0021	N-acetylmuramoyl-L-alanine amidase	465.17
BCp0008	putative cytoplasmic protein	459.33
BCp0005	type B DNA polymerase	419.79
BCp0014	putative cytoplasmic protein	410.71
BCp0018	putative cytoplasmic protein	409.89
BCp0009	putative cytoplasmic protein	387.00
BCp0019	hypothetical protein	384.77
BCp0002	hypothetical protein	327.30
BCp0012	hypothetical protein	325.97
BCp0004	putative cytoplasmic protein	313.03
BCp0003	putative cytoplasmic protein	289.84
BCp0011	hypothetical protein	190.48
BCp0016	N-acetylmuramoyl-L-alanine amidase	174.74
BC0917	putative cytoplasmic protein	10.40
BC0521	16S ribosomal RNA	7.41
BC4890	tRNA-His	6.43
BC0525	tRNA-Glu	5.03
BC2906	hypothetical protein	5.00
BC2387	hypothetical protein	4.52
BC4877	tRNA-Thr	4.52
BC3002	hypothetical protein	4.51
BC3715	hypothetical protein	4.04
BC3767	hypothetical protein	4.03
BC0181	tRNA-Lys	4.02
BC4878	tRNA-Phe	4.01

*

3.3.3 BcL25 dramatically up-regulates numerous genes relative to WT at neutral pH.

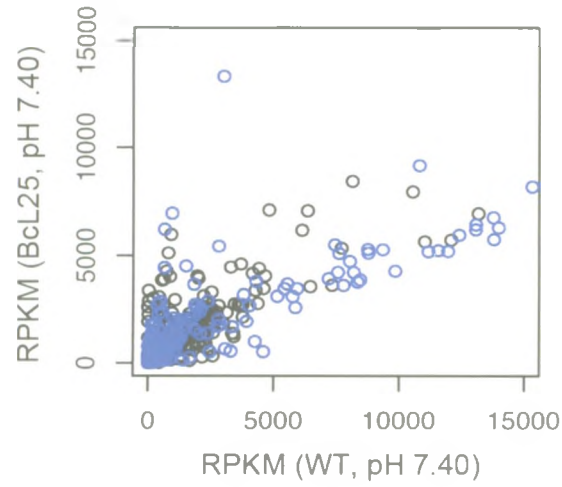
We compared the transcriptomes of IPTG-induced BcL25 (σ5816 over-expression) and WT when both strains were grown at neutral pH in an attempt to identify genes in BcL25 whose expression may be regulated by σ5816. A plot of RPKM values in BcL25 and WT show that numerous genes are substantially up-regulated in BcL25, and that the transcriptional profiles are dramatically different overall between these two strains (Fig. 13A).

As mentioned in the previous section, the transcriptomes of WT and BcL1 were highly similar to each other under conditions of both neutral pH and acid stress. Except for the genes encoded on the plasmid (pClin15), the overwhelming majority of gene expression, as quantified by RPKM, did not substantially differ between these two strains. Thus, when analyzing gene expression in BcL25, the read count data from BcL25 was compared to the read count data of not only WT, but also BcL1 at neutral pH. We then worked with partial replicates: no replicate in the first condition (IPTG-treated BcL25), but one replicate in the second condition (WT and BcL1 at neutral pH). We were thus able to use the statistical R package DESeq to call differential gene expression with statistical power, which cannot be done with RPKM ratios alone. DESeq is a widely used package for calling changes in gene expression with partial replicates, and the error model it applies to the count data (derived from variance within the replicate set) is able to assign a P value adjusted for a false discovery rate (FDR), set at 10%, to control for Type I error (false positives).

Using DESeq, numerous genes in BcL25 were identified as more highly expressed relative to WT under the condition of neutral pH. Fifteen genes with the highest expression ratio (expression in BcL25 relative to WT) are listed ($P << 1^{-10}$), of which five are hypothetical (Fig. 13B). The two genes with the highest expression ratios include σ5816 itself (842 fold increase) and the immediate down-stream gene encoding the proteolytic subunit of an ATP-dependent Clp protease (759 fold increase). Additional genes with high expression ratios include isoflavone reductase (152 fold increase), oxalate/formate antiporter (134 fold increase), and superoxide dismutase (18 fold increase) (Fig. 13C). These genes were also dramatically up-regulated in acid-treated WT relative to untreated WT. When comparing RPKM values between BcL25 and WT alone, these genes listed in Fig. 13B are also found to be among the most-up-regulated, though the actual values of fold change in expression slightly varied.

Figure 13. Expression of numerous genes is up-regulated in BcL25 relative to WT. Panel A. Plotting of RPKM values in BcL25 and WT show the transcriptional profiles of both strains are substantially different, with substantial up-regulation in numerous genes. Blue and black dots correspond to genes encoded on the forward and reverse strands, respectively. **Panel B.** Treating BcL1 at neutral pH as a replicate of WT at neutral pH, DESeq is used to call differential expression between this replicated condition and the unreplicated condition of IPTG-induced BcL25. Genes in BcL25 are plotted by their read count and the log2 change in expression, with red dots and black dots representing statistically significant (at FDR of 10%) and insignificant hits respectively. **Panel C.** The most highly up-regulated genes in BcL25 relative to both WT and BcL1, as determined by DESeq, are listed. P value is adjusted via Benjamani-Hochberg method to account for an FDR of 10%.

A



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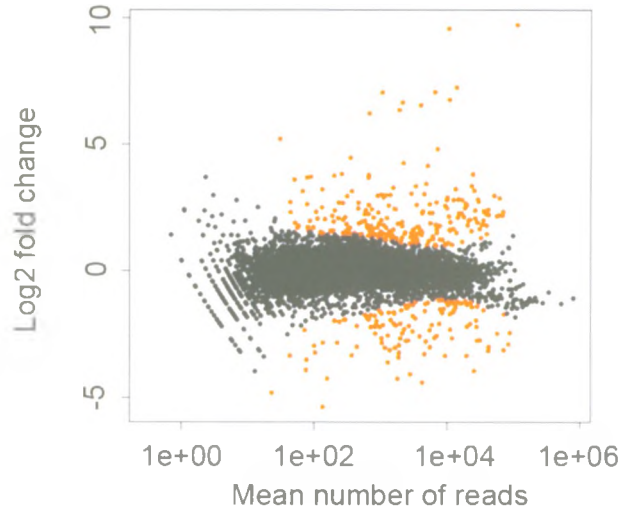

C

Gene	Function	Fold Change	Adjusted P
BC2794	RNA polymerase factor sigma-70	841.87	4.99E-82
BC2793	ATP-dependent Clp protease	758.58	1.28E-192
BC3031	isoflavone reductase	152.17	7.25E-133
BC2300	oxalate/formate antiporter	133.56	7.38E-131
BC3966	hypothetical protein	133.08	1.17E-70
BC3030	hypothetical protein	108.62	2.38E-123
BC3964	hypothetical protein	100.73	3.46E-91
BC3965	hypothetical protein	93.52	3.34E-110
BC5438	antiholin-like protein LrgB	81.86	4.99E-82
BC5439	murein hydrolase regulator LrgA	74.89	1.66E-51
BC2792	glycine betaine-binding protein	37.02	5.83E-15
BC0579	malate-sodium symport	28.09	2.65E-73
BC2463	hypothetical protein	22.11	9.81E-29
BC0580	NAD-dependent malic enzyme	19.13	2.29E-46
BC5445	superoxide dismutase [Mn]	17.69	2.39E-55

3.4 Candidates for σ5816 regulon and validation of their expression via qRT-PCR

Genes with the highest potential of being candidates for the σ5816 regulon were identified based on i) each gene being up-regulated in WT upon acid treatment, and ii) each gene being up-regulated in the σ5816 over-expression strain BcL25 relative to WT (Fig. 13A). A third criterion, that each gene be substantially up-regulated in acid-treated WT relative to acid-treated BcL1, could not be applied as no genes that fit this requirement were found (Fig. 12B). Aside from the *BC2794* gene encoding σ5816 itself, we picked four candidate genes for further testing: *BC2793*, encoding the proteolytic subunit of an ATP-dependent Clp protease; *BC5438*, encoding antiholin-like protein LrgB; *BC2300*, encoding an oxalate/formate antiporter; and *BC5445*, encoding a superoxide dismutase [Mn] (Fig. 14A).

In order to confirm that these genes were indeed being up-regulated, as found through SOLiD sequencing, qRT-PCR validation experiments were performed. Using both an aliquot of the same RNA that was sent for sequencing as well as a new RNA preparation, we confirmed that these genes were being substantially up-regulated in acid-treated WT (Fig. 14B). We also confirmed via qRT-PCR that these genes were being up-regulated in IPTG-induced BcL25 relative to WT (Fig. 14C). While the values of the induction ratios of genes varied between the data from transcriptome sequencing and qRT-PCR, the overall direction in the change of expression was the same from both experiments. The qRT-PCR data was normalized to *rpoB* gene expression. Interestingly, the RNA sequencing data indicates that *rpoB* expression decreased approximately twofold in acid-induced WT, and 1.67 fold in IPTG-induced BcL25. For qRT-PCR

experiments, the primers for each queried gene were validated by ensuring they all had amplification efficiencies approximately equal to our house-keeping gene *rpoB*.

Figure 14. Possible σ5816 regulon candidates and transcriptome validation via qRT-PCR. Panel A. Data from SOLiD sequencing show that four genes in addition to σ5816 itself are up-regulated substantially i) acid-stressed WT relative to untreated WT (acid/neutral) and ii) IPTG-induced BcL25 (+IPTG/WT) relative to WT at neutral pH. **Panel B.** The up-regulation of these genes in acid-treated WT relative to untreated WT, as determined by SOLiD sequencing data, is validated by qRT-PCR. Data was derived from either the original RNA preparation that was sent for sequencing (superscript A) or RNA prepared from an independent pH shift experiment (superscript B). **Panel C.** The up-regulation of these genes in BcL25 relative to WT at neutral pH, as determined by SOLiD sequencing data, is validated also by qRT-PCR. All data were obtained using the original RNA preparation sent for RNA-seq (superscript A).

A

Gene	Function	Induction Ratio	
		WT $\frac{acid}{neutral}$	BcL25 $\frac{+IPTG}{WT}$
BC2794	RNA polymerase factor sigma-70	2.62	841.87
BC2793	ATP-dependent Clp protease proteolytic subunit	5.36	758.58
BC2300	oxalate/formate antiporter	122.89	133.56
BC5438	antiholin-like protein LrgB	46.30	81.86
BC5445	superoxide dismutase [Mn]	30.20	17.69

B

Gene	Function	Induction Ratio		
		SOLiD[A]	qRT-PCR[A]	qRT-PCR[B]
BC2794	RNA polymerase factor sigma-70	2.62	16.21	18.72
BC2793	ATP-dependent Clp protease proteolytic subunit	5.36	15.97	17.53
BC2300	oxalate/formate antiporter	122.89	123.78	221.19
BC5438	antiholin-like protein LrgB	46.30	191.56	113.57
BC5445	superoxide dismutase [Mn]	30.20	79.02	334.11

C

Gene	Function	Induction Ratio	
		SOLiD[A]	qRT-PCR[A]
BC2794	RNA polymerase factor sigma-70	841.87	27,081.12
BC2793	ATP-dependent Clp protease proteolytic subunit	758.58	4091.27
BC2300	oxalate/formate antiporter	81.86	230.45
BC5438	antiholin-like protein LrgB	133.56	292.04
BC5445	superoxide dismutase [Mn]	17.69	31.67

3.4.1 Enhanced σ5816 activity in BcL23 is unlikely the reason for up-regulation of some of the candidate genes.

To further test whether the up-regulation of these candidate genes in IPTG-induced BcL25 was due specifically to σ5816 over-expression, qRT-PCR was used to measure the change in expression of these genes in induced BcL25 relative to induced BcL31, a strain which has the plasmid pCCEXP5 without any gene insert. While the expression of σ5816 and the proteolytic subunit of ATP-dependent Clp protease was substantially higher in BcL25 relative to BcL31 (8335 fold increase and 1243 fold increase respectively), surprisingly the expression for each of antiholin-like protein LrgB, oxalate/formate antiporter, and superoxide dismutase [Mn] differed by less than 2 fold between these two strains (Fig. 15A).

To test whether the up-regulation of the candidate genes in BcL31 is simply due to induction of the $P_{hyperspank}$ promoter with IPTG, a qRT-PCR experiment was performed to compare the gene expression between uninduced BcL31 and BcL31 induced for 30 min with 1mM IPTG. While the expression of σ5816 and the proteolytic subunit of ATP-dependent Clp protease differs by less than 2 fold between the two conditions, the expression of antiholin-like protein LrgB, oxalate/formate antiporter, and superoxide dismutase [Mn] substantially increases, albeit this increase in expression is less than the increase seen between BcL25 and WT (Fig. 15B).

Figure 15. IPTG-induced BcL31 up-regulates σ5816 regulon candidates. Panel A. Although candidate gene expression is substantially up-regulated in IPTG-induced BcL25 relative to WT, *BC2300, BC5438,* and *BC5445* are not substantially up-regulated in IPTG-induced BcL25 relative to IPTG-induced BcL31 (expression plasmid backbone alone) **Panel B.** Induction of $P_{hyperspank}$ in the pCCEXP5 plasmid, without any gene insert, is causing an up-regulation of *BC2300, BC5438,* and *BC5445*.

A

Gene	Function	Induction Ratio	
		BcL25/WT	BcL25/BcL31
BC2794	RNA polymerase factor sigma-70	27,081.12	8335.19
BC2793	ATP-dependent Clp protease proteolytic subunit	4091.27	1242.62
BC2300	oxalate/formate antiporter	230.45	1.02
BC5438	antiholin-like protein LrgB	292.04	1.26
BC5445	superoxide dismutase [Mn]	31.67	1.77

B

Gene	Function	Induction Ratio
BC2794	RNA polymerase factor sigma-70	1.83
BC2793	ATP-dependent Clp protease proteolytic subunit	1.91
BC2300	oxalate/formate antiporter	75.41
BC5438	antiholin-like protein LrgB	42.1
BC5445	superoxide dismutase [Mn]	24.76

CHAPTER 4: DISCUSSION

4.1 An effective, regulatable expression system for *B. cereus*

A regulatable promoter that can drive the expression of a gene of interest is a useful molecular tool; however, a standardized and characterized expression system had not yet been developed for *B. cereus*. In an attempt to identify the functions of ECF σ factors σ5816 and σ7615, we looked for a phenotype in BcL23 (σ7615 over-expression) and BcL25 (σ5816 over-expression). Additionally, we attempted to identify a set of genes regulated by σ5816 by comparing the transcriptome of BcL25 to that of WT. To take both approaches, an expression system had to be first created. We created two expression vectors: pCCEXP4 and pCCEXP5, containing the promoters P_{spank} and $P_{hyperspank}$ respectively. To first test whether this expression system would even work in *B. cereus*, the *lacZ* reporter gene was inserted into each vector and its expression monitored by performing β-galactosidase assays. Upon induction with IPTG, both promoters were able to drive the expression the *lacZ* reporter gene, and *lacZ* transcripts were able to be translated to provide measurable signal (Fig. 4A and 4B).

The original hybrid promoter created from both the SP01 phage promoter and the *lac* operon (P_{spac}) was modified to create the P_{spank} promoter, which has high intrinsic affinity for RNAP. However, expression from P_{spank} is tightly controlled as two operator sites, which each bind the *lacI* repressor, flank the -35 and -10 elements (Fig. 4A). The absence of IPTG inducer results in only a small amount of basal *lacZ* transcription, as active *lacI* repressor molecules are binding these operator sites and preventing efficient

RNAP binding to P_{spank} via steric hindrance; the presence of IPTG, however, results in *lacI* repressor inactivation, allowing RNAP to freely bind P_{spank}.

The $P_{hyperspank}$ promoter is identical to P_{spank} except for a G to T base substitution six bases downstream of the -10 element, which is also one base before the downstream operator (Fig. 3B). Through an unknown mechanism, this substitution results in a greater amount of gene transcription from induced $P_{hyperspank}$ relative to P_{spank}. Two possibilities include enhanced open promoter complex formation and/or enhanced transcription initiation, as the substitution occurs within the region predicted to include the transcription start site. The absence of IPTG results in a greater amount of leaky transcription driven from $P_{hyperspank}$ in comparison to P_{spank}. Thus, $P_{hyperspank}$ is less tightly regulated.

To identify the σ5816 regulon, we wanted to find genes that were more highly expressed in a σ5816 over-expression strain relative to WT. Additionally, we wanted to see if a phenotype could be found in strains that over-expressed σ5816 and σ7615. For both objectives, strains that could produce the greatest amounts of σ5816 or σ7615 protein had to be made. Thus, the $P_{hyperspank}$ promoter was chosen instead of P_{spank} to over-express the σ factors of interest. Noteworthy, future experiments involving not only σ factors but also other proteins in *B. cereus* may depend more on having tight regulation rather than high absolute levels of protein expression. In these cases, the P_{spank} promoter in the pCCEXP4 expression vector may be more useful than $P_{hyperspank}$ in pCCEXP5 for driving protein expression.

After the BcL23 (σ5816 over-expression) and BcL25 (σ7615 over-expression) strains were created, qRT-PCR showed that IPTG induction of $P_{hyperspank}$ was indeed causing a substantial increase in σ factor mRNA (Fig. 5A and 5B). Expression of mRNA was normalized to the expression of the *rpoB* gene, which encodes the β subunit of core RNAP. Normalization to *rpoB* is suitable, as not only is this house-keeping gene constitutively expressed, but the encoded protein is also a limiting subunit of core RNAP assembly. Thus, the ratio of σ factor to *rpoB* expression can be used to directly infer the relative proportion of σ factor available to potentially bind core RNAP and form holoenzyme. While the qRT-PCR experiments demonstrated that the induction of $P_{hyperspank}$ can dramatically up-regulate σ factor mRNA in BcL23 and BcL25, they did not give any information as to the levels of translated σ factor protein being expressed in these strains.

Purified His-σ5816 and His-σ7615 were sent to Pro-Sci Inc. to develop rabbit anti-serum with polyclonal antibodies specific for these σ factors. Using this serum, σ5816 could be detected via Western blot. Intense bands of approximately the correct size were seen in samples of i) purified His-σ5816 (26.4 kDa, lane A; Fig. 7), ii) whole-cell lysate of *E. coli* BL-21 made to express His-σ5816 using the pET system (26.4 kDa, lane B; Fig. 7), and iii) whole-cell lysate of *E. coli* 2523 NEB Express made to express MBP-σ5816 using the pMAL system (71 kDa, lane C; Fig. 7). Untreated WT and untreated BcL25 (σ5816 in pCCEXP5 for over-expression) had bands of approximately equal intensity that migrated in distance similarly to purified His-σ5816, and these bands likely represent σ5816 (Fig. 7). Surprisingly, treatment of BcL25 with 1mM IPTG did not result in a more intense band than untreated WT or untreated BcL25 (Fig. 7). This

suggests that despite an increase in σ5816 mRNA following $P_{hyperspank}$ induction with IPTG, the greater levels of mRNA may not correlate with greater levels of protein. There are a few possibilities that may explain this result.

First, BcL25 may have acquired a nonsense mutation in the σ5816 gene of the p5-σ5816 plasmid. While no mutation was seen in a sequenced plasmid preparation shortly after the creation of BcL25, we cannot rule out that a sequencing error occurred, or more likely that a mutation occurred after the transformation of the plasmid into strain 14579. To examine whether the lack of σ5816 protein increase in IPTG-treated BcL25 was due to a problem with the expression plasmid itself, the whole-cell lysate of IPTG-treated *E. coli* XL1-Blue with the p5-σ5816 plasmid was analyzed. Via Western blot, no band of approximately the right size was seen (data not shown). This may suggest that the mRNA expressed from $P_{hyperspank}$ induction is not being translated. Importantly, the start codon of the σ5816 gene is UUG rather than AUG, and this may contribute to inefficient σ5816 translation in *E. coli* XL1-Blue. Despite the absence of a band showing σ5816 in *E. coli* XL1-Blue, we believe it is unlikely that the σ5816 mRNA driven by $P_{hyperspank}$ is not being translated in *B. cereus,* as induction specifically and substantially increases the mRNA of the proteolytic subunit of a Clp protease (Fig. 15A, Section 4.4, ATP-dependent Clp protease may be involved in σ5816 regulation).

Second, it may be that proteolytic machinery in strain 14579 prevents the over-expression of σ5816. While no dramatic differences in σ5816 protein levels are apparent between WT and either untreated or IPTG-treated BcL25, it is unclear whether there may still be differences in σ5816 activity as it is not known how σ5816 is regulated. Numerous alternative σ factors have been described whose activities are not dependent

on protein levels but rather dependent on post-translational modifications and/or interactions with other mediators. Many ECF σ factors such as *E. coli* σ^{FecI} and σ^{E} are repressed by negative regulators, including anti-σ factors (Brutsche and Braun, 1997). IPTG-treated BcL25 could potentially have greater σ5816 activity, at least temporarily, than either untreated BcL25 or WT if the inhibitory systems have not repressed newly translated σ5816 protein. Therefore, even though σ5816 protein turnover may limit overall σ5816 protein levels, IPTG-treated BcL25 could have a greater fraction of active σ5816 at least temporarily, and this may be sufficient to cause changes in gene expression. As mentioned above, IPTG-treatment of BcL25 specifically and substantially increases the mRNA levels for the proteolytic subunit of a Clp protease (Fig. 15A).

Third, it may be possible that the polyclonal antibodies in the rabbit anti-sera raised against His-σ5816 are cross-reacting with a different σ factor or protein of similar size to σ5816 in strain 14579. Thus, the band representing this protein may mask any differences in σ5816 protein levels between IPTG-treated and untreated BcL25, as well as WT, as analyzed by Western blot.

The levels of σ7615 in WT and either untreated or IPTG-treated BcL23 (σ7615 inserted into pCCEXP5) still have to be assayed.

4.2 σ5816 and σ7615 function has not yet been determined by searching for a phenotype

One reason BcL8 ($\Delta\sigma^{B}$, Δσ5816, Δσ7615) may not have exhibited a dramatic growth or survival defect relative to WT when both strains were treated with acid is because even though σ5816 and σ7615 regulate genes important for an acid tolerance

response (ATR), strain 14579 possesses an additional regulatory system(s) that is able to compensate for a loss of transcription of these genes by σ5816 or σ7615, and thus maintain the ATR. We subsequently tried an alternative approach and tested if the IPTG-induced over-expression of either of these σ factors in BcL23 or BcL25 respectively could allow for enhanced growth under acid stress; the genes important for the ATR in BcL23 and BcL25 could potentially be up-regulated, giving rise to a hyper-resistant phenotype. Growth curve experiments involving BcL23 and BcL25, however, show that the over-expression of these σ factors does not allow for better growth relative to WT under acidic conditions (Fig. 6A and 6B). These experiments were conducted before anti-sera specific for σ5816 and σ7615 was obtained. In light of the Western blot that was unable to demonstrate an increase in σ5816 protein levels in IPTG-treated BcL25 relative to untreated BcL25 and WT (Fig. 7), it may be the lack of a phenotype is due to unchanged levels of σ5816 protein. As previously mentioned, σ7615 levels have yet to be assayed in WT and either untreated or IPTG-treated BcL23. In the case that σ7615 is indeed over-expressed in BcL23, it may be that under the acidic conditions tested, WT amply expresses ATR genes such that further up-regulation of these genes through σ7615 over-expression is not beneficial.

Prior to these experiments, the growth of BcL23 and BcL25 in both the presence and absence of IPTG was, importantly, found to be indistinguishable from WT (data not shown). These data suggest that the potential over-expression of σ7615 and σ5816 is not adversely affecting growth of BcL23 and BcL25 respectively.

We next looked for a growth-defective phenotype in BcL8 ($\Delta\sigma^B$, Δσ5816, Δσ7615) in the presence of various agents that cause cell envelope stress. The rationale

for this series of experiments comes from the fact that even though σ5816 and σ7615 are up-regulated under acidic conditions, perhaps they are instead responding directly to a secondary effect of low pH. At pH levels of below 5, strain 14579 cells begin to lyse; therefore, perhaps σ5816 and σ7615 are induced to cope with the increasing amount of cell envelope stress that arises as the pH approaches 5.

We looked for growth defects in BcL8 relative to WT when both strains were challenged with antibiotic, bile salt, and osmotic stresses. The two antibiotics tested, bacitracin and vancomycin, elicit cell envelope stress by affecting the integrity of the bacterial cell wall. Bacitracin inhibits the activity of C55-isoprenyl pyrophosphate, an important lipid carrier for peptidoglycan building blocks (Stone, 1971), while vancomycin prevents the incorporation of N-acetylglucosamine and N-acetylmuramic acid into the peptidoglycan matrix (Hammes, 1974). Studies in *B. subtilis* have previously shown that σ^M is induced by and mediates resistance to bacitracin (Cao and Helmann, 2002). Additionally, vancomycin was previously shown to induce *B. subtilis* σ^W (Cao *et al.*, 2002). These σ factors have since been shown to be important in maintaining cell envelope homeostasis (Cao *et al.*, 2002). From our results, however, σ5816 and σ7615 likely do not play roles in responding to the cell envelope stress caused by bacitracin or vancomycin, as no sensitivity was found in BcL8 (Fig. 7A and 7B). Interestingly, BcL8 grew slightly better than WT at 20 μg/mL and 30 μg/mL bacitracin, as well as 0.75 μg/mL vancomycin. This is not the first report of a σ factor deletion resulting in a hyper-resistant phenotype. Deletion of σ^B in strain 14579 has been previously shown to cause hydrogen peroxide hyper-resistance (van Schaik *et al.*, 2005).

Bile salts act as a detergent and are able to impart cell-envelope stress by causing a disaggregation of the cell membrane bilayer (Kristofferson, 2007). The bile salt stress response in strain 14579 has been examined previously (Kristofferson, 2007); however, the regulation of this response has yet to been studied. From our data, we were unable to see any difference in growth between BcL8 and WT in the presence of bile salts, suggesting σ5816 and σ7615 do not play roles in alleviating stresses caused by bile salt (Fig. 9A). A relatively high concentration of NaCl is also able to adversely affect the cell envelope via osmotic shock. Like the previously tested stimuli, NaCl-mediated salt stress did not reveal a growth-defect phenotype in BcL8 relative to WT (Fig. 9B).

In conclusion, no phenotype associated with either the loss or gain of σ5816 and σ7615 function was found, at least under the conditions tested. We believe the lack of a phenotype is most likely due to *B. cereus* encoding other ECF σ factors that have functional redundancy for either or both of σ5816 and σ7615. The potential existence of the regulatory overlap and functional redundancy in a set of ECF σ factors has been described in other bacteria. For example, *B. subtilis* encodes seven ECF σ factors, yet a quadruple mutant (Δ*sigV*, Δ*sigY*, Δ*sigZ*, Δ*ylaC*) was indistinguishable from WT when screening for defects in motility, multicellular differentiation, and sensitivity to more than 200 chemicals (Mascher *et al.*, 2007). Additionally, only a triple mutant missing *sigM*, *sigW*, and *sigX*, but not mutants missing any one or two of these σ factors, showed a growth and developmental phenotype. In the same triple mutant, antibiotic sensitivity was greatest relative to mutants lacking only one or two of these σ factors in various combinations (Mascher *et al.*, 2007). Due to the strong possibility that functional redundancy exists among the 10 ECF σ factors encoded in strain 14579, approaches other

than trying to identify a phenotype may be more productive to determine σ5816 and σ7615 function.

4.3 Transcriptome analysis using the SOLiD system

To the best of our knowledge, this study is the first to use NGS to analyze transcriptomes in *B. cereus*. Traditionally, microarrays have been largely used for studying genome-wide transcription (Wang *et al.*, 2009). However, microarray technology requires that fluorescently-labeled cDNAs hybridize to probes, and this has fundamental disadvantages. First, microarrays can be subject to a high level of background noise, as cDNAs can cross-hybridize with different probes representing different genes. Second, microarrays have a limited dynamic range in signal detection that results from i) the high level of background noise previously described, and ii) saturation of fluorescent signal on the microarray (Okoniewski and Miller, 2006). The sequence-based approach of NGS technologies overcomes these disadvantages: the discrimination between closely related cDNAs results in low background noise, and because expression is based directly upon the number of sequenced reads that represent a gene transcript, a high dynamic range is achievable (Wang *et al.*, 2009). Consequently, NGS is increasingly being recognized as superior to microarrays for studying gene expression (Metzker, 2010). Of the different NGS technologies used in transcriptomics, the SOLiD system in particular is gaining rapid popularity (Metzker, 2010). First, relative to other NGS technologies, SOLiD allows for larger experiments involving many samples, due to its large multiplexing capability (Metzker, 2010). Second, SOLiD sequences with high accuracy, as it makes use of two-base-encoded probes that

eventually query each base in a fragment twice (Metzker, 2010). This also allows for improved detection of single-nucleotide polymorphisms (Metzker, 2010).

One of the most important considerations when using NGS for transcriptomics is the number of high-quality reads able to be mapped. A greater number of reads results in higher confidence when analyzing differences in gene expression. In our study, a large number of reads were called: each condition that was sequenced yielded over 20 million reads that were mapped to the strain 14579 reference genome using the alignment software Bowtie. A second important consideration is the availability of replicate data. Due to the costs associated with NGS, researchers often undertake experiments with unreplicated samples (Anders and Huber, 2010). This poses a problem when trying to compare gene expression between two samples, as the count data cannot be statistically modeled to account for biological, experimental, and technical variation (Anders and Huber, 2010). Although unreplicated data cannot be used to i) observe marginal differences in gene expression and ii) measure differences in gene expression with high accuracy, general trends regarding genome-wide expression can be identified (Anders and Huber, 2010). In this study, data from unreplicated samples are used only to identify extremely differentially expressed genes, and then qRT-PCR is performed to validate the results.

4.3.1 Analysis of BcL1 does not reveal candidates for the σ5816 regulon

When WT is treated with acid, numerous genes were found to be up-regulated that have been previously described as part of the ATR or general stress response. The most highly up-regulated gene under acid stress was found to be long-chain fatty acid

CoA ligase (195 fold increase, Fig. 9B). In bacteria well-adapted to acidic environments such as *Streptococcus mutans*, the ATR changes the cell membrane composition to include a higher level of longer-chain fatty acids (Cotter and Hill, 2003). A previous study that used microarrays to analyze the ATR in strain 14579 found that long-chain fatty acid CoA ligase and other enzymes involved in lipid synthesis and metabolism were highly up-regulated (van Melis *et al.*, 2011). Additionally, a number of other genes were found to be up-regulated under acid stress, and these genes have also been described as part of the ATR in *B. subtilis* (Wilks *et al.*, 2009). Examples include *lrgA* and *lrgB* (encoding antiholin-like protein A and B, respectively), *alsS* and *alsD* (encoding α-acetolactate synthase and acetolactate decarboxylase, respectively), and *yflT* (encoding general stress protein 17M) (Fig. 7B).

We had hoped that the transcriptome analysis of acid-treated BcL1 (Δσ5816) would identify genes that were not as highly expressed relative to acid-treated WT (greater than tenfold difference). Unfortunately, no obvious candidates were identified (Fig. 10B). One gene, *BC0917*, was found to be expressed at a level tenfold lower in BcL1 relative to WT; however, this gene is hypothetical and has yet to be annotated. As previously mentioned during the search for a phenotype in BcL8 ($\Delta\sigma^B$, Δσ5816, Δσ7615), it is possible that the functional redundancy between some of the ECF σ factors in strain 14579 allows for normal or near normal transcription of genes in the σ5816 regulon despite a σ5816 deletion in BcL1. It may still be that selected genes, which are part of the σ5816 regulon, had only a slightly lower expression (less than threefold difference) in acid-treated BcL1 relative to WT; however, these genes could not have been readily identified.

A discrepancy that arose from the RNA sequencing data must be noted. Unexplainably, a number of reads mapped to the σ5816 gene (*BC2794*) in the BcL1 mutant using Bowtie. A second program called the Burrows-Wheeler Aligner (BWA) was tried also. This program is able to better map reads corresponding to insertions and deletions; however, BWA also yielded reads that mapped to this gene. This result suggests that BcL1 does not contain a deletion of the σ5816 gene. However, numerous PCR experiments have been performed that contradict this explanation. Using aliquots of the same BcL1 and WT RNA preparations that were sent for sequencing, qRT-PCR did not show amplification of σ5816 mRNA in BcL1 but did in WT. Additionally, gDNA was obtained from the very plates BcL1 and WT were streaked out on to initially start cultures for the RNA sequencing experiments. Using this gDNA, PCR was performed on both the mutant and WT with three different sets of primers. First, primers flanking the σ5816 gene revealed a completely truncated product of the correct size in BcL1, while the same primers yielded a band representing the full size gene in WT. Second, a primer pair located entirely within the σ5816 gene was unable to detect any gene product in BcL1, suggesting it is unlikely the gene moved elsewhere in the genome by an unanticipated recombination event. Contrastingly, WT gDNA yielded an appropriate sized band. Third, a primer pair recognizing a unique, truncated sequence (the junction where σ5816 was deleted) as well as sequence up-stream of the truncated gene yielded an appropriately sized band in BcL1. Contrastingly, WT with the unaltered σ5816 gene did not yield this band. In all, the products produced by PCR with all three primer sets are consistent with BcL1 carrying the expected deletion of the σ5816 gene. Currently, we do not have an explanation for the RNA sequencing data.

Surprisingly, transcriptome analysis revealed 22 genes that appeared to be expressed substantially higher in WT relative to BcL1 under both untreated and acid-treated conditions. Looking closer, BcL1 was seen to have only a very low number of reads that mapped to these genes. Further, all 22 of these genes are encoded on the pBClin15 plasmid present in WT. This strongly suggests that BcL1 has lost the pBClin15 plasmid present in WT, and the low number of reads that mapped to the plasmid were likely due to sequencing errors. The loss of pBClin15 in BcL1 went unnoticed until now, and an associated phenotype has yet to be seen. BcL1 grows similar to WT at the temperatures and conditions used for all experiments. The loss of pBClin15 in BcL1 was most likely an unintentional event which occurred during the creation of the chromosomal σ5816 deletion.

4.3.2 Analysis of BcL25 revealed strongly up-regulated genes relative to WT

We had hoped that over-expressing σ5816 in BcL25 would reveal genes that were more highly expressed relative to expression levels in WT, and that these genes may have defined the σ5816 regulon. Although an obvious increase in σ5816 protein levels was not seen in IPTG-treated BcL25 relative to WT (Fig. 7), transcriptome analysis did reveal numerous genes that were substantially up-regulated; this may be indicative that σ5816 activity was increased in IPTG-treated BcL25 relative to WT. Regulon candidates were chosen based also on their up-regulation in acid-treated WT relative to WT at neutral pH. Four candidates were chosen for further analysis: the proteolytic subunit of ATP-dependent Clp protease (ClpA), superoxide dismutase [Mn] (SOD), oxalate/formate antiporter (OFA), and antiholin-like protein LrgB (LrgB).

Clp proteases have been described in other bacteria. The ClpA of *E. coli* has intrinsic ATP activity and is active only in the presence of a second component, ClpB (Katayama *et al.*, 1988). ClpA was found to degrade abnormal canavanine-containing proteins, as well as casein. Another *E. coli* Clp protease, ClpXP, plays a role in sig^E activation (Heinrich and Wiegert, 2009).

SOD is a well-studied protein, and its role in alleviating oxidative stress has been known for decades (McCord and Fridovic, 1969; McCord, 1993). The enzyme catalyzes the reaction $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ where superoxide radicals and protons are converted to hydrogen peroxide and oxygen gas. SOD can help alleviate both acidic and oxidative stresses, as the two are often related: acidic environments can adversely affect metabolic pathways, resulting in the production of superoxide radicals. Expectedly, the enzyme catalase, which breaks down hydrogen peroxide into water and hydrogen gas, is up-regulated also in both BcL1 and WT at low pH.

The role of oxalate and formate in the ATR of *B. subtilis* has been previously studied. One major mechanism by which *B. subtilis* removes excess protons is through the decarboxylation of oxalate through OxdC through the reaction: oxalate + $H^+ \rightarrow$ formate + CO_2 (Tanner and Bornemann, 2000; MacLellan *et al.*, 2009b). Thus, up-regulation of OFA in the ATR of *B. cereus* presumably helps to remove an excess of cellular formate. Interestingly, oxalate decarboxylase itself is not up-regulated in acid-treated WT or BcL1. It may be that this enzymatic reaction is not limited by oxalate decarboxylase, but rather a shortage of oxalate or an accumulation of cellular formate.

LrgB has been previously found to work in conjunction with LrgA to inhibit the expression and activity of murein hydrolases, which are extracellular proteins involved in peptidoglycan cleavage (Groicher *et al.*, 2000; Archibald *et al.*, 1993). LrgA and LrgB have been previously believed to play roles in both modulating the proton motive force (PMF) and responding to environmental stress, although it is not known exactly how (Groicher *et al.*, 2000). LrgA was found to be up-regulated to a similar extent as LrgB in both i) acid-treated WT relative to untreated WT, and ii) IPTG-treated BcL25 relative to untreated WT.

Unfortunately, we cannot say with any certainty whether the up-regulation of these four candidate genes is specifically due to σ5816 over-expression in BcL25. SOD, OFA and LrgB were all up-regulated in IPTG-induced BcL25 and acid-stressed WT according to RNA sequencing data. While qRT-PCR also showed dramatic up-regulation of these genes in BcL25 thus validating the RNA sequencing experiment, we observed that the BcL31 control strain, which has pCCEXP5 without any inserted gene, also up-regulated SOD, OFA, and LrgB when induced with 1mM IPTG. Therefore, the increased expression of σ5816 in BcL25 is unlikely the reason for SOD, OFA, and LrgB up-regulation, and thus it is not clear if these genes are part of the σ5816 regulon.

Although terminators are present upstream of $P_{hyperspank}$ in pCCEXP5, it is not known whether transcriptional terminators exist immediately downstream of the MCS. The high level of transcription initiation from $P_{hyperspank}$ may allow for some read through of RNAP past the σ5816 gene into downstream genes on the plasmid. These genes include *lacI*, the replication initiation protein pTA1060, and chloramphenicol acetyltransferase (Fig. 4C). However, it is not clear how increased expression of any of

these plasmid genes would up-regulate SOD, OFA, and LrgB. Another possibility is that the high level of RNAP recruitment to $P_{hyperspank}$ is outcompeting recruitment to chromosomal promoters, and insufficient levels of transcription initiation from these chromosomal promoters are causing some cellular stress. Again, how exactly this would specifically up-regulate SOD, OFA, and LrgB is not known. It is important to note, however, that full induction of $P_{hyperspank}$ in pCCEXP5 (BcL31) does not cause a growth defect relative to WT.

4.4 ATP-dependent Clp protease may be involved in σ5816 regulation

ClpA is specifically up-regulated in IPTG-induced BcL25 and acid-treated WT, while its expression is unaffected in IPTG-induced BcL31. The *BC2793* gene encoding ClpA is 22 bases downstream of *BC2794*, which encodes σ5816. Both genes are on the reverse strand and potentially form an operon. As previously mentioned, an ECF σ factor often autoregulates transcription of its own gene. Therefore, it is possible that in IPTG-induced BcL25, the over-expression of plasmid-encoded σ5816 driven by the $P_{hyperspank}$ promoter is increasing the transcription of this operon located on the chromosome. Although Western blots did not reveal greater protein levels of σ5816 in IPTG-treated BcL25 relative to untreated BcL25, the substantial increase of ClpA mRNA expression in IPTG-treated BcL25 relative to untreated BcL25 and WT argues that at least some of the σ5816 mRNA from $P_{hyperspank}$ induction is being translated and the σ factor is active.

Many of the previously studied ECF σ factors are co-transcribed with an anti-σ factor which sequesters and thus inhibits the σ factor often at the membrane. In the presence of an appropriate stress, the anti-σ factor is degraded through regulated

intramembrane proteolysis (RIP), a step-wise process whereby a series of proteases completely degrade the anti-σ factor (Heinrich and Wiegert, 2009). The well-studied *E. coli* ECF σ factor σ^E, for example, is regulated by this process, and its anti-σ factor RseA is degraded by DegS, RseP, and an ATP-dependent Clp protease ClpXP. It is unclear how σ5816 activity is regulated, as no putative anti-σ factor is found adjacent to this gene. The role of ClpA as part of σ5816 regulation is unclear. ClpA could be involved in the positive-regulation of σ5816 if it plays a role in the RIP of σ5816-specific anti-σ factor encoded elsewhere in the genome. Alternatively, ClpA could directly activate σ5816 through a proteolytic processing event; *B. subtilis* σ^E or σ^K, which are involved in the sporulation pathway, are regulated in this fashion (Carlson *et al.*, 1996). However, ClpA could be responsible for the negative regulation of σ5816 by degrading the σ factor in the absence of an appropriate stimulus. If ClpA is involved in σ5816 degradation, its up-regulation in IPTG-treated BcL25 may explain why the σ5816 protein level of this same condition is not substantially greater relative to untreated BcL25 or WT, as shown by Western blot (Fig. 7). Elucidating σ5816 regulation would be of future interest.

4.5 Conclusions and Future work regarding ECF σ5816 and σ7615

Although previous experiments identified acid stress as a condition which induces σ5816 and σ7615 expression, we have been unable to show that these ECF σ factors are needed for the ATR of *B. cereus*. We were unable also to find evidence that these σ factors are responding to various aspects of cell envelope stress rather than acid stress. While the RNA sequencing experiments in this study provided the most in-depth transcriptome analysis in strain 14579 to date, a core set of genes representing a part of

the σ5816 regulon was unable to be identified. However, the proteolytic subunit of an ATP-dependent Clp protease was found to be selectively up-regulated in IPTG-induced BcL25 and acid-stressed WT. ClpA may be one potential regulator of σ5816 and may be co-transcribed in an operon with σ5816 itself. Tools that may be useful in further study of these σ factors have now been developed, including an expression system and σ-specific antibodies. We believe the most promising strategy for elucidating the functions of σ5816 and σ7615 is to still determine the regulon of these σ factors. Additional strategies can be used aside from whole-transcriptome sequencing.

4.5.1 Promoter determination

A more complicated approach to determine the regulon of an ECF σ factor exploits the fact that these σ factors often auto-regulate their expression. An upstream sequence is analyzed to find the promoter sequence which is recognized by the ECF σ factor gene of interest. For example, by inserting a reporter gene under the control of the upstream sequence of the σ5816 gene, one can use site-directed mutagenesis to find which bases dramatically affect reporter gene expression. In this painstaking approach, a putative promoter sequence can ultimately be determined. Once this promoter sequence is known, genes or operons elsewhere in the genome preceded by a similar promoter can be identified. Further experiments can examine whether these genes are indeed part of the ECF σ factor regulon. Genes regulated by *B. subtilis* σ^X were identified in this manner (Cao and Helmann, 2004).

4.5.2 *In vitro* transcription

Another method for determining the function of a given ECF σ factor involves *in vitro* transcription. This requires highly purified σ factor protein. Genomic DNA template, core RNAP, ECF σ factor, as well as necessary reagents needed for transcription can produce σ-specific mRNA transcripts. The identities of mRNA molecules produced can then be determined via microarray analysis. This process, coined run-off transcription-microarray analysis (ROMA), has been used effectively for defining σ factor regulons in *B. subtilis* (MacLellan *et al.*, 2009a). With the emergence of affordable NGS technologies, mRNA transcripts could be analyzed via sequencing, and this may prove superior to ROMA.

4.5.3 Chromatin immunoprecipitation

As σ factors are DNA-binding proteins, chromatin immunoprecipitation (ChIP) can be used to identify target DNA sequences if σ-specific antibodies are available. Subsequently, these DNA targets can be analyzed via microarray analysis (ChIP-chip) or by sequencing (ChIP-seq) to define the σ factor regulon. ChIP-chip has been successfully used to define regulons of σ factors in *E. coli* (Rhodius and Wade, 2009). As anti-sera containing polyclonal σ5816- and σ7615-specific antibodies are now available, ChIP-based methods may prove effective in elucidating the functions of these ECF σ factors. Further purification of σ-specific polyclonal antibodies from the rabbit anti-sera may be required; one method would be to use affinity purification.

4.6 Research significance and applications

While much work and effort has been put into elucidating the functions of ECF σ factors in a variety of bacterial species, including *E. coli* and *B. subtilis*, the extent as to the range of functions this group of σ factors controls remains unknown. For example, σ factors in Gram-positive bacteria have been best studied in the model bacterium *B. subtilis*; however, out of the seven total ECF σ factors encoded, the functions of only four are known. In the case of *S. coelicolor,* elucidating the functions of all fifty ECF σ factors seems a daunting task. Yet, understanding the physiological mechanisms by which bacteria survive stressful environments has a wide range of applications. In clinical microbiology, understanding how pathogenic bacteria employ ECF σ factors to regulate virulence and virulence-associated genes may give insight on how these organisms are able to efficiently colonize their hosts.

B. cereus is an emerging pathogen whose role as the causative agent of not only food-borne illness but also non-gastrointestinal infections is becoming more apparent. *B. cereus* type strain ATCC 14579 serves as a safe model relative to more virulent strains in which we can elucidate the functions of ECF σ factors, many of which may play a role in pathogenesis. Moreover, these ECF σ factors may have orthologs with conserved functions in other members of the *Bacillus cereus* group of bacteria. Thus, the ultimate goal of understanding fundamental biological processes involving ECF σ factors in *B. cereus* may have relevance to other species, including the highly virulent *B. anthracis*. Increasing our knowledge of fundamental bacterial biology is the first step in developing treatments for infections as well as for exploiting bacterial biology for a host of applications in the biotechnology sector.

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