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Alternative sigma factors F, E, G, and K in *Clostridium botulinum* sporulation and stress response

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ACADEMIC DISSERTATION

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Abstract

Clostridium botulinum presents a risk to food safety through the production of endospores. These spores are highly heat-resistant and may withstand temperatures used in food processing. Despite this, the process of spore formation is poorly understood in *C. botulinum*. This study aimed to analyse in Group I *C. botulinum* ATCC 3502 the role of sigma (σ) factors σ^F, σ^E, σ^G, and σ^K. The role of these σ factors is well known in other spore formers, activating in an ordered cascade to regulate gene transcription during sporulation. To study gene expression during sporulation in *C. botulinum* ATCC 3502, we identified a suitable normalisation reference gene for reverse-transcription real-time PCR (RT-qPCR). Mutants of *sigF*, *sigE*, *sigG*, and *sigK* were examined on the transcriptional level during sporulation, and each strain was characterised for growth and spore formation. Furthermore, the role of σ^K in stress tolerance was investigated under cold, NaCl, and pH stresses.

Transcriptional analysis, from exponential to stationary phases of growth, of eight candidate reference genes was performed. The candidate genes were 16S ribosomal RNA (*rrn*), the ATP metabolism enzymes adenosine kinase (*adK*) and glutamate dehydrogenase (*gluD*), the DNA-binding protein gyrase (*gyrA*), and ribosome-related proteins alanyl-tRNA synthetase (*alaS*), GTP-binding Era (*era*), RNA polymerase β' subunit (*rpoC*) and 30S ribosomal protein S10 (*rpsJ*). Of these candidates, only 16S *rrn* was stable during the study period. 16S *rrn* was used as the normalisation reference gene for RT-qPCR analysis of *spo0A*, *sigF*, *sigE*, *sigG*, and *sigK* expression during the same growth period. Expression of *spo0A* was highest during exponential growth, suggesting a role in early sporulation. Induction of *sigF*, *sigE*, and *sigG* expression occurred on entry into stationary growth, indicating a role in sporulation. Expression of *sigK* appeared biphasic, being expressed in both exponential and stationary phases, suggesting σ^K may play a dual role in sporulation.

The genes of σ^F, σ^E, σ^G, and σ^K were mutated using the ClosTron tool. RT-qPCR analysis of the *sigF* and *sigE* sense mutants suggested that the sporulation pathway was disrupted in the early stages. This was confirmed by electron microscopy, which showed that all *sigF* and *sigE* mutants were unable to form spores. They halted sporulation after asymmetric cell division, stage II of the seven-stage sporulation cycle. The *sigG* sense mutant showed delayed transcription of the sporulation pathway and both *sigG* mutants possessed a thin spore coat but no cortex. This indicated that σ^G may be responsible for cortex, but not coat, formation in *C. botulinum*. The *sigK* sense mutant did not express the early-sporulation genes *spo0A* and *sigF*. Both *sigK* mutants appeared to halt sporulation early. Sporulation was restored by complementing the *sigK* mutation *in trans*. These results suggested that σ^K plays an essential role in early sporulation of *C. botulinum* ATCC

3502, and adds further weight to the possibility of a dual role in sporulation overall in this strain.

Expression of *sigK* was assessed in *C. botulinum* ATCC 3502 after cold, osmotic (NaCl), and acidic shock. After cold and osmotic shock, expression of *sigK* was induced. Both sense and antisense *sigK* mutants were then grown under stress conditions of low temperature, high NaCl, and low pH. Under low temperature and high NaCl conditions, but not in low pH, growth of the mutant strains was negatively affected compared to parent strain growth, suggesting that σ^K may play a role in tolerance to low temperature and high salinity stress conditions.

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List of original publications

This thesis is based on the following publications:

I. Kirk DG, Palonen E, Korkeala H, Lindström M. Evaluation of normalization reference genes for RT-qPCR analysis of *spo0A* and four sporulation sigma factor genes in *Clostridium botulinum* Group I strain ATCC 3502. Anaerobe. 2014. 26:14-19.

II. Kirk DG, Zhang Z, Korkeala H, Lindström M. Alternative sigma factors SigF, SigE, and SigG are essential for sporulation in *C. botulinum* ATCC 3502. Appl Environ Microbiol. 2014. 80:5141-5150.

III. Kirk DG, Dahlsten E, Zhang Z, Korkeala H, Lindström M. Involvement of *Clostridium botulinum* ATCC 3502 sigma factor K in early-stage sporulation. Appl Environ Microbiol. 2012. 78:4590-4596.

IV. Dahlsten E, Kirk D, Lindström M, Korkeala H. Alternative sigma factor SigK has a role in stress tolerance of group I *Clostridium botulinum* ATCC 3502. Appl Environ Microbiol. 2013. 79:3867-3869.

The publications are referred to in the text by their roman numerals.

Abbreviations

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BoNT	Botulinum neurotoxin
bp	Base pair
Ca^{2+}	Calcium ion
cDNA	Complementary DNA
CO_2	Carbon dioxide
C_q	Quantification cycle
CV	Coefficient of variation
DMP	Protein complex of SpoIID, SpoIIM, and SpoIIP
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid
E	Primer binding efficiency
ECF	Extracytoplasmic function
EDTA	Ethylenediaminetetraacetic acid
G	G-force
GI	Gastrointestinal
GTP	Guanosine triphosphate
H_2	Hydrogen (molecular)
HA	Haemagglutinin
HCl	Hydrochloric acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobases
kDa	Kilodalton
LB	Luria Bertani
MAP	Modified atmosphere packaging
min	Minute(s)
mg	Milligram(s)
ml	Millilitre(s)
mM	Milli-Molar
µg	Microgram(s)
µl	Microlitre(s)
N_2	Nitrogen (molecular)
NaCl	Sodium chloride
NTNH	Non-toxin non-haemagglutinin
OD_{600} nm	Optical density at 600 nanometers
Pb	Lead
PCR	Polymerase chain reaction
R	Relative expression ratio
RNA	Ribonucleic acid
RNAP	RNA polymerase
mRNA	Messenger RNA
rRNA	Ribosomal RNA
RT	Reverse transcription
RT-qPCR	Quantitative reverse transcription real-time PCR
s	Second(s)
SASP	Small acid-soluble protein
TEM	Transmission electron microscopy
TPGY	Tryptone-peptone-glucose-yeast extract
U	Units
UV	Ultra-violet

1. Introduction

In the wake of the Napoleonic wars, a mysterious and fatal food-poisoning illness causing paralysis was cropping up in the German region of Württemberg. In 1817, Justinus Kerner linked these outbreaks to improperly-prepared fermented blood sausages. Kerner studied many cases between 1817 and 1822, and further hypothesised that the "sausage poisoning" or "Kerner's Disease" (as it became known in medical bulletins of the day) was caused by intoxication rather than infection. He even suggested potential therapeutic uses of the toxin due to its ability to cause paralysis.

The bacterium behind Kerner's Disease was not discovered until 1897, when Emile-Pierre Van Ermengem isolated *Bacillus botulinus* (now known as *Clostridium botulinum*), so named from the Latin *botulus*, meaning "sausage" following an outbreak in Belgium (Van Ermengem, 1897, translation 1979; Cato *et al*., 1986; Torrens, 1998). Kerner's Disease thus became known as botulism. Van Ermengem went on to identify the causative agent of botulism as the botulinum neurotoxin (BoNT), the production of which became the defining characteristic of *C. botulinum* strains (Prévot, 1953). This has resulted in *C. botulinum* representing a large, heterogeneous species divided across four metabolically distinct groups. Two of these groups pose a significant food safety risk to humans.

Prior to its designation as a *Clostridium*, *C. botulinum* was defined as a *Bacillus* due to its rod-shaped morphology. Members of the *Clostridium* genus are obligate anaerobes, which differentiate them from the obligate and facultative aerobes of the *Bacillus* genus (Cato *et al*., 1986). Despite this, the clostridia and bacilli share a common ancestry and both can form highly durable endospores (Stackebrandt and Hippe, 2001). It is the endospores of *C. botulinum* that survive heat treatment in food processing and germinate into BoNT-producing vegetative cells. Endospores were noticed as early as 1838, but were properly defined in the *Bacillus anthracis* species in 1876 by Ferdinand Cohn and Robert Koch (Asimov, 1975; Gould, 2006). Koch discovered that spore formation occurred in cycles of sporulation, germination, and multiplication in 1888. Cohn noticed that spores were resistant to heat and only germinated in fresh medium. The morphological stages of sporulation were eventually categorised into seven stages (Ryter, 1966). This became the basis of the *B. subtilis* model of sporulation and many of the clostridia follow the same morphological stages in spore formation.

In modern food processing, foods are pasteurised, treated with preservatives, packaged under modified atmospheres, and stored at low temperatures in order to prevent microbial growth. Spores of *C. botulinum* are capable of withstanding many of these treatments, particularly where pasteurisation is insufficient, leading to outbreaks of botulism. These outbreaks are usually due to improper storage of food or improper home-canning/cooking of foods.

The molecular mechanisms behind sporulation and stress response in the clostridia have recently come under investigation as new techniques in genetic manipulation have been developed (Chen *et al*., 2007a; Heap *et al*., 2007, 2010, 2012; Ng *et al*., 2013). Due to the relative ease with which *B. subtilis* is genetically manipulated (Spizizen, 1958), it has served as the model organism for understanding systems, including sporulation and stress response, in clostridia. The sporulation regulators, σ factors, are largely conserved between *C. botulinum* and *B. subtilis*; however, *C. botulinum* lacks a homologue of the major stress-response σ factor of *B. subtilis*. Since stress responses typically require a large change in gene expression, other σ factors may be involved in the *C. botulinum* stress response. Sporulation and the σ factors associated with it have not been evaluated in *C. botulinum* previously. Understanding the molecular mechanisms behind sporulation and stress response in *C. botulinum* may yield novel approaches to food safety and prevention of botulism.

2 Review of the literature

2.1 *Clostridium botulinum* and botulism

2.1.1 *Clostridium botulinum*

Clostridium botulinum belongs to the genus *Clostridium* which derives its name from the Greek *kloster* (κλωστήρ), meaning 'small spindle'. Clostridia are a group of anaerobic, Gram-positive, rod-shaped, endospore-forming bacteria that typically contain a low GC nucleotide content (Cato *et al*., 1986). Many species are capable of fermentation, solvent production, and toxin production. *C. botulinum* strains were so named for the common ability to produce botulinum neurotoxin (BoNT), the causative agent in botulism (Stackebrandt and Rainey, 1997).

C. botulinum strains are highly heterogeneous and are traditionally separated into four metabolically distinct Groups (I-IV) (Table 1). Characteristics such as lipase production, the ability to ferment carbohydrates, and proteolytic activity were commonly used to distinguish strains of a particular group. However, PCR, amplified fragment length polymorphism, pulsed fragment gel electrophoresis, and 16S ribosomal RNA (*rrn*) sequencing are more precise methods of identifying toxin types and grouping strains (Lindström *et al*., 2001; Keto-Timonen *et al*., 2005; Leclair *et al*., 2006; Hill *et al*., 2007; Dahlsten *et al*., 2008). There are seven known toxin types designated by a letter (A-G), and recently a possible eighth type (H) was discovered. Neurotoxin type varies among the groups of *C. botulinum* (Cato *et al*., 1986; Smith and Sugiyama, 1988 – cited by Peck, 2009; Hatheway, 1990). Group I and II *C. botulinum* strains produce BoNT types A, B, E, and F which cause botulism in humans. Group III *C. botulinum* strains are linked primarily to animal botulism and toxin types C and D (Eklund and Dowell, 1987 – cited by Hatheway, 1990; Lindström *et al*., 2004; Takeda *et al*., 2005; Myllykoski *et al*., 2009). Group IV *C. botulinum* is also known as *C. argentinense* and produces type G neurotoxin. Type G toxin has been experimentally associated with botulism in animals, including primates, suggesting humans may be at risk (Giménez and Ciccarelli, 1970; Ciccarelli *et al*., 1977; Suen *et al*., 1988a). Additionally, some BoNT-producing strains of *C. butyricum* and *C. baratii* have been identified and produce types E and F toxin, respectively (Hall *et al*., 1985; McCroskey *et al*., 1986). Of particular importance to the food industry are BoNT-producing strains causing human botulism, namely Group I and II *C. botulinum* strains, and Group III *C. botulinum* strains which may cause botulism in farmed animals.

Table 1 Characteristics of BoNT-producing clostridia

Strains	Toxin types[a]	Growth temperatures (°C)[b]	Proteolytic activity	Lipase production	Carbohydrate fermentation	References
Group I *C. botulinum*	A, B, F, (H)	Optimum: 37 Minimum: 10-12	+	+	+	Hatheway (1990), Peck (2009), Barash and Arnon (2014)
Group II *C. botulinum*	B, E, F	Optimum: 25 Minimum: 3	-	+	+	Schmidt *et al.* (1961), Hatheway (1990), Graham *et al.* (1997), Peck (2009)
Group III *C. botulinum*	C, D	Optimum: 40 Minimum: 15	+/-	+	+	Hatheway (1990), Peck (2009)
Group IV *C. botulinum* (*C. argentinense*)	G	Optimum: 37 Minimum: NA	+	-	-	Hatheway (1990), Peck (2009)
Other						
C. butyricum	E	Optimum: 30-37 Minimum: 12	-	-	+	Hatheway (1990), Peck (2009)
C. baratii	F	Optimum: 30-45 Minimum: 10-15	-	-	+	Hatheway (1990), Peck (2009)

[a] Toxin type H has yet to be verified as a distinct toxin type (Johnson, 2014).
[b] NA represents unknown data.

2.1.2 Botulinum neurotoxins and botulism

Botulinum neurotoxins

Botulinum neurotoxins (BoNTs), the causative agents of botulism, are produced by *C. botulinum* and some strains of *C. butyricum* and *C. baratii*. Eight types of BoNT molecule have been identified (A-H) in *C. botulinum* and dual toxin-producing strains have also been found. Following the discovery of the botulinum neurotoxin in the late 1800s by Van Ermengem, Leuchs (1910) identified the existence of different BoNT types when antibodies that were raised against one serotype failed to protect against BoNT isolated from another botulism case (Erbguth, 2004). These were designated toxin types A and B by Burke (1919). In 1922, Seddon and Bengston separately discovered two subtypes of a new type, C (Gunnison and Meyer, 1929). Type D was discovered by Meyer and Gunnison (1928). Interestingly, it has since been shown that type C and D neurotoxins exhibit enough molecular homology for antibodies raised against one to protect against the others, calling into question whether they should be regarded as different types at all (Oguma *et al*., 1980). Strains producing a mosaic of types C and D neurotoxins exist, furthering the argument of similarity between these two toxin types (Moriishi *et al*., 1996). Type E was identified by Gunnison *et al*. (1936) and type F was identified by Møller and Scheibel (Møller and Schiebel, 1960 – cited by Hatheway, 1990). The latest confirmed type, G, was discovered by Giménez and Ciccarelli (1970a). Recently, a new neurotoxin type, designated type H, was reported by Barash and Arnon (2014) existing in a dual toxin-producing strain with type B neurotoxin. Some controversy exists over the recent type H discovery as the genetic information has not been published (ostensibly for security reasons), with some critics suggesting it may not be a new type at all (Johnson, 2014). Other dual toxin-producing strains exist that include types AB, Ab, Af, Ba, and Bf, where the upper-case toxin type denotes the dominant toxin produced (Poumeyerol *et al*., 1983; Giménez, 1984; Giménez and Ciccarelli, 1970b; Barash and Arnon, 2003).

The BoNT molecule consists of a light (~50 kDa) and heavy chain (~100 kDa), and exists in a neurotoxin complex with several non-toxic molecules. The non-toxin non-haemagglutinin (NTNH) and haemagglutinins (HA) associate with BoNT. They are thought to play a role in absorption and protection of the toxin complex (Sugii and Sakaguchi, 1975; Matsumura *et al*., 2008; Gu *et al*., 2012). Not all *C. botulinum* strains possess HA proteins, however. Some type A- and E-producing strains lack obvious HA genes. Instead, *orfx* genes may be present in the toxin gene cluster, although the functions of their products are unknown at present (Chen *et al*., 2007b; Jacobson *et al*., 2008). The genes encoding the BoNT complex typically exist close together in the genome and gene transcription is primarily regulated by BotR. BotR is an alternative sigma (σ) factor that

acts with RNA polymerase (RNAP) as a positive regulator of genes in the neurotoxin cluster of many *C. botulinum* strains (Marvaud *et al*., 1998; Dupuy and Matamouros, 2005). Recently, a two-component system located 11 kb from the neurotoxin cluster was identified as a negative regulator of the neurotoxin complex in a type A strain of *C. botulinum* (Zhang *et al*., 2013). It acts by binding to the promoter regions of *ha* and *ntnh*/*bont* genes, thus preventing BotR from binding with RNAP, thereby inhibiting gene transcription. This two-component system is the only negative regulator of the neurotoxin cluster identified in a *C. botulinum* strain to date.

Botulism

Botulism is a rare paralytic disease that is caused by blockage of acetylcholine transmission at the neuromuscular junctions by the botulinum neurotoxin in mammals and birds. In severe cases, paralysis of the respiratory muscles may occur. Mechanical ventilation is required until new nerve endings grow, allowing normal muscular function (Peck, 2009). Several types of botulism exist. These are foodborne (classical) botulism, in which the toxin is ingested; infant or intestinal botulism, where the gastrointestinal (GI) tract is colonised by toxin-producing cells; wound botulism, where a deep cut is colonised by toxin-producing cells (Hall, 1945; Merson and Dowell, 1973); iatrogenic botulism, where therapeutic BoNT-based treatments cause systemic disease (Chertow *et al*., 2006); and inhalation botulism, where disease is caused by absorption of the neurotoxin through the lungs (Park and Simpson, 2003). The latter form of botulism has been posited as a potential bioterrorist threat though only one incident in humans has been reported (Holzer, 1962; Arnon *et al*., 2001). Currently foodborne, infant, and wound botulism are the more common varieties of the disease.

Foodborne botulism is the most widely recognised form of botulism as the disease was first identified as a food poisoning illness. Many cases are associated with improper home-preparation of foods. Group I-associated botulism cases are typically linked to bottled goods and canned (often home-canned) foods (Peck, 2006). Group II *C. botulinum* strains particularly pose a threat in minimally heated, chilled foods and are most often related to smoked, dried, or fermented meat and fish products (Korkeala *et al*., 1998; Peck and Stringer, 2005; Lindström *et al*., 2006; Peck, 2006). The toxin complex is heat labile and sufficient heating (80 °C) will destroy it during cooking (Wright, 1955). Improved food preparation and storage, coupled with strategies to prevent microbial growth, has resulted in a reduction in the incidence of foodborne botulism.

Infant botulism is currently the most prevalent reported form of botulism. It occurs when an infant, typically less than a year old, ingests spores of a neurotoxin-producing *Clostridium* (Koepke *et al*., 2008). Spores of *C. botulinum* are ubiquitous in the environment, and both honey and dust have been identified as sources of spores (Arnon *et al*., 1979; Nevas *et al*., 2005; Derman *et al*., 2014). Recently, the keeping of aquatic

reptiles, specifically terrapins, has also been linked to cases of infant botulism, suggesting their artificial habitats may be another source of spores (Grant *et al*., 2013). In infants under a year old, the bacterial flora of the GI tract is less diverse than that of an adult and is thus more susceptible to colonisation by *C. botulinum* (Mackie *et al*., 1999). In healthy adults, spores pose little threat as they do not get the opportunity to germinate and outgrow into toxin-producing cells among the microbiota of the GI tract (Wilcke *et al*., 1980). If the intestinal microbiota is severely compromised and *C. botulinum* infects the GI tract, botulism can manifest similar to infant botulism. In adults, this is known as intestinal botulism (McCroskey and Hatheway, 1988). In addition to *C. botulinum*, *C. butyricum* and *C. baratii* have both been associated with infant botulism (Aureli *et al*., 1986; Suen *et al*., 1988b).

Wound botulism is a rare form of botulism whereby a deep cut becomes infected by spores. These may germinate and outgrow into toxin-producing cells under the anaerobic conditions generated in a healing wound. In recent years, incidence of wound botulism has become more frequent with a rise in intravenous drug abuse using contaminated syringes (Passaro *et al*., 1998; Brett *et al*., 2004).

2.2 Sigma factors

2.2.1 Sigma (σ) factors and regulation

σ factors

Growth and development characteristics, such as toxin production and sporulation, are mediated by changes in gene transcription. Gene transcription is performed by the RNA polymerase (RNAP) holoenzyme, the core of which is made up of five subunits (two α subunits, β, β', and ω). A sixth, dissociable subunit known as the σ subunit directs gene transcription. The σ subunit recognises gene promoter sequences (-10 and -35 positions upstream of the transcription initiation site) and is involved in DNA melting, allowing RNAP to initiate transcription. Upon elongation of the gene transcript, the σ subunit is released and, upon termination of transcription, the RNAP core is free to take up another σ factor (Mooney *et al*., 2005; Raffaelle *et al*., 2005). This results in a cyclical relationship between the RNAP core and available σ factors (Paget and Helmann, 2003; Österberg *et al*., 2011). There are two major families of σ factors present in Gram-positive bacteria, the σ^{54} and σ^{70} families. The σ^{54} family members are typically involved in nitrogen metabolism. These also require ATP and additional proteins to assist in DNA melting (Sonenshein *et al*., 2005). In contrast, the σ^{70} family σ factors have large regulons and are

capable of melting DNA without assistance at the promoter region (Lonetto *et al*., 1992; Paget and Helmann, 2003; Helmann, 2009).

The σ^{70} family consists of primary and alternative σ factors divided into five groups (I-V) based on the function of the σ factor and its protein structure (Helmann, 2002). All σ factors have conserved DNA-binding and promoter-recognition sites. The general protein structure of a σ^{70} factor consists of four regions (1-4). Regions 1 and 3 are structural regions involved in σ factor auto-inhibition of DNA binding and structural linkage, respectively. Regions 2 and 4 are the most conserved regions within σ factors. Region 2 contains the RNAP core-binding domain (known as region 2.1) and the DNA-binding domain (2.3). Regions 2.4 and 4.2 recognise the -10 and -35 promoter sites, respectively. Variations within regions 2.4 and 4.2 differentiate the σ factors (Paget and Helmann, 2003; Österberg *et al*., 2011).

Not all of regions 1-4 are present in each σ factor, but they play a role in categorising σ factors into one of the five σ factors groups (I-V). Group I σ factors are known as primary σ factors and are essential for growth. All four regions are present in group I σ factors. Groups II-V contain alternative σ factors that coordinate areas of metabolism, developmental changes, and stress responses, but are not essential for growth. Group II σ factors are closely related to those of group I. They consist of four regions and are involved in bacterial growth and stress response. Group III σ factors are involved in developmental changes, such as sporulation and certain stress responses (e.g. heat shock). The group III σ factors possess regions similar to regions 2, 3 and 4 of the group I σ factors. Group IV σ factors are also known as extracytoplasmic function (ECF) σ factors. Only regions 2 and 4 are conserved in these σ factors. Members of this group are associated with metabolic activity, responding to extracytoplasmic signals. Group V σ factors closely resemble those of group IV; however, they are unique to clostridia and are involved in toxin gene regulation (Helmann, 2002).

Regulation of σ factors

The ability of σ factors to fundamentally alter the transcriptional dynamic in the cell, and thus cell development, requires tight regulation. The amount of free RNAP in the cell regulates σ factor activity via competition (Helmann, 2011; Österberg *et al*., 2011). Competitive binding for core RNAP by alternative σ factors is influenced by the free concentration of the σ factor in question, as well as the affinity of that σ factor to the core complex compared to that of the primary σ factor, which is almost always present (Maeda *et al*., 2000). On a transcriptional level, σ factors may be temporally regulated under the influence of a transcription factor. This is the case for sporulation, where a cascade of σ factors is triggered upon nutrient limitation at the end of exponential growth. The activity of some σ factors can be further regulated post-translation. In a process known as "partner switching", σ factors may be bound and inactivated by antagonistic proteins called anti-σ

factors. The anti-σ factor/σ factor complex may then recognise an anti-anti-σ factor under certain phosphorylation-dependent conditions. The anti-anti-σ factor binds the anti-σ factor (effectively switching partners) thus releasing and activating the σ factor. Such an arrangement is seen in *B. subtilis* σ^B and σ^F during stress response and sporulation, respectively (Sonenshein *et al*., 2005; Österberg *et al*., 2011). Another feature of some σ factors is an N-terminal pro-sequence which must be cleaved prior to activation of the σ factor. Sporulation σ factors σ^E and σ^K in *B. subtilis* possess an N-terminal pro-sequence that regulates their activity within the sporulation pathway (Hilbert and Piggot, 2004).

The number of alternative σ factors encoded in the genome appears to reflect the lifestyle of a species. This is likely due to the diversity of environments bacteria must adapt to on a transcriptional level in order to survive. For example, the gut bacterium, *Escherichia coli*, has seven alternative σ factors, while *Streptomyces coelicolor*, a soil bacterium, contains more than 60. Bacteria such as bacilli and clostridia, which can live in environments such as soil, water, and the gut, have more than 10 alternative σ factors, varying between species (Haldenwang, 1995; Helmann, 2003; Sonenshein *et al*., 2005; Österberg *et al*., 2011).

2.2.2 The σ^{70} family in bacilli and clostridia

The σ^{70} family of σ factors has been extensively studied in *B. subtilis*, which is the model for Gram-positive spore formers (Haldenwang, 1995). In *B. subtilis*, group I, III and IV σ factors have been identified. A single group I (primary) σ factor, nine group III σ factors, and seven group IV σ factors are encoded in *B. subtilis* (Haldenwang, 1995, Luo and Helmann, 2010). These σ factors typically have large regulons and can profoundly change the transcriptional dynamic of the cell (Helmann, 2009). In contrast, the variety of group III and IV σ factors appears to differ greatly in the major clostridia: *C. acetobutylicum*, *C. botulinum*, *C. difficile*, *C. perfringens* and *C. tetani* (Table 2). In this context, the major clostridia are clostridia of medical/industrial importance and of which genome annotations are currently further advanced than other clostridial species.

Conserved between the major clostridia and *B. subtilis* are the primary σ factor, σ^A, and the alternative σ factors, σ^H, σ^F, σ^E, σ^G and σ^K (Sauer *et al*., 1995). These σ factors are highly conserved in the promoter recognition regions 2.4 and 4.2 and are arranged in the genome similar to *B. subtilis*. As such, they are thought to play similar developmental roles in clostridia. The flagellation and motility regulator, σ^D, is also conserved in many clostridia, but is not present in *C. perfringens* and *C. tetani*. A notable difference between the major clostridia and *B. subtilis* is the lack of obvious stress response σ factors in many species of clostridia. The general stress-response σ factor of *B. subtilis*, σ^B, has been identified only in *C. difficile* while the heat-shock response σ factor, σ^I, is present in *C. acetobutylicum* (Boylan *et al*., 1993; Paredes *et al*., 2005; Tseng *et al*., 2011).

The group IV ECF σ factors of *B. subtilis* are involved in antibiotic resistance, production of sublancin (an antimicrobial glycopeptide), and lysozyme resistance (Luo and Helmann, 2009; Luo *et al*., 2010; Oman *et al*., 2011; Hastie *et al*., 2013). Largely, the group IV σ factors are not conserved in the major clostridia. However, clostridia possess the unique group V ECF-like σ factors involved predominantly in toxin regulation (Helmann, 2002). This group consists of toxin regulators such as TcdR (*C. difficile*) and UviA (*C. perfringens*), TetR (*C. tetani*) and BotR (*C. botulinum*) (Moncrief *et al*., 1997; Marvaud *et al*., 1998a, 1998b; Mani and Dupuy, 2001; Raffestin *et al*., 2005; Dupuy and Matamouros, 2006; Dupuy *et al*., 2006). Regions 2 and 4 are highly similar in these σ factors, to the extent that swapping these regions from one σ factor with another (i.e. BotR with TetR or UviA with TcdR) results in functional transcription of the target toxin genes *in vitro* (Dupuy *et al*., 2006). These σ factors do not appear to have counterparts in *B. subtilis* which suggests that the σ factors of the clostridia have diverged in function from those studied in the *B. subtilis* model.

Table 2 σ^{70} family σ factors of *Bacillus subtilis* and the major clostridia

σ factor	Function[a]	Bacteria[b]	Notable regulation[c]	References
Group I				
σ^{A}	Regulation of essential genes	*B. subtilis* and all clostridia	Constitutively expressed	Haldenwang (1995), Nölling *et al*. (2001), Brüggemann *et al*. (2003), Myers *et al*., (2006), Sebaihia *et al*. (2006, 2007), Helmann (2011)
Group III				
σ^{B}	General stress	*B. subtilis* and *C. difficile*	Anti-σ factor; partner switching	Haldenwang (1995, 2011), Sebaihia *et al*. (2006), Helmann (2011)
σ^{C}	Post-exponential growth (suggested)	*B. subtilis*	Unknown	Johnson *et al*. (1983), Haldenwang (1995)
σ^{D}	Flagellar motility, chemotaxis	*B. subtilis*, *C. acetobutylicum*, *C. difficile*, and *C. botulinum*	Anti-σ factor	Haldenwang (1995), Nölling *et al*. (2001), Sebaihia *et al*. (2006, 2007), Helmann (2011)
σ^{E}	Sporulation (early)	*B. subtilis* and all clostridia	σ cascade; pro-σ^{E} processing	
σ^{F}	Sporulation (early)	*B. subtilis* and all clostridia	σ cascade; anti-σ factor; partner switching	Haldenwang (1995), Nölling *et al*. (2001), Brüggemann *et al*. (2003), Myers *et al*., (2006), Sebaihia *et al*. (2006, 2007), Helmann (2011)
σ^{G}	Sporulation (late)	*B. subtilis* and all clostridia	σ cascade; possible anti-σ factor	
σ^{H}	Sporulation	*B. subtilis* and all clostridia	Transcription repressed by AbrB	
σ^{I}	Heat shock, cell envelope	*B. subtilis* and *C. acetobutylicum*	Unknown	Haldenwang (1995), Nölling *et al*. (2001), Helmann (2011)

Table 2 Continued

σ factor	Function[a]	Bacteria[b]	Notable regulation[c]	References
σ^K	Sporulation (late)	*B. subtilis* and all clostridia	σ cascade; pro-σ^K processing; *skin* element[d]	Haldenwang (1995), Nölling *et al.* (2001), Brüggemann *et al.* (2003), Myers *et al.*, (2006), Sebaihia *et al.* (2006, 2007), Helmann (2011)
Group IV				
σ^M	Cell wall biosynthesis, stress resistance	*B. subtilis*	Anti-σ factor	Haldenwang (1995), Luo *et al.* (2009, 2010), Helmann (2011)
σ^V	Lysozyme resistance	*B. subtilis*, *C. tetani*, *C. perfringens*, *C. difficile*, and *C. botulinum*	Anti-σ factor	Haldenwang (1995), Brüggemann *et al.* (2003), Myers *et al.*, (2006), Sebaihia *et al.* (2006, 2007), Hastie *et al.*, (2013)
σ^W	Antibiotic resistance	*B. subtilis*, *C. acetobutylicum*, *C. perfringens*, and *C. difficile*	Anti-σ factor	Haldenwang (1995), Nölling *et al.* (2001), Myers *et al.*, (2006), Sebaihia *et al.* (2006), Luo *et al.* (2009, 2010), Helmann (2011)
σ^X	Antimicrobial peptide resistance	*B. subtilis* and putatively identified in *C. acetobutylicum*	Anti-σ factor	Haldenwang (1995), Nölling *et al.* (2001), Luo *et al.* (2009, 2010), Helmann (2011)
σ^Y	Sublancin production	*B. subtilis*	Anti-σ factor	Haldenwang (1995), Helmann (2011), Mendez *et al.* (2012)
σ^Z	Unknown	*B. subtilis*	Anti-σ factor	
σ^{YlaC}	Unknown	*B. subtilis* and *C. tetani*	Anti-σ factor	Haldenwang (1995), Brüggemann *et al.* (2003), Helmann (2011)

Table 2 Continued

σ factor	Function[a]	Bacteria[b]	Notable regulation[c]	References
Group V				
BotR	Neurotoxin production	*C. botulinum*	Function blocked by two-component system 786/787	Marvaud *et al.* (1998a), Sebaihia *et al.* (2007), Zhang *et al.* (2013)
TcdR	Toxin production	*C. difficile*	Anti-σ factor	Moncrief *et al.* (1997), Sebaihia *et al.* (2006)
TetR	Neurotoxin production	*C. tetani*	Unknown	Marvaud *et al.* (1998b), Brüggemann *et al.* (2003)
UviA	Bacteriocin production	*C. perfringens*	Unknown	Dupuy *et al.* (2005), Myers *et al.* (2006)

[a] As determined in *B. subtilis* for σ factor groups I-IV, and in clostridia for group V.
[b] Bacteria investigated were *B. subtilis*, *C. acetobutylicum*, *C. perfringens*, *C. tetani*, *C. difficile*, and *C. botulinum.*
[c] Post-translational regulation of σ factors as determined for *B. subtilis* for σ factor groups I-IV.
[d] The *skin* element is only found in *B. subtilis*, *C. difficile* and *C. tetani*.

2.3 Spores and sporulation

2.3.1 Spore formation and structure

A common trait of the bacilli and clostridia is the formation of resistant endospores (spores). Spores are extremely durable and can resist high temperatures, desiccation, and both UV and γ radiation. They are capable of surviving exposure to digestive enzymes and may remain dormant for long periods of time. Spores suggested to have formed some 250 million years ago were found to be viable (Vreeland *et al*., 2000; Setlow, 2011). The formation of spores has been described as a bet-hedging survival strategy. As bacteria grow and reach a point of nutrient limitation, a subpopulation enters into sporulation. Sporulation requires the bacteria to invest much of their time and energy, delaying the process of cell division and eventually reaching a point of no return. This strategy allows the sporulating subpopulation to survive a variety of stresses upon the loss of nutrients, which would otherwise result in the bacteria entering a vegetative dormant state or dying off (Veening *et al*., 2008, Reder *et al*., 2012a). In *B. subtilis*, sporulation may be synchronised with >90% of cells entering sporulation within 24 hours (Piggot and Coote, 1976). In clostridia, sporulation is asynchronous, meaning cells enter into sporulation at different times, and is triggered ostensibly by nutrient limitation (Brown *et al*., 1957; Perkins and Tsuji, 1962). Spores play a pivotal role in infectious forms of clostridial diseases such as infant botulism, pseudomembranous colitis, and tetanus. Foodborne botulism and clostridia-associated food poisoning are also caused by contamination of foods by spores of *C. botulinum* and *C. perfringens*. In cases of foodborne botulism, *C. botulinum* spores germinate into vegetative cells and produce toxin *ex vivo*, whereas *C. perfringens* vegetative cells are ingested, contaminating the GI tract, which then release enterotoxin during sporulation in the gut (Huang *et al*., 2004; Mallozzi *et al*., 2010; Lindström *et al*., 2011). Much of what is known about sporulation has been studied in the model organism, *B. subtilis*.

There are seven morphological stages of sporulation (I-VII) which are identical in bacilli and most clostridia (Ryter *et al*., 1966) (Fig. 1). Prior to sporulation, the cell is in a vegetative state often referred to as stage 0. During stage I, the DNA rearranges to form axial filaments (Hilbert and Piggot, 2004). These filaments are not always visible, thus stage I is visually indistinct from stage 0 (Waites *et al*., 1970). During stage I, sporulation is reversible if fresh nutrients are introduced to the growth medium, allowing vegetative cell division to continue (Narula *et al*., 2012). When the sporulating cell reaches stage II, it divides asymmetrically forming the larger mother cell and the smaller forespore. The forespore is engulfed by the asymmetric membrane at stage III of sporulation. At this point, sporulation becomes irreversible (Hilbert and Piggot, 2004). At stage IV, the spore cortex, a thick layer of modified peptidoglycan, forms around the forespore. Stage V is characterised by the assembly of the spore coat around the pre-formed cortex. In some species, the exosporium also develops at this point. During stage VI, the spore cortex and coat proteins mature, and the spore core loses water and takes up calcium. Finally, at stage VII, the spore is released from

the mother cell (Labbé, 2005; Henriques and Moran, 2007). Notable differences to this model in the clostridia are *C. perfringens* and *C. pasteurianum*. In the case of *C. perfringens*, stage VII also results in the release of enterotoxin. In *C. pasteurianum*, the order of stages IV and V is reversed and cortex peptidoglycan appears after spore coat formation (Mackey and Morris, 1971; Hilbert and Piggot, 2004).

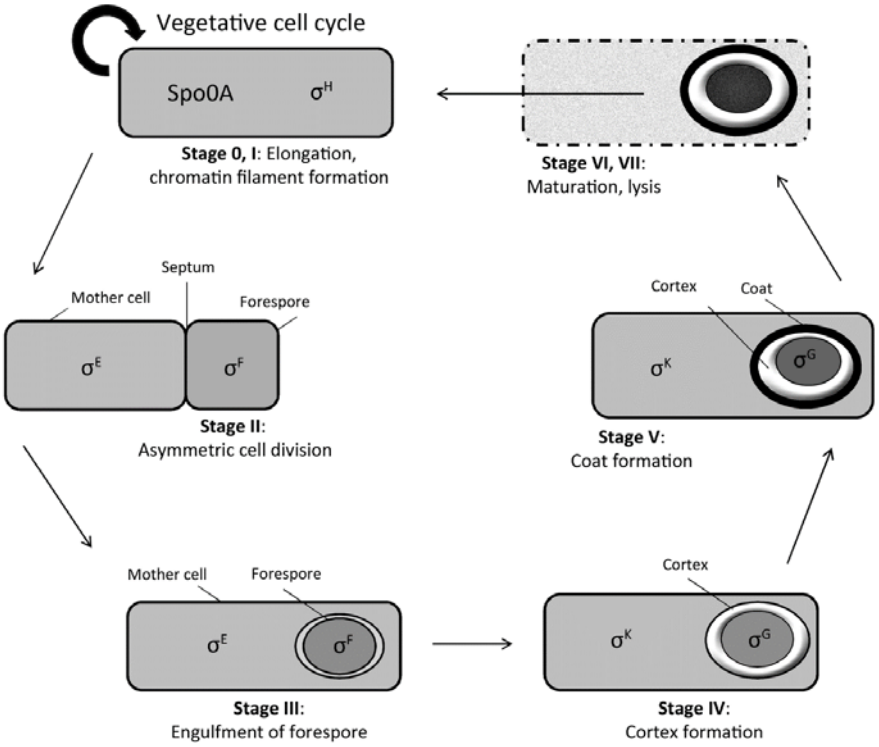


Figure 1 The morphological stages of the sporulation cycle with σ factor activity in *B. subtilis*. Adapted from Errington (2003).

The result of the sporulation process is a mature endospore (Fig. 2). The spore core becomes dehydrated during sporulation. Within the core, DNA is saturated by small acid-soluble proteins (SASPs) which form approximately 10% of the spore protein in the core. The core also contains RNA, enzymes, high levels of dipicolinic acid (DPA), and divalent metal ions, such as Ca^{2+} (Setlow, 2011). Due to the low water content, metabolic activity in the core is minimal, although RNA can be degraded and synthesised in the core for several days after the spore has formed. This has been suggested as an adaptation mechanism allowing the spore to prepare for germination in a new environment (Desser and Broda, 1968; Segev *et al*., 2012). Surrounding the core is the inner membrane, consisting of the forespore cell membrane. Germination receptors are embedded in this membrane (Korza and Setlow, 2013). The inner membrane is surrounded by a thick layer of cortex peptidoglycan which is thought to play a role in dehydration of the spore core and confers wet heat

resistance to the spore (Setlow, 2011). A backbone of muramic δ-lactam and muramic acid-L-alanine differentiates cortex peptidoglycan from cell wall peptidoglycan. Additionally, cortex peptidoglycan has a low level of cross-linking and fewer side-chains than cell wall peptidoglycan due to the muramic δ-lactam, which are thought to contribute to spore desiccation and heat resistance (Setlow and Johnson, 2007).

Surrounding the cortex is the spore coat. More than 70 proteins have been identified in the *B. subtilis* spore coat and this number may vary considerably between species. The coat is composed of two insoluble layers in which protective enzymes are embedded. These enzymes prevent lytic enzyme activity and inactivate toxic chemicals (Henriques and Moran, 2007). The spore may also be surrounded by an additional layer known as the exosporium. Exosporia are comprised of proteins, including enzymes and glycoproteins, and are thought to play a role in adherence of spores to mammalian cells and other surfaces (Panessa-Warren *et al*., 1997; Paredes-Sabja and Sarker, 2012). The exosporium is not essential to the spore for viability and not all spore-formers possess exosporia after sporulation, notably *C. perfringens* and *B. subtilis* (Labbé, 2005; Henriques and Moran, 2007).

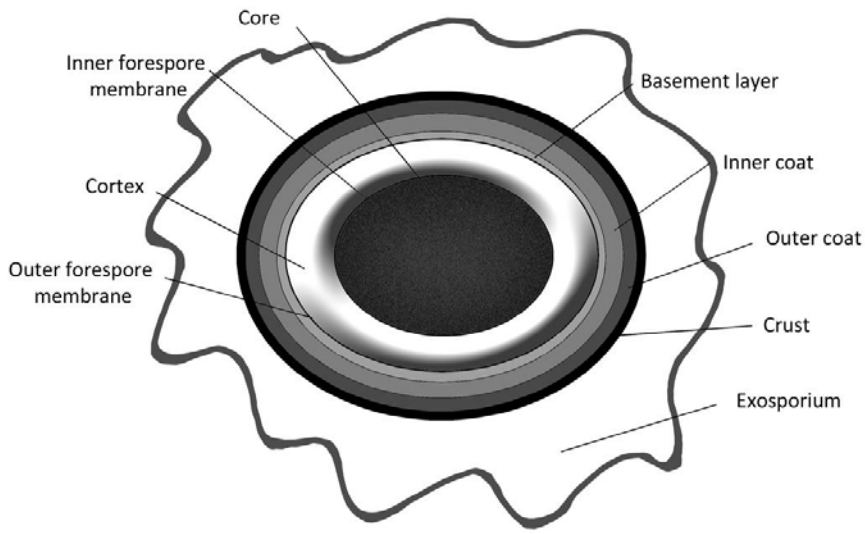


Figure 2 Mature endospore structure depicting exosporium, crust, coat layers, peptidoglycan cortex, forespore membranes and spore core. Exosporia may vary considerably in size and shape. Figure adapted from McKenney *et al*. (2013).

2.3.2 Initiation of sporulation

Sporulation on the molecular level has been studied in great detail in *B. subtilis* (Paredes *et al*., 2005). In the *B. subtilis* model, sporulation is initiated under nutrient limitation and high cell density (Sonenshein, 2000). The sporulation pathway is primarily initiated by the transcription

factor Spo0A. Spo0A contains a DNA-binding domain and a phospho-acceptor domain (Burbulys *et al*., 1991; Grimsley *et al*., 1994). Phosphorylation of Spo0A to its active form (Spo0A~P) is regulated by five orphan sensor kinases (KinA-KinE) which are embedded in the cell membrane. The kinases detect signals prompting the activation of the sporulation pathway, and then interact directly with Spo0A or indirectly via a phosphorelay system. Phosphorylation of Spo0A causes a conformational change, allowing Spo0A~P to bind DNA (Jiang *et al*., 2000).

The phosphorelay can be initiated by kinases KinA, KinB, and KinE. These phosphorylate Spo0F, which in turn phosphorylates Spo0B. Spo0B~P interacts directly with Spo0A, transferring the phosphoryl group. Phosphorylation of Spo0A can be performed directly by KinC, and KinD is thought to play an inhibitory role in Spo0A phosphorylation (Fabret *et al*., 1999; Errington, 2003; Piggot and Hilbert, 2004). Spo0A~P, in addition to positively regulating gene transcription, can act as an inhibitor. Spo0A~P inhibits transcription of *abrB*, the product of which is an inhibitor of *sigH*. σ^H can then drive transcription of *spo0A*. In this way, Spo0A~P indirectly promotes transcription of its own gene, allowing Spo0A~P to accumulate in the cell and begin the process of sporulation. In opposition to Spo0A~P is Spo0E. Spo0E dephosphorylates Spo0A~P, thereby inactivating it. This process aids in the decision making process of the cell entering into sporulation (Ohlsen *et al*., 1994; Fujita and Sadaie, 1998). Sporulation may be aborted prior to stage III by adding fresh nutrients to the culture medium (Piggot and Coote, 1976).

Initiation of sporulation in the clostridia is not well understood, as members of the clostridia appear to lack distinct genes homologous to *spo0F*, *spo0B*, and the sensor kinase genes of the *B. subtilis* phosphorelay system. Genes encoding Spo0A, σ^H and, in some strains, AbrB are conserved in clostridia (Paredes *et al.*, 2005). While a distinct phosphorelay system has not yet been elucidated, Spo0A of clostridia possess similar phosphor-acceptor domains to the Spo0A of *B. subtilis* (Wörner *et al*., 2006). Candidate orphan kinases have been found in *C. acetobutylicum* and *C. botulinum* that may phosphorylate Spo0A directly (Paredes *et al*., 2005; Wörner *et al*., 2006). These studies were limited, however, and the activity of these orphan kinases remains to be demonstrated by mutational study in clostridia. The difference between *B. subtilis* and the clostridia in terms of the Spo0A phosphorylation mechanism may account for differences in sporulation, such as synchronicity. Sporulation is asynchronous in clostridia, including *C. botulinum* and *C. difficile*, whereas the process can be synchronised in *B. subtilis* under nutrient limitation (Brown *et al*., 1957; Perkins and Tsuji, 1962; Piggot and Coote, 1976; Burns and Minton, 2011).

The trigger of sporulation amongst clostridia has been of much debate. Traditionally, spore formation is initiated under conditions of nutrient limitation (Paredes *et al*., 2005). Quorum sensing mechanisms, such as the AgrBD system, may play a role in cell density dependent triggering of sporulation in clostridia (Cooksley *et al*., 2010). Until recently, the processes of sporulation and solventogenesis were previously thought to be dependent in clostridia. Unlike in *B. subtilis*, many clostridia produce metabolites such as butyrate and acetate which accumulate during growth. During stationary-phase growth, at the same time as the initiation of sporulation, these metabolites are converted to butanol and acetone (Alsaker and Papoutsakis 2005; Jones *et al*., 2008). Under continuous culture, solventogenesis can be induced in *C. acetobutylicum* without initiating

sporulation, implying the two processes are not dependent on each other (Grimmler *et al*., 2011). While the trigger for sporulation amongst clostridia remains unknown, the molecular mechanisms regulating spore development appear to be largely similar to those of the *B. subtilis* model.

2.3.3 σ cascade

Sporulation is regulated by a cascade of four major σ factors (F, E, G, and K) in the *B. subtilis* model. Operon structures containing the genes *sigF*, *sigE*, *sigG*, and *sigK* of the σ cascade are present in all bacilli and clostridia (Paredes *et al*., 2005). Sporulation is divided into early sporulation (forespore and mother cell development) and late sporulation (spore cortex and coat formation, and spore maturity). Early sporulation is regulated predominantly by σ^F and σ^E, while σ^G and σ^K regulate late sporulation. Consequently, the expression of the σ factor genes is highly regulated during sporulation. Activation of the first σ factor aids in the activation of the next, thus forming the σ cascade which coordinates timing of gene expression during sporulation (Stragier and Losick, 1990). The σ cascade has been thoroughly studied in the *B. subtilis* model and has recently come under observation in *Clostridium* species due to improved genome sequencing and manipulation techniques (Paredes *et al*., 2005; Heap *et al*., 2007). The presence of the σ factors F, E, G, and K in clostridia suggests a σ cascade mechanism similar to that of *B. subtilis* exists and regulates sporulation in clostridia. However, recent sporulation studies in *C. acetobutylicum* (Jones *et al*., 2011; Tracy *et al*., 2011; Al-Hinai *et al*., 2014), *C. perfringens* (Harry *et al*., 2009; Li and McClane, 2010), and *C. difficile* (Fimlaid *et al*., 2013; Pereira *et al*., 2013; Saujet *et al*., 2013) suggest the regulons of these σ factors and the timing in which they are activated differ considerably from the *B. subtilis* model, particularly between the forespore and mother cell. The regulatory network of sporulation in *C. difficile* has been likened to a less advanced version of the *B. subtilis* model with less strict controls, allowing adaptation to the environment in the gut during colonisation (Saujet *et al*., 2014).

σ^F

In early sporulation of *B. subtilis*, Spo0A~P acts in conjunction with σ^H to transcribe *sigF*, the first σ factor of the σ cascade (Fujita and Sadaie, 1998; Fujita *et al*., 2005). The *sigF* gene (*spoIIAC*) is located in the *spoIIA* operon (Losick *et al*., 1986). Also encoded in this operon are the genes for the anti-anti-σ factor SpoIIAA and the anti-σ factor SpoIIAB, which are located upstream of *sigF* (Duncan and Losick, 1992; Min *et al*., 1993). σ^F activation is regulated by a partner-switching mechanism (Sonenshein *et al*., 2005; Österberg *et al*., 2011). Upon translation, σ^F is held inactive by SpoIIAB-ATP and is released when SpoIIAB-ATP binds to dephosphorylated SpoIIAA. SpoIIAA is dephosphorylated by SpoIIE, the gene for which is also positively regulated by Spo0A~P and σ^H (Min *et al*., 1993; Duncan *et al*., 1995; Piggot and Losick, 2002; Hilbert and Piggot, 2004). Thus, Spo0A~P regulates both transcription of *sigF* and activation of σ^F. In early-stage sporulation, genes in the forespore are regulated by σ^F. The activity of σ^F and its related proteins in *B. subtilis* occurs exclusively in the forespore compartment following asymmetric cell

division (Schmidt *et al*., 1990). Important genes in the σ^F regulon are *spoIIR*, *spoIIQ*, and *sigG*. SpoIIR localises to the forespore membrane and plays a role in activating σ^E, the next σ factor in the σ cascade (Karow *et al*., 1995; Errington, 2003). SpoIIQ combines with SpoIIA-H to form a cross-membrane channel, described as a "feeding tube", which is conserved among endospore formers and is essential for forespore development (Camp and Losick, 2009; Crawshaw *et al*., 2014). Finally, *sigG* encodes pro-σ^G (Wang *et al*., 2006).

In clostridia, the complex structure of the *spoIIA* operon is conserved. This suggests that the role of σ^F and its mechanism of activation are similar to that of *B. subtilis* (Stragier, 2002). *B. subtilis*, mutants of *sigF* halt sporulation during stage II, with varying degrees of asymmetric cell division (Piggot and Coote, 1976; Errington and Mandelstam, 1983; Schmidt *et al*., 1990). Similar phenotypes were observed in mutational studies on *sigF* in *C. acetobutylicum* (Jones *et al*., 2011), *C. perfringens* (Li and McClane, 2010) and *C. difficile* (Fimlaid *et al*., 2013; Pereira *et al*., 2013). In *C. acetobutylicum*, mutation of *sigF* results in reduced transcription of downstream σ factor genes *sigE*, *sigG*, and *sigK*. Sporulation halts prior to stage II during asymmetric cell division, suggesting σ^F may play an earlier role in sporulation in *C. acetobutylicum* than in *B. subtilis* (Jones *et al*., 2011). Like *sigF* mutants of *B. subtilis*, sporulation halts at stage II in *sigF* mutants of *C. perfringens* and *C. difficile* (Li and McClane, 2010; Fimlaid *et al*., 2013, Pereira *et al*., 2013). These mutational studies suggest that σ^F is essential for early sporulation in these clostridia.

σ^E

In the mother cell of *B. subtilis* during early-sporulation, gene expression is regulated by σ^E. Like *sigF*, transcription of *sigE* is driven by Spo0A~P (Fujita and Sadaie, 1998; Fujita *et al*., 2005). The *sigE* gene is found at the *spoIIG* locus and encodes the pro-σ^E protein (Losick *et al*., 1986; LaBell *et al*., 1987). Pro-σ^E contains an N-terminal pro-sequence that must be cleaved in order to activate σ^E (Hilbert and Piggot, 2004). This is performed by a membrane-bound protease, encoded immediately upstream of *sigE*, called SpoIIGA (Stragier *et al*., 1988). SpoIIGA requires the σ^F-dependent SpoIIR in order to activate. SpoIIR localises to the membrane of the forespore and activates SpoIIGA from inside the intermembrane space between the forespore and mother cell (Stragier *et al*., 1988; Karow *et al*., 1995; Errington, 2003). In this way, σ^F indirectly induces σ^E activation. The σ^E regulon contains *spoIIIA* and *sigK*. SpoIIIA activates σ^G and *sigK* encodes the late-stage sporulation σ factor K. σ^E also transcribes the genes encoding SpoIID, SpoIIM, and SpoIIP, which form the so-called 'DMP' complex (Stragier *et al*., 1989; Illing and Errington, 1991). This complex is responsible for the engulfment of the forespore by the mother cell, progressing sporulation to stage III (Abanes-De Mello *et al*., 2002).

The arrangement of *spoIIGA* followed by *sigE* is conserved in clostridia, as is *spoIIR*, the product of which activates SpoIIGA (Paredes *et al*., 2005). This suggests that the mechanism of pro-σ^E cleavage is similar to that of *B. subtilis*. Mutational studies of *sigE* in *B. subtilis* result in a phenotype that halts sporulation at stage II, after asymmetric cell division (Piggot and Coote, 1976). Similarly, sporulation was disrupted in the early stages in *sigE* mutants of *C. acetobutylicum* (Tracy *et al*., 2011), *C. perfringens* (Harry *et al*., 2009) and *C. difficile* (Fimlaid *et al*., 2013; Pereira *et al*.,

2013). This suggests that the role of σ^E in spore development is similar in *B. subtilis* and clostridia. Regulation of the DMP complex genes, associated with engulfment and the σ^E regulon in *B. subtilis*, appears to be affected by both σ^F and σ^E in *C. difficile* (Saujet *et al*., 2013). This indicates that sporulation in the clostridia, while outwardly similar to *B. subtilis*, is regulated differently from *B. subtilis* sporulation by the σ factors within the pathway.

σ^G

Late-stage sporulation in the forespore of *B. subtilis* is regulated by σ^G (Hilbert and Piggot, 2004). The *sigG* gene is located immediately downstream of *sigE* in *B. subtilis* and is regulated by σ^F in the forespore (Masuda *et al*., 1988; Karmazyn-Campelli *et al*., 1989; Wang *et al*., 2006). Upon translation, σ^G is thought to be held inactive by an anti-σ factor. Activation of σ^G is dependent on σ^E via SpoIIIAG and SpoIIIAH, encoded in the *spoIIIA* operon. These are involved in releasing σ^G from an anti-σ factor, resulting in σ^G activation (Higgins and Dworkin, 2012). Active σ^G regulates transcription of cortex-related genes and *spoIVB*, which is involved in σ^K activation. Thus, σ^G is necessary for the completion of stage IV and progression to stage V of sporulation in *B. subtilis* (Cutting *et al*., 1990).

In the clostridia, *sigG* is also located downstream of *sigE* (Paredes *et al*., 2005). However, several differences have been observed between *sigG* mutants of clostridia and *B. subtilis*. In *sigG* mutants of *B. subtilis*, sporulation clearly halts at stage III. The forespore is engulfed by the mother cell, and no spore coat or cortex is present (Cutting *et al*., 1990). In contrast, sporulation appears to halt at stage V in *sigG* mutants of *C. acetobutylicum*. Disruption of *sigG* results in spore coat formation and minimal cortex development (Tracy *et al*., 2011). *C. difficile sigG* mutants exhibit a phenotype wherein the spore coat, but not the cortex, forms (Fimlaid *et al*., 2013; Pereira *et al*., 2013). Furthermore, σ^G activation does not rely on σ^E in *C. difficile* (Fimlaid *et al*., 2013; Saujet *et al*., 2013). This may indicate a substantial regulatory difference between the clostridia and *B. subtilis* regarding σ^G in late-stage sporulation.

σ^K

During late sporulation, σ^K regulates genes in the mother cell in *B. subtilis* (Kunkel *et al*., 1990). In many *B. subtilis* strains, pro-σ^K is encoded in two parts, *spoIVCB* and *spoIIIC*, separated by a *sigK* intervening element called "*skin*" (Stragier *et al*., 1989; Kunkel *et al*., 1990; Takemaru *et al*., 1995). The *skin* element is ~48 kb in length and is spliced out by a site-specific recombinase, SpoIVCA, in the mother cell genome. This results in *spoIVCB* and *spoIIIC* combining to form *sigK* (Stragier *et al*., 1989; Sato *et al*., 1990). The combined *sigK* gene is expressed under the control of σ^E in the mother cell, but requires forespore-specific σ^G in order to activate. σ^G regulates expression of *spoIVB*, the product of which localises in the forespore membrane with BofA, SpoIVFA, and SpoIVFB (Gomez and Cutting, 1996; Hilbert and Piggot, 2004). These proteins form a complex in the mother-cell side of the membrane which cleaves pro-σ^K into active σ^K (Ricca *et al*., 1992; Doan and Rudner, 2007; Higgins and Dworkin, 2012). This elaborate mechanism of regulation ensures

that σ^K acts at the appropriate time, however, it has been demonstrated that the *skin* element is unnecessary for *B. subtilis* to form viable spores (Kunkel *et al*., 1990).

In the clostridia, the arrangement of *sigK* is more diverse. The majority of clostridia, including *C. botulinum* (Sebaihia *et al*., 2007), contain a pro-sequence for σ^K and lack a *skin* element; however, strains of *C. difficile* and *C. tetani* are among those that possess *B. subtilis*-like *skin* elements. In *C. tetani*, this is a ~47-kb fragment and appears to closely resemble the *B. subtilis* skin element (Sonenshein *et al*., 2005). The *skin* element of *C. difficile* ($skin^{Cd}$) is much smaller at ~15 kb, and is in the opposite orientation within the gene, compared to the *B. subtilis skin* element (Haraldsen and Sonenshein, 2003). Unlike in *B. subtilis*, $skin^{Cd}$ appears to be essential for sporulation in *C. difficile* and its disruption halts sporulation prior to asymmetric cell division. Additionally, *sigK* of *C. difficile* does not include a pro-sequence (Haraldsen and Sonenshein, 2003; Pereira *et al*., 2013).

Disruption of *sigK* results in different sporulation phenotypes in some clostridia compared to *B. subtilis*. In *B. subtilis*, σ^K is exclusively active in late sporulation and mutation results in sporulation halting at the earliest stages of spore cortex development (Piggot and Coote, 1976; Kunkel *et al*., 1990). Similarly, mutation of *sigK* in *C. difficile* results in a phenotype that possesses a cortex but no spore coat (Fimlaid *et al*., 2013; Pereira *et al*., 2013). However, activation of σ^K in *C. difficile* is not dependant on σ^G, as it is in *B. subtilis* (Fimlaid *et al*., 2013; Saujet *et al*., 2013). This is likely due to the lack of a pro-sequence for *sigK* in *C. difficile*. Despite this, σ^K appears to play a major role in regulating late sporulation in *C. difficile*. In contrast, mutation of *sigK* in *C. perfringens* and *C. acetobutylicum* results in early-stage sporulation disruption, prior to asymmetric cell division (Harry *et al*., 2009; Al-Hinai *et al*., 2014). In the case of *C. acetobutylicum*, σ^K is also essential for late-stage sporulation (Al-Hinai *et al*., 2014). This suggests that σ^K may play a dual role in sporulation, perhaps limited to clostridia in which *sigK* lacks a skin element, unlike the late-stage-restricted σ^K of *B. subtilis*.

2.4 Environmental stress and stress response

2.4.1 Food safety and processing-induced stresses

Modern food processing faces a difficult challenge in maintaining the safety of foods as consumers demand high-quality, minimally processed foods that are low in sodium and have few additives. In addition to ensuring food safety, maintaining food quality is of great importance. Currently, a variety of stresses are employed to reduce the numbers of bacteria present in food and to prevent their growth during storage. The use of multiple stresses in foods is referred to as “hurdle technology”. With hurdle technology, different stresses are applied in a milder fashion than if each stress was employed alone. This maintains a high standard of food safety while having a less detrimental effect on food quality. The most common stresses utilised in hurdle technologies are heat (pasteurisation), reduction of water activity via osmotic stresses including salts and sugars,

acidification (lowering pH), modified atmosphere packaging (MAP), and storing foods at refrigeration temperatures (Leistner and Gould, 2002).

Pasteurisation and storage under refrigeration temperatures play major roles in food safety. Destruction and fatal injuring of bacterial cells occurs during pasteurisation and high temperature treatments. Low temperature storage aims to reduce or prevent growth by affecting metabolism, membrane fluidity and integrity, DNA integrity, and protein stability (Lim and Gross, 2011). Growth is further inhibited by acid stress. Acid stress causes cell metabolic activity to focus on proton homeostasis, resulting in growth cessation and even cell death as the cell becomes overwhelmed by high proton levels (Leistner and Gould, 2002). Osmotic stress causes a reduction in water activity, negatively affecting cellular metabolism as water leaves the cell and excess ions enter. In particular, NaCl contains ions disruptive to cellular metabolism (Wood, 2011). Ethanol was been shown to reduce growth and toxin production of *C. botulinum* and appeared to have a reversible sporostatic/bacteriostatic effect, preventing spore germination or cell growth (Daifas *et al*., 2003). These stresses may be overcome, however, as bacteria have adopted several strategies to survive environmental stress.

2.4.2 Stress tolerance in *C. botulinum*

The notorious foodborne pathogen *C. botulinum* may resist pasteurisation and sterilisation procedures by producing heat-resistant spores. Spores of Group I *C. botulinum* strains can survive temperatures up to 121 °C. The "botulinum cook" for canned goods (121 °C for 3 min) inactivates spores of *C. botulinum*, however if this is not fully applied, spores may survive and germinate (Stumbo *et al*., 1975). Spores of the Group II *C. botulinum* strains are typically less temperature resistant than those of Group I and have a *D*-value of 2.4 minutes at 82 °C (90% reduction of naturally germinating spores) (Stumbo *et al*., 1975; Peck, 1990; Peck *et al*., 1992). If heat-treatment fails, spores may remain viable and germinate into vegetative cells which may then overcome measures used to control bacterial growth, such as acidic environments and cold temperatures, and produce BoNT. BoNT production has been shown to occur at temperatures as low as 3 °C by some Group II strains (Graham *et al*., 1997). This is a cause for concern in minimally processed chilled foods, in which heat-treatment is minimal, such as ready-to-eat meals and chilled meat products (Peck and Stringer, 2005; Lindström *et al*., 2006; Peck, 2006). Additionally, the use of low-oxygen MAP methods may permit *C. botulinum* growth and toxin production (Austin *et al*., 1998; Del Torre *et al*., 1998; Larson and Johnson, 1999).

The growth limitations of both Group I and II *C. botulinum* strains under common food processing-induced stress conditions have been well characterised (Peck, 2009). Specific heat stress response mechanisms, such as DnaK and the *hrcA* operon, are present in *C. botulinum* and are conserved in many Gram-positive organisms including *C. acetobutylicum*, *B. subtilis*, and *Listeria monocytogenes*, (Narberhaus and Bahl, 1992; Narberhaus *et al*., 1992; Hanawa *et al*., 1995; Hecker *et al*., 1996; Schulz and Schumann, 1996; Hu *et al*., 2007; Selby *et al*., 2011). Cold stress response mechanisms, such as cold-shock proteins, are found in Group I *C. botulinum* strains (Söderholm *et*

al., 2011). Group II *C. botulinum* strains are psychrophilic and lack the cold-shock proteins found in Group I strains and many other Gram-positive organisms (Wouters *et al*., 2000; Söderholm *et al*., 2013). These specific heat- and cold-stress response proteins protect and repair DNA, or act as chaperones to prevent protein mis-folding under stress conditions (Lim and Gross, 2011).

Despite the wide conservation of specific stress-response proteins among Gram-positive organisms, a major feature of stress-response regulator, σ^B, is absent in many clostridia including *C. botulinum* (Paredes *et al*., 2005; Sebaihia *et al*., 2007). In other Gram-positive bacteria (such as *B. subtilis*, *Staphylococcus aureus*, and *L. monocytogenes*), σ^B is involved in regulating responses to osmotic, temperature, and acidic stresses, and is regarded as the general stress response mechanism (Boylan *et al*., 1993; Becker *et al*., 1998; Chan *et al*., 1998; Price, 2011). However, in a Group I *C. botulinum* strain, heat-shock response genes were shown to be induced under pH and NaCl stress, suggesting there may be an overlap between stress response mechanisms in this strain (Selby *et al*., 2011). Regulation by two-component systems (consisting of a sensor kinase and a response regulator) also appears to play a large role in temperature stress-response pathways of *C. botulinum* (Lindström *et al*., 2012; Derman *et al*., 2013; Mascher *et al*., 2014). Additionally, stress-response proteins in the clostridia have been linked to solventogenesis and sporulation, suggesting there may be a link or shared regulatory mechanism between these pathways (Bahl *et al*., 1995; Alsaker and Papoutsakis, 2005; Dahlsten *et al*., 2014).

2.4.3 Sporulation and stress response

The formation of highly resistant spores is a survival strategy utilised by bacteria primarily to survive periods of low nutrient availability. The spore has evolved to be a highly durable structure, capable of resisting temperature, osmotic, acidic, enzymatic, and radiation stresses (Setlow, 2011). Though the spore itself is almost completely dormant, some RNA synthesis can occur within the core (Segev *et al*., 2012). This suggests the spore can prepare itself for germination in a new environment. Despite this, the spore is unable to repair membranes, proteins, or DNA. DNA is protected against radiation and desiccation by SASPs and DPA which bind to it in the spore core (Setlow, 2006). Spore cortex peptidoglycan is protected from hydrolysis by the spore coat. Coat proteins contain enzymes involved in detoxifying chemicals and preventing digestion from cortex-lytic enzymes and lysozyme (Henriques and Moran, 2007; Setlow, 2011). Thus, sporulation is an effective mechanism for survival in stressful conditions.

Recent evidence in *B. subtilis* suggests there is some cross-talk between the sporulation and general stress response pathways (Reder *et al*., 2012a, 2012b, 2012c). The decision to sporulate is effectively made by the amount of Spo0A~P in the cell. Phosphorylation of Spo0A is positively regulated by several kinases, and negatively regulated by Spo0E. The gene encoding Spo0E is under σ^B regulation, the general stress regulator. Under stress conditions, including nutrient limitation, σ^B is present and transcribes *spo0E* in *B. subtilis*, suggesting a role in sporulation (Voelker *et al*., 1995; Reder *et al*., 2012b, 2012c). Overexpression of *sigB* results in the inhibition of sporulation. Reder *et al*. (2012c) have suggested that this is due to the cell favouring the low-

energy cost of vegetative dormancy over the high-energy cost and commitment of sporulation. It is also of interest to note that σ^B is tightly regulated by a partner-switching mechanism highly similar to that of sporulation σ factor, σ^F (Sonenshein *et al*., 2005; Österberg *et al*., 2011; Setlow *et al*., 2011). Cross-talk between the stress response and sporulation pathways has not yet been identified in *C. botulinum*.

3 Aims of the study

1. Accurate RT-qPCR analysis requires the use of a normalisation reference gene with stable transcript levels during all phases of growth. We identified a suitable normalisation reference gene for studying sporulation gene expression in *C. botulinum* ATCC 3502 (I).

2. The roles of sporulation-related σ factors σ^F, σ^E, σ^G, and σ^K have been well characterised in the model organism, *B. subtilis*. Evidence from recent studies in clostridia suggests that regulation of sporulation by these σ factors occurs differently to the *B. subtilis* model. We aimed to evaluate the roles of σ^F, σ^E, σ^G, and σ^K in sporulation of *C. botulinum* ATCC 3502 (II-III).

3. *C. botulinum* lacks the general stress response regulator σ^B, found in many Gram-positive organisms. A link between sporulation and stress response via σ^B was recently proposed. We aimed to investigate the role of sporulation regulator σ^K in stress tolerance of *C. botulinum* ATCC 3502 (IV).

4 Materials and methods

4.1 Bacterial strains, plasmids, and culture (I-IV)

Bacterial strains and plasmids are listed in Table 3. The Group I *C. botulinum* ATCC 3502 strain (Sebaihia *et al*., 2007) was examined in all studies and was used as the parent strain for mutant construction. Mutant strains of σ factor genes *sigF* (*cbo3087*), *sigE* (*cbo2532*), *sigG* (*cbo2533*) and *sigK* (*cbo2541*) were created using the ClosTron insertional knockout tool developed at the University of Nottingham by Heap *et al*. (2007, 2010). Standard cultivation was performed at 37 °C in an atmosphere of 85% N_2, 10% CO_2, and 5% H_2 (MG1000 Anaerobic Work Station, Don Whitley Scientific Ltd, Shipley, UK). Strains were cultured in anaerobic tryptone-peptone-glucose-yeast extract (TPGY; BD, Franklin Lakes, NJ, USA; VWR International, Radnor, PA, USA; Merck, Whitehouse Station, NJ, USA) broth and anaerobic TPGY plates (1.5% agar), which were supplemented with thiamphenicol, D-cycloserine, or erythromycin where appropriate. The plasmid conjugation donor, *E. coli* CA434 (Purdy *et al.*, 2002), was grown aerobically at 37 °C in Luria-Bertani (LB) broth (Sigma Aldrich, St. Louis, MO, USA) supplemented with chloramphenicol and kanamycin (Sigma Aldrich). All experiments were performed in triplicate.

4.2 Gene expression analysis (I-IV)

4.2.1 Culture sampling (I-IV)

Cultures of *C. botulinum* ATCC 3502 parent and sense-oriented mutant strains were sampled for subsequent RNA isolation for RT-qPCR analysis to determine gene expression of normalisation reference gene candidates and sporulation genes throughout growth (I-III). Overnight cultures were inoculated into TPGY broth at a 1:100 dilution and grown at 37 °C. Growth was measured by optical density (OD_{600} nm) compared to a blank of fresh TPGY broth. One-ml samples for RNA isolation were taken during the exponential, late-exponential, transition, and stationary phases of growth (I-III).

Samples of *C. botulinum* ATCC 3502 culture were taken for RT-qPCR analysis of gene expression under stress (IV). *C. botulinum* overnight culture was inoculated into TPGY broth as above and grown to the early-exponential growth phase. At this point the culture was split and a cold shock (temperature decrease from 37 °C to 15 °C), an osmotic shock (0% NaCl to 4.5% NaCl weight/volume), or an acid shock (pH downshift from pH 6.9 to pH 5.0) was administered to the cultures to induce a stress response. One-ml culture samples for RNA isolation were taken pre-shock and 1 min, 15 min, 30 min, 2 h, and 5 h post-shock for use in RT-qPCR (IV).

Table 3 Strains and plasmids

Strain[a] or plasmid	Description	Source
Strain		
C. botulinum ATCC 3502	Parent strain	ATCC, Manassas, VA, USA
*C. botulinum sigF*522s::CT	ClosTron insertional mutant of *sigF* in sense orientation	II
*C. botulinum sigF*487a::CT	ClosTron insertional mutant of *sigF* in antisense orientation	II
*C. botulinum sigE*441s::CT	ClosTron insertional mutant of *sigE* in sense orientation	II
*C. botulinum sigE*385a::CT	ClosTron insertional mutant of *sigE* in antisense orientation	II
*C. botulinum sigG*546s::CT	ClosTron insertional mutant of *sigG* in sense orientation	II
*C. botulinum sigG*546s::CT	ClosTron insertional mutant of *sigG* in antisense orientation	II
*C. botulinum sigK*427s::CT	ClosTron insertional mutant of *sigK* in sense orientation	III
*C. botulinum sigK*296a::CT	ClosTron insertional mutant of *sigK* in antisense orientation	III
C. botulinum ATCC 3502/pMTL82151	Parent strain with empty vector	III
*sigK*427s::CT/pMTL82151	*sigK*427s::CT with empty vector	III
*sigK*296a::CT/pMTL82151	*sigK*296a::CT with empty vector	III
*sigK*427s::CT/pMTL82151::*sigK*	*sigK*427s::CT with complementation vector	III
*sigK*296a::CT/pMTL82151::*sigK*	*sigK*296a::CT with complementation vector	III
E. coli 5-alpha	Chemically competent cloning strain	Invitrogen, Carlsbad, CA, USA
E. coli CA434	Conjugation donor strain containing R702 plasmid	Purdy *et al.* 2002
Plasmid		
pMTL007	*Clostridia* mutagenesis vector	Heap *et al.* 2007
pMTL007C-E2	*Clostridia* mutagenesis vector	Heap *et al.* 2010
pMTL007C-E2::Cbo-*sigF*522s::CT	pMTL007C-E2 targeting *sigF* in sense orientation	II; DNA 2.0, Menlo Park, CA, USA

Table 3 Continued

Strain[a] or plasmid	Description	Source
pMTL007C-E2::Cbo-*sigF*487a::CT	pMTL007C-E2 targeting *sigF* in antisense orientation	II; DNA 2.0
pMTL007C-E2::Cbo-*sigE*441s::CT	pMTL007C-E2 targeting *sigE* in sense orientation	II; DNA 2.0
pMTL007C-E2::Cbo-*sigE*385a::CT	pMTL007C-E2 targeting *sigE* in antisense orientation	II; DNA 2.0
pMTL007C-E2::Cbo-*sigG*546s::CT	pMTL007C-E2 targeting *sigG* in sense orientation	II; DNA 2.0
pMTL007C-E2::Cbo-*sigG*457a::CT	pMTL007C-E2 targeting *sigG* in antisense orientation	II; DNA 2.0
pMTL007::Cbo-*sigK*427s::CT	pMTL007 targeting *sigK* in sense orientation	III
pMTL007C-E2::Cbo-*sigK*296a::CT	pMTL007C-E2 targeting *sigK* in antisense orientation	III; DNA 2.0
pMTL82151	Modular plasmid	Heap *et al.* 2010
pMTL82151::*sigK*	pMTL82151 containing *sigK* gene for complementation	III

[a] Mutant strains named according to Kuehne and Minton (2012), strains/plasmids from study III have been updated accordingly.

4.2.2 RNA isolation and cDNA synthesis (I-IV)

Culture samples collected as described above were added directly to an ice-cold stop solution of phenol and ethanol (1:9). Samples were kept on ice for 30 min prior to centrifugation (8000 x G) at 4 °C for 5 min. Cell pellets were then frozen at -70 °C until RNA extraction. The thawed pellets were re-suspended and in a lysis buffer of lysozyme (50 mg/ ml, Sigma Aldrich), mutanolysin (1000 U/ml, Sigma Aldrich) and Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and incubated at 37 °C for 30 min prior to isolation. RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with a second DNase treatment after RNA elution (Ambion DNase-free kit, Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was evaluated by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and RNA purity (A_{260}/A_{280}) was examined using the NanoDrop spectrophotometer (Thermo Fisher Scientific).

Reverse transcription (RT) of RNA was performed on a standardised amount of RNA. The DyNAmo cDNA Synthesis Kit (Thermo Fisher Scientific) with random hexamer primers was used according to the instructions provided. Reverse transcription (RT) to cDNA was performed in duplicate (I, II, IV) or triplicate (III) for each RNA sample. No-RT controls, consisting of five pooled RNA samples without reverse transcriptase, were included in each run to control DNA contamination. The cDNA and no-RT control samples were frozen at -20 °C before use in qPCR.

4.2.3 Quantitative real-time reverse transcription-PCR (RT-qPCR) (I-IV)

The Maxima SYBR Green qPCR Master Mix kit (Thermo Fisher Scientific) (I, II) and DyNAmo Flash SYBR Green kit (Thermo Fisher Scientific) (III, IV) were used for RT-qPCR reaction mixtures. Reaction mixtures were prepared according to kit instructions. The Rotor-Gene 3000 Real Time Thermal Cycler (Qiagen) was used to perform RT-qPCR runs. Each run included two technical replicates of each cDNA replicate, the no-RT controls, and a non-template control for each primer pair to ensure no DNA contamination was present. Primers for each target gene were designed using Primer3 (Koressaar and Remm, 2007; Untergrasser *et al.,* 2012). Primer efficiencies were calculated from slopes of standard curves constructed from pooled, serially-diluted cDNA using the Rotor-Gene 6 software. Primer efficiencies varied between 94-104% for all genes. The quantification cycle (C_q) values (Livak and Schmittgen, 2001) were determined from primer efficiencies and prepared standard curves for each gene. Each RT-qPCR run consisted of an enzyme activation step at 95 °C for 10 min, followed by 40 cycles of a denaturation step at 95 °C for 15 s, and annealing and extension at 60 °C for 60 s. A final extension of 60 °C for 1 min was performed followed by a melt curve analysis from 60 °C to 99 °C at 0.5 °C per 5 s at the end of each run.

4.2.4 RT-qPCR analysis (I-IV)

Normalisation reference gene analysis (I)

Seven candidate reference genes were selected from two comprehensive reference gene studies (Nieto *et al*., 2009; Metcalf *et al*., 2010) along with 16S *rrn* (16S rRNA), previously used as a reference gene in *C. botulinum* (Broussolle *et al*., 2002; Lövenklev *et al*., 2004; Kouguchi *et al*., 2006; Chen *et al*., 2008; Artin *et al*., 2010; Cooksley *et al*., 2010; Selby *et al*., 2011; Lindström *et al*., 2012). These candidate genes were *adK* (*cbo3460*, adenosine kinase), *alaS* (*cbo2564*, alanyl-tRNA synthetase), *era* (*cbo2946*, GTP-binding protein Era), *gluD* (*cbo1811*, glutamate dehydrogenase), *gyrA* (*cbo0007*, DNA gyrase subunit A), *rpoC* (*cbo3487*, RNA polymerase β' subunit), and *rpsJ* (*cbo3481*, 30S ribosomal protein S10). The transcript stability of each candidate reference gene was evaluated by RT-qPCR during the exponential to stationary phases of growth. Stability of the C_q values for each gene over time were analysed using ANOVA and pairwise comparisons with Predictive Analytics SoftWare Statistics 18 (IBM, Armonk, NY, USA). A statistical cut-off of $P < 0.05$ was used. The coefficient of variation (CV) for the C_q values of each gene was determined such that $CV = \frac{\sigma}{\mu}$ where σ is the standard deviation of the C_q values of a candidate reference gene and μ is the mean C_q value (de Jonge *et al*., 2007). CVs were determined for each gene in both exponential and stationary growth phases separately, and for overall growth.

Gene expression analysis during sporulation and stress (I-IV)

C_q values of the target sporulation genes *spo0A* (*cbo1872*), *sigF* (*cbo3087*), *sigE* (*cbo2533*), *sigG* (*cbo2532*), and *sigK* (*cbo2541*) were normalised to 16S *rrn* C_q values. Expression levels at all time points were calibrated to expression levels in the exponential growth phase (I-III) or prior to stress shock (IV). Relative expression (R) was calculated using the Pfaffl method (Pfaffl, 2001) (I, II, IV). The simplified version of this method, the $\Delta\Delta C_q$ method (Livak and Schmittgen, 2001), was used to calculate R in study III as the primer efficiencies differed by less than 5%.

The Pfaffl equation was thus $R = \frac{\left(1 + E_{gene}\right)^{\Delta C_{q,gene}(\text{calibrator-sample})}}{\left(1 + E_{16Srrn}\right)^{\Delta C_{q,16Srrn}(\text{calibrator-sample})}}$, where E_{gene} is the efficiency of the target gene primers and E_{16Srrn} is the efficiency of 16S *rrn* primers. $\Delta C_{q,\ gene}$ is the difference between the target gene C_q at the calibration time and time point under investigation. $\Delta C_{q,16Srrn}$ is the difference between the 16S *rrn* C_q at the calibration time point and time point under investigation.

In the $\Delta\Delta C_q$ equation, the R value was calculated such that $R = 2^{\Delta\Delta C_q}$ where $\Delta\Delta C_q$ is $(C_{q,Target} - C_{q,16Srrn})_{Time\ x} - (C_{q,Target} - C_{q,16Srrn})_{Time\ 0}$. Here, '$C_{q,\ target}$' is the target gene C_q value, '$C_{q,\ 16Srrn}$' is the 16S *rrn* C_q value, 'time x' is the time point under investigation, and 'time 0' is the calibration time point. A two-tailed Student's *t*-test was performed on expression data normalised by log2 transformation. The statistical cut-off was $P < 0.05$.

4.3 Construction of mutant strains and complementation (II, III)

4.3.1 Construction of mutant strains (II, III)

ClosTron plasmids pMTL007C-E2 (Heap *et al*., 2010, II) and pMTL007 (Heap *et al*., 2007, III) were used for mutant construction. These plasmids differ in that pMTL007 requires isopropyl β-D-1-thiogalactopyranoside (IPTG) in order to express a mobile intron element from *Lactococcus lactis* (Ll.*ltrB*) while pMTL007C-E2 does not (Karberg *et al*., 2001; Heap *et al*., 2007, 2010). This mobile intron was targeted in each ClosTron plasmid to insert into each of the genes *sigF*, *sigE*, *sigG*, (II) and *sigK* (III) in both the sense and antisense orientations. The intron also carried an erythromycin resistance gene which activated upon insertion into the target gene (Heap *et al*., 2007, Heap *et al*., 2010).

Insertion sites were determined using the Perutka algorithm (Perutka *et al*., 2004). ClosTron plasmid vectors were constructed at the University of Helsinki and by DNA 2.0 (Menlo Park, CA, USA). The plasmids were cloned into *E. coli* CA434 and conjugated into *C. botulinum* ATCC 3502 under anaerobic conditions on non-selective agar plates. Transconjugant colonies of *C. botulinum* were streaked to purity on agar plates containing the antibiotic D-cycloserine to kill off remaining *E. coli*. IPTG was added to cultures of *C. botulinum* containing the pMTL007 mutant plasmid to induce expression of the ClosTron intron. Purified transconjugant *C. botulinum* colonies were streaked on plates containing erythromycin. Erythromycin resistant colonies were selected for integrant screening. Successful integrants were confirmed by PCR. Southern blotting was applied to confirm single insertions of the intron using HindIII-digested DNA from parent and mutant strains and a digoxigenin-labelled probe specific for the erythromycin resistance gene on the insertion, performed according to Palonen *et al*. (2011). Sense-oriented mutants were used in all experiments, while antisense-oriented mutants were assayed to confirm the non-sporulating (II, III) and cold-sensitive (IV) phenotypes.

4.3.2 Complementation of *sigK* mutation (III)

The modular plasmid variant pMTL82151 (Heap *et al*., 2009) was used in complementation of the *sigK* mutation. A fragment comprising the *sigK* coding sequence plus ~420 bp upstream and ~140 bp downstream of the gene was inserted into plasmid pMTL82151 resulting in the complementation plasmid, pMTL82151::*sigK*. This was transformed into *E. coli* CA434 and conjugated into the *sigK* mutant. Successful transconjugants were selected by thiamphenicol resistance. *C. botulinum* ATCC 3502 containing the empty pMTL82151 vector was used as a complementation control. Complementation of the *sigF*, *sigE*, and *sigG* mutants was not successful. However, to demonstrate that the mutant phenotypes were the result of gene disruption rather than polar effects caused by the ClosTron insertion, mutant strains were constructed with the ClosTron insertion in both sense and antisense orientations for each of *sigF*, *sigE*, *sigG*, and *sigK*.

4.4 Characterisation of mutant strains (II-IV)

4.4.1 Sporulation (II, III)

Spore staining and viability assay

The *C. botulinum* ATCC 3502 parent, *sigF*, *sigE*, *sigG*, and *sigK* mutant strains were grown on TPGY plates at 37 °C for seven days to allow sufficient spore formation (Burns *et al.*, 2010). Cells were stained with malachite green on a glass slide and counter-stained with safranin (Lechtman *et al.*, 1965). Spores possessing a spore coat stained green/blue in colour. Viability of spores was evaluated by administering a heat-treatment and observing growth after germination. Parent and mutant strains were cultured for seven days at 37 °C in TPGY broth. One-ml samples of each culture were heated to 80 °C for 15-20 min and serially diluted in 10 ml of fresh TPGY broth. Growth indicated the presence of viable spores capable of germination.

Transmission electron microscopy (III)

C. botulinum ATCC 3502 parent strain and sense-oriented *sigF*, *sigE*, and *sigG* mutants were incubated in TPGY broth at 37 °C for seven days. Fixation of *C. botulinum* spores and cells for TEM imaging was based on the protocol developed by Stevenson *et al*. (1972). The cells were fixed in a solution of glutaraldehyde and formaldehyde at room temperature and embedded in Epon 812 epoxy resin. Ultrathin sectioning was performed using a diamond knife. Each section was stained on a carbon mesh grid with uranyl acetate and Pb citrate. Imaging was performed with the FEI Tecnai 12 transmission electron microscope (FEI, Hillsboro, OR, USA).

4.4.2 Stress tolerance of the *sigK* mutants (IV)

Stress tolerance of the *C. botulinum* ATCC 3502 parent and *sigK* mutant strains was evaluated by monitoring growth under three different stress conditions. Growth curves of the parent and *sigK* mutants were tracked under a cold stress (17 °C), an osmotic stress (4.5% NaCl), or an acidic stress (pH 6.0, 5.3 and 5.0) using the Bioscreen C™ (Growth Curves, Helsinki, Finland) under an atmosphere of 85% N_2, 10% CO_2, and 5% H_2. The growth rates of each strain were determined from fitted growth curves in accordance with Baranyi and Roberts (1994) using the DMFit v2.0 Microsoft Excel add-on (Institute of Food Research, Norwich, United Kingdom).

5 Results

5.1 Normalisation reference gene analysis (I)

Eight reference gene candidates, 16S *rrn*, *adK*, *alaS*, *era*, *gluD*, *gyrA*, *rpoC*, and *rpsJ*, were assessed for stability of expression in *C. botulinum* ATCC 3502 from the exponential to stationary growth phases. RT-qPCR analysis revealed that 16S *rrn* was the only stable candidate reference gene overall. ANOVA and pair-wise comparisons showed that, overall, 16S *rrn* transcript levels did not significantly change during both phases of growth. 16S *rrn* demonstrated stable C_q values and a desirably low CV of 1.7-2.0% during the experimental period (Fig. 3). Thus, 16S *rrn* was chosen as the normalisation reference gene for subsequent RT-qPCR analyses of sporulation gene expression. The other candidate genes exhibited significant ($P < 0.05$) variation in gene expression, as determined by ANOVA and pairwise analysis, particularly after the transition from exponential to stationary growth. During the exponential phase, CVs of less than 4% were noted for *alaS*, *era*, *gluD*, and *gyrA*, however, the overall CVs for these genes were higher (9-14%). Thus, these were unsuitable for studying gene expression during sporulation.

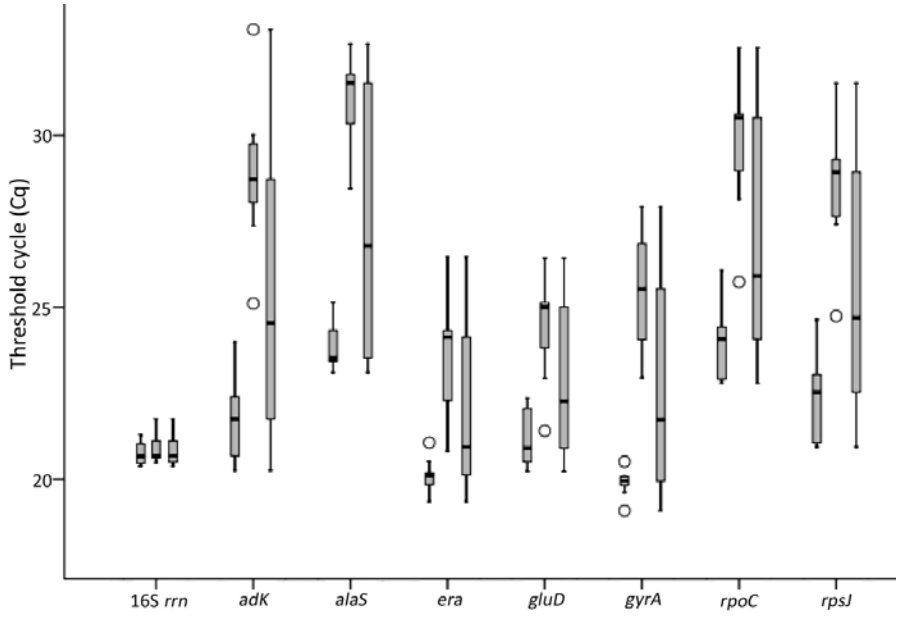


Figure 3 Boxplot of C_q values during the (from left to right) exponential phase, stationary phase, and overall growth for each reference gene candidate. CVs were: 16S *rrn* (1.7%, 2.0%, 1.8%), *adK* (5.8%, 7.5%, 16.0%), *alaS* (2.8%, 4.6%, 13.9%), *era* (2.5%, 7.5%, 10.1%), *gluD* (3.9%, 6.2%, 9.1%), *gyrA* (2.0%, 6.9%, 13.7%), *rpoC* (5.1%, 6.5%, 12.6%), *rpsJ* (5.7%, 6.5%, 14.0%), for exponential, stationary, and overall growth, respectively (I).

5.2 Gene expression analysis

5.2.1 Expression of sporulation regulator genes in *C. botulinum* ATCC 3502 (I, II)

The roles of the transcriptional regulators Spo0A, σ^F, σ^E, σ^G, and σ^K in *C. botulinum* ATCC 3502 sporulation were investigated by measuring relative expression of *spo0A*, *sigF*, *sigE*, *sigG*, and *sigK* during the exponential, late-exponential, transition, and stationary phases of growth (Fig. 4). Relative expression levels were normalised to 16S *rrn* expression and calibrated to the exponential phase time point.

Relative expression levels of *spo0A* in the parent strain (Fig. 4A) decreased after the late-exponential phase of growth. Induction of *sigF* (Fig. 4E), *sigE* (Fig. 4H), and *sigG* (Fig. 4K) expression levels occurred in the late-exponential phase. Relative expression levels of *sigE* and *sigG* expression remained high until the stationary growth phase. Relative *sigK* expression levels (Fig. 4N) decreased after the exponential growth phase and increased greatly during stationary growth, demonstrating a biphasic pattern of expression.

5.2.2 Expression of sporulation regulator genes in *C. botulinum* ATCC 3502-derived σ factor mutants (II, III)

Relative expression levels of *spo0A*, *sigE*, *sigF*, *sigG*, and *sigK* were evaluated during the exponential, late-exponential, transition, and stationary phases of growth in the *C. botulinum* ATCC 3502 parent strain and *sigF*, *sigE*, and *sigG* mutants to determine the relationship between these regulators during sporulation (II). Statistical comparisons of gene expression over time were made for each individual strain (Fig. 4 and 5). Additionally, comparisons of gene expression over time between the parent and mutant strains were performed (II). Of these comparisons, *spo0A* expression differed significantly between the parent and mutant strains during the exponential phase and the results are depicted separately (Fig. 5; II). Relative expression levels of *spo0A* and *sigF* were evaluated and compared between the parent strain and *sigK* mutant during the exponential, late-exponential, and transition phases of growth (Fig. 6; III). As before, relative expression levels were normalised to 16S *rrn* expression and calibrated to the exponential phase time point. The parent and mutant strains grew similarly at 37 °C.

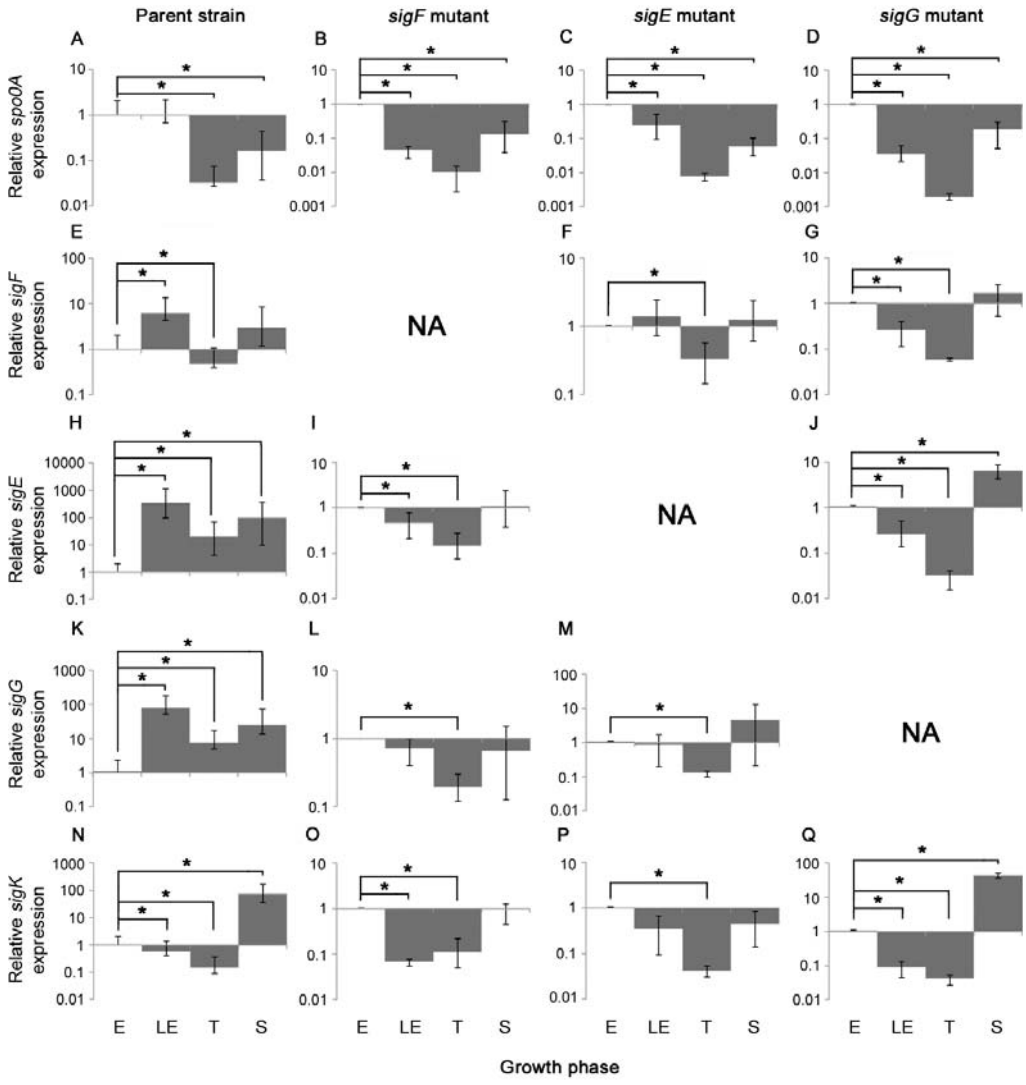


Figure 4 Relative expression analysis of sporulation genes *spo0A*, *sigF*, *sigE*, *sigG*, and *sigK* in the *C. botulinum* ATCC 3502 parent strain (A, E, H, K, N), *sigF* mutant (B, I, L, O), *sigE* mutant (C, F, M, P), and *sigG* mutant (D, G, J, Q). Expression levels were measured during exponential, late-exponential, transition, and stationary growth phases, abbreviated to E, LE, T, and S, respectively. Relative expression levels were calibrated to the exponential growth phase levels and normalised to 16S *rrn* expression. Error bars denote maximum and minimum expression values from three biological replicates. Statistical significance ($P < 0.05$) is represented by an asterisk. NA refers to non-applicable relative expression analysis (II).

In all mutants tested, the relative expression levels of *spo0A* during the late-exponential growth phase were significantly lower than in the parent strain (Fig. 5). In the *sigF* and *sigE* mutants, expression of *sigF* (Fig. 4F), *sigE* (Fig. 4I), *sigG* (Fig. 4L, M), and *sigK* (Fig. 4O, P) failed to be induced from the late-exponential phase onwards. Expression levels of these genes were also significantly reduced in the *sigF* and *sigE* mutants compared to the parent strain (II). In the *sigG* mutant, induction of *sigE* expression was delayed until the stationary growth phase (Fig. 4J). Relative *sigK* expression was observed in a biphasic manner, however expression levels were significantly lower than those of the parent strain (Fig. 4Q; II). In the *sigK* mutant, *spo0A* (Fig. 6A) and *sigF* (Fig. 6B) expression levels were lower than in the parent strain during exponential and late-exponential growth (III).

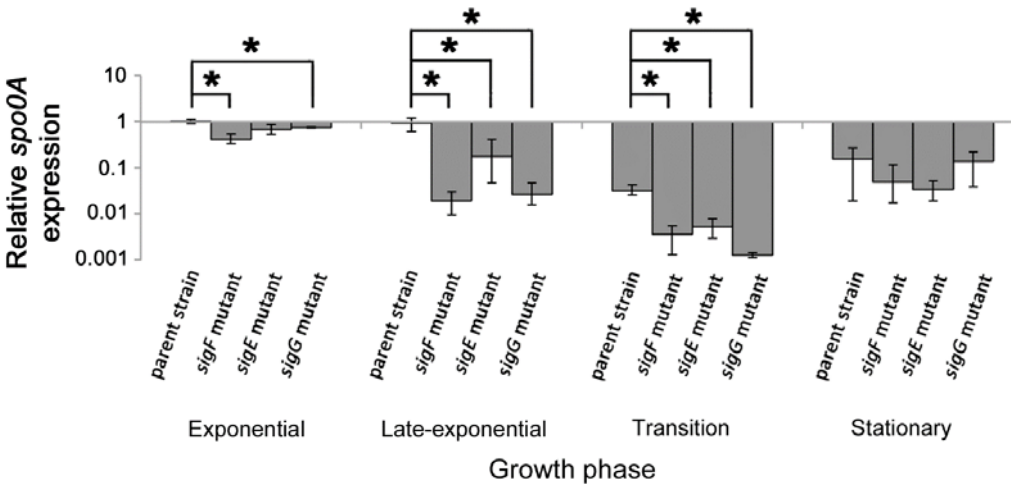


Figure 5 Relative expression analysis of *spo0A* in *C. botulinum* ATCC 3502 parent and *sigF, sigE,* and *sigG* sense-oriented mutant strains during exponential, late-exponential, transition, and stationary growth. Relative expression levels were calibrated to the exponential growth phase levels and normalised to 16S *rrn* expression. Error bars denote maximum and minimum expression values from three biological replicates. Statistical significance ($P < 0.05$) is represented by an asterisk (II).

5.2.3 Expression of *sigK* under stress (IV)

The role of *sigK* in the *C. botulinum* ATCC 3502 stress response was investigated by measuring expression levels of *sigK* after exposure to either cold, osmotic, or acidic stress. Expression levels were calibrated to the pre-shocked cultures and normalised to 16S *rrn* expression levels (Fig. 7). As observed in the sporulation study above (5.2.1), *sigK* was expressed during the exponential phase and down-regulated significantly on entering the late-exponential phase. In the cold-shocked culture (temperature downshift from 37 °C to 15 °C), a slight but immediate induction of *sigK* was observed. Relative *sigK* expression levels then decreased to pre-shock levels between 15 min and 1 h after cold shock. At 2 and 5 h after the shock, relative expression levels were significantly up-

regulated compared to pre-shock levels. Osmotic shock of NaCl (0% to 4.5% weight/volume) also induced an immediate increase in relative *sigK* expression levels. Relative expression levels of *sigK* decreased between 15 min and 2 h post-shock but increased significantly at 5 h. Acid shock of the culture, (pH reduction from pH 6.9 to pH 5.0) resulted in no significant changes to relative *sigK* expression.

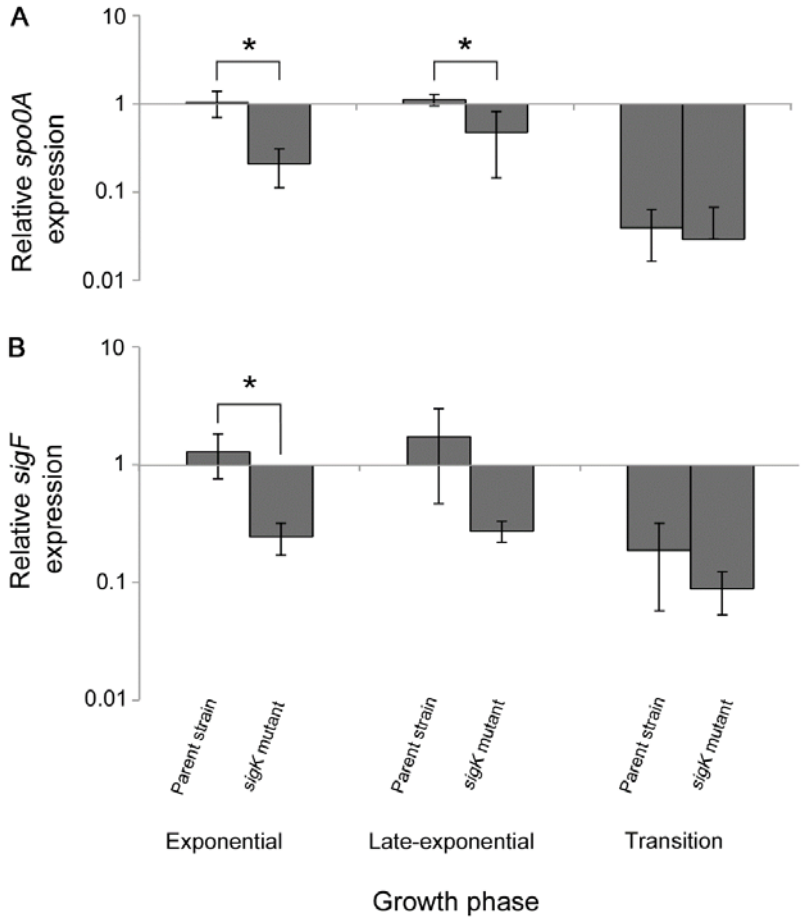


Figure 6 Relative expression analysis of *spo0A* (A) and *sigF* (B) in *C. botulinum* ATCC 3502 parent and *sigK* mutant strains during exponential, late-exponential, and transition phases of growth. Relative expression levels were calibrated to the exponential growth phase levels and normalised to 16S *rrn* expression. Error bars denote standard deviation of expression values from three biological replicates. Statistical significance ($P < 0.05$) is represented by an asterisk (III).

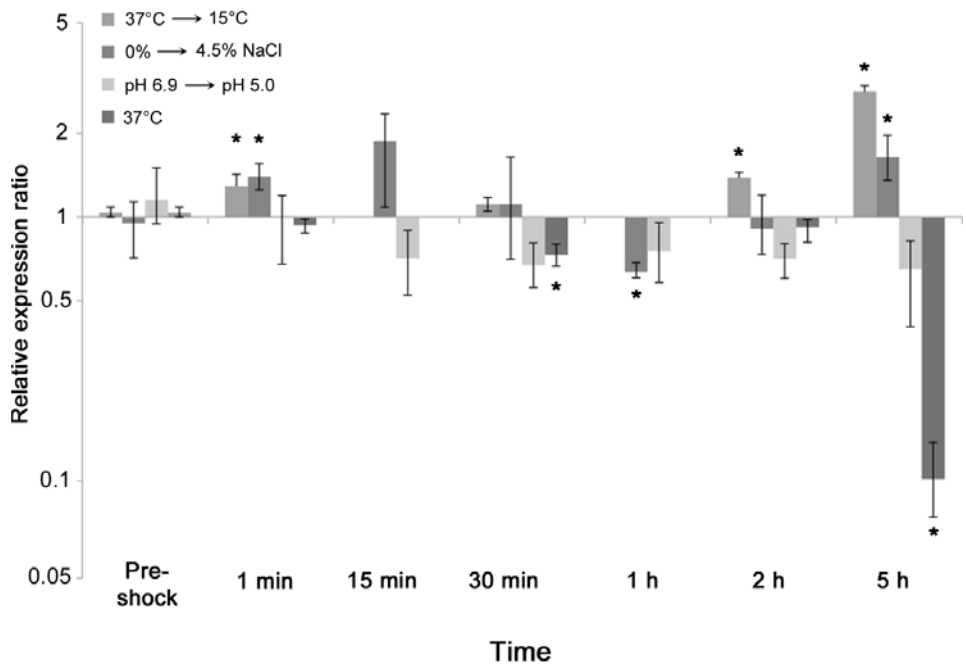


Figure 7 Relative expression analysis of *sigK* in *C. botulinum* ATCC 3502 at 1 min, 15 min, 30 min, 2 h, and 5 h after cold shock (temperature downshift from 37 °C to 15 °C), osmotic shock (exposure to 4.5% NaCl), acid shock (pH downshift from pH 6.9 to pH 5.0), and normal growth at 37 °C. Relative expression levels were calibrated to the pre-shock culture and normalised to 16S *rrn* expression. Error bars denote maximum and minimum expression values from three biological replicates. Statistical significance ($P < 0.05$) is represented by an asterisk (IV).

5.3 Sporulation and *sigK* mutant growth under stress (II-IV)

5.3.1 Sporulation assessment of *C. botulinum* ATCC 3502 and derived σ factor mutants (II, III)

Sporulation was assessed in both sense and antisense *sigF*, *sigE*, *sigG*, and *sigK* mutants by malachite green staining and viability assay. All of the mutant strains failed to germinate after heat-treatment at 80 °C and thus did not form viable spores (Table 4). The *sigG* mutants, however, stained positive for spores. Light microscopy and staining revealed that the *sigK* mutants showed an elongated phenotype. The *C. botulinum* ATCC 3502 parent and *sigK* complement strains formed viable, heat-resistant spores. TEM images of the parent and sense-oriented mutant strains were taken after an appropriate seven-day sporulation period. The parent strain formed identifiably mature spores during this time (Fig. 8A). TEM revealed that the *sigF* mutant halted sporulation during asymmetric cell division, corresponding to stage II of sporulation (Fig. 8B). The *sigE* mutant also showed disruption of sporulation at stage II (Fig. 8C). The *sigG* mutant halted sporulation

following the engulfment of the forespore by the mother cell (Fig. 8D). A thin spore coat was visible surrounding the forespore cell membrane in this mutant, although no spore cortex was visible.

Table 4 Sporulation assay results for parent, mutant, and complementation strains (II, III)

Strain	Assay	
	Heat treatment	Malachite green stain
C. botulinum ATCC 3502	Positive	Positive
C. botulinum ATCC 3502-pMTL82151	Positive	Positive
*sigF*522s::CT	Negative	Negative
*sigF*487a::CT	Negative	Negative
*sigE*441s::CT	Negative	Negative
*sigE*385a::CT	Negative	Negative
*sigG*546s::CT	Negative	Positive
*sigG*546s::CT	Negative	Positive
*sigK*427s::CT	Negative	Negative
*sigK*296a::CT	Negative	Negative
*sigK*427s::CT/pMTL82151	Negative	Negative
*sigK*296a::CT/pMTL82151	Negative	Negative
*sigK*427s::CT/pMTL82151::*sigK*	Positive	Positive
*sigK*296a::CT/pMTL82151::*sigK*	Positive	Positive

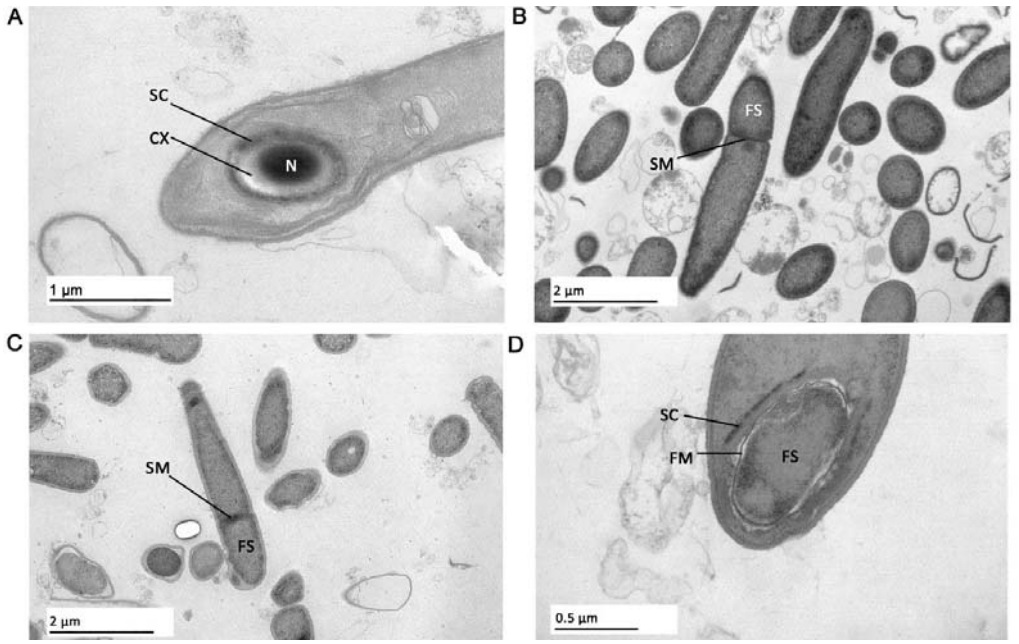


Figure 8 Transmission electron micrographs of ultrathin sections of *C. botulinum* ATCC 3502 parent strain (A), *sigF* mutant (B), *sigE* mutant (C), and *sigG* mutant (D) grown in TPGY broth for seven days at 37 °C. Indicated are the nucleoid area (N), spore coat (SC), spore cortex (CX), possible forespore membrane (FM) and forespore (FS), and possible septation membranes (SM) (II).

5.3.2 Growth assessment of *C. botulinum* ATCC 3502 and derived σ factor mutants (I-IV)

The *C. botulinum* ATCC 3502 parent and all mutant strains grew similarly at 37 °C in TPGY during all growth phases. In order to assess the role of σ^K under stress conditions, the *sigK* mutant was compared to the parent strain under cold, osmotic, and acidic growth stress conditions (IV). Under cold stress at 17 °C, the parent strain reached a significantly higher maximum optical density than the *sigK* mutants. Under osmotic stress, evaluated at 4.5% NaCl, the lag phase of the *sigK* mutants was notably longer than the parent strain lag phase. The growth rates of the *sigK* mutants peaked 6-12 hours after the peak of the parent strain growth rate, though the growth rates of the *sigK* mutants were slightly higher than those of the parent strain. Under acidic stress, no significant differences between the parent and *sigK* mutant strains were observed at pH 6.0 or pH 5.3. No growth was observed for the parent or *sigK* mutant strains at pH 5.0.

6 Discussion

6.1 Normalisation reference gene analysis (I)

RT-qPCR is a common and reliable method for studying changes in gene expression. This involves converting mRNA to cDNA, thus target gene expression must be normalised for every individual cDNA sample. This requires a normalisation reference gene which has stable transcript levels under all test conditions in order to provide accurate analysis of the target gene expression (Bustin, 2002). An ideal normalisation reference gene should possess a low (< 4%) coefficient of variation of the C_q values representing transcript levels, and a maximum fold change of < 2 during the experimental period (de Jonge *et al*., 2007).

In order to accurately study gene expression in *C. botulinum* ATCC 3502 during sporulation, a suitable normalisation reference gene was required. From the exponential phase to the stationary phase of growth, the latter phase representing the onset of sporulation, transcript level stability of eight candidate normalisation reference genes (16S *rrn*, *adK*, *alaS*, *era*, *gluD*, *gyrA*, *rpoC*, and *rpsJ*) was assessed. 16S *rrn* fulfilled the stability criteria above and was determined to be the only suitable normalisation reference for the study of gene expression during sporulation in *C. botulinum* ATCC 3502. Ribosomal RNA (rRNA) has several disadvantages as a normalisation reference gene, however. The high percentage of rRNA in the cell, comprising >90% of total cellular RNA, requires the cDNA sample to be substantially diluted, leading to a higher risk of error (Solanas *et al*., 2001; Kozera and Rapacz, 2013). Furthermore, synthesis of rRNA may decline in stationary phase as the cell multiplication rate decreases (Hansen *et al*., 2001), thus the mRNA to rRNA ratio may not be identical in cells experiencing the exponential and stationary phases. However, no deterioration of 16S *rrn* was observed throughout these studies suggesting rRNA levels remained stable during all growth phases. 16S *rrn* has traditionally been used in RT-qPCR in *C. botulinum* under a variety of conditions (Broussolle *et al*., 2002; Lövenklev *et al*., 2004; Kouguchi *et al*., 2006; Chen *et al*., 2008; Artin *et al*., 2010; Cooksley *et al*., 2010; Selby *et al*., 2011; Lindström *et al*., 2012; Mascher *et al*., 2014). In *C. difficile* and *C. acetobutylicum*, *dnaF* (Fimlaid *et al*., 2013), *rpoC* (Saujet *et al*., 2013), and *fabZ* (Al-Hinai *et al*., 2014) were utilised in RT-qPCR analyses, though the stability of their transcript levels was not reported. Reporting of the transcript stability of normalisation reference genes was recently recommended to better verify results of gene expression studies (Kozera and Rapacz, 2013).

Due to the aforementioned drawbacks of using 16S *rrn*, mRNA-based normalisation reference genes are preferable for RT-qPCR analysis. Several of the candidate reference genes were selected for their use in *C. difficile* (Metcalf *et al*., 2010). The candidate reference genes tested did not meet the stability criteria for all growth phases. However, *alaS*, *era*, *gluD*, and *gyrA* demonstrated stable transcript levels during exponential growth. Therefore, these genes may be of use in RT-qPCR analyses of *C. botulinum* ATCC 3502 restricted to this growth phase. Transcript levels of these genes became unstable on entering the stationary growth phase, ruling them out for the study of

sporulation. This is likely due to the slowing down of metabolic activity during stationary growth and sporulation.

The identification of stable 16S *rrn* transcript levels during the entire experimental time window and stable transcript levels of *alaS*, *era*, *gluD*, and *gyrA* during exponential growth in *C. botulinum* ATCC 3502 may be useful for designing future RT-qPCR studies in this organism. Accurately detecting changes in gene expression under a variety of conditions, such as stress conditions used in the food processing industry, is of major importance to understanding the fundamental biology and mechanisms of development and adaptation in this notorious food pathogen.

6.2 Sporulation gene expression analysis and characterisation of sporulation phenotypes in *C. botulinum* ATCC 3502 and derived σ factor mutants (I-III)

C. botulinum can be found globally in soils and aquatic sediments. In these environments *C. botulinum* may persist as spores for decades, until it enters the food chain triggering disease via neurotoxin production (Yule *et al*., 2006; Espelund and Klaveness, 2014). Neurotoxin production and sporulation are both triggered during early stationary phases of growth, and a link between these processes through quorum sensing has been proposed (Cooksley *et al*., 2010). Regulation of the sporulation pathway, however, is poorly understood in *C. botulinum*. Genes coding for sporulation regulators Spo0A, σ^F, σ^E, σ^G, and σ^K are conserved in clostridia, including *C. botulinum*, and in *B. subtilis*, the model organism for sporulation study. In the *B. subtilis* model, Spo0A is responsible for the initiation of sporulation. σ^F and σ^E regulate early-sporulation gene expression in the forespore and mother cell, respectively, while σ^G and σ^K regulate late-sporulation gene expression in the forespore and mother cell, respectively (Piggot and Hilbert, 2004). In this discussion, the findings on sporulation of *C. botulinum* ATCC 3502 are described in the known context of the *B. subtilis* model.

6.2.1 Sporulation and related σ factor gene expression in *C. botulinum* ATCC 3502 (I, II)

The relative expression levels of the genes *spo0A*, *sigF*, *sigE*, *sigG*, and *sigK*, which encode the major sporulation regulators, of *C. botulinum* ATCC 3502 were analysed from exponential- to stationary-phase growth. This time frame was chosen to observe the gene transcripts accumulate in the first cells entering sporulation, as sporulation is asynchronous in *C. botulinum* (Brown *et al*., 1957; Perkins and Tsuji, 1962). 16S *rrn*, identified as having stable transcript levels during the examined growth phases, was used as the normalisation reference gene for expression analysis. Expression of *spo0A* was observed during exponential and late-exponential growth, consistent with its role in the *B. subtilis* model of initiating sporulation (Brown *et al*., 1994; Fujita and Sadaie, 1998). Expression of early-sporulation related genes *sigF* and *sigE*, and late-sporulation related

sigG occurred during late-exponential growth. Expression levels of *sigE* and *sigG* remained high after their initial induction, likely due to the asynchronous nature of sporulation in *C. botulinum*. The simultaneous expression of these σ factor genes has also been observed in *C. acetobutylicum* (Jones *et al*., 2008) and *C. difficile* (Fimlaid *et al*., 2013; Pereira *et al*., 2013), which may be attributed to the conserved operon structure of these genes amongst the clostridia.

Interestingly, expression levels of *sigF* were not induced much higher than their initial levels throughout growth. This may suggest that σ^F functions at comparatively low levels to the other σ factors, further indicated by the need for heavy post-translational regulation of σ^F (Sonenshein *et al*., 2005; Österberg *et al*., 2011). Expression levels of *sigE* and *sigG* appeared to follow each other proportionally. These genes are located next to each other at the *spoIIG* locus, suggesting they may be co-transcribed in *C. botulinum* ATCC 3502. Expression of *sigK* was notably different in *C. botulinum* compared to the *B. subtilis* model, in which it is solely expressed in late-stage sporulation. Transcript levels decreased after the exponential phase, indicating *sigK* was expressed at low levels during exponential growth. Similar observations of early *sigK* expression have been made recently in other *C. acetobutylicum* and *C. perfringens* (Jones *et al*., 2008; Harry *et al*., 2009; Al-Hinai *et al*., 2014). During the stationary phase, *sigK* expression was re-induced to high levels. This indicates that σ^K may have a dual role in *C. botulinum* ATCC 3502 – one during exponential growth, and the typical late-stage sporulation regulatory role. Harry *et al*. (2009) suggested that σ^K plays a role in early sporulation in *C. perfringens* and a recent study in *C. acetobutylicum* suggested that σ^K is responsible for correct Spo0A regulation in early sporulation (Al-Hinai *et al*., 2014). Our findings show that all four σ factors are actively induced during the growth period corresponding with the onset of sporulation in a similar manner observed in other clostridia. This suggests that while the sporulation pathway of *C. botulinum* ATCC 3502 may possess the same regulators as the *B. subtilis* model, they may behave differently.

6.2.2 Sporulation and related σ factor gene expression in *C. botulinum* ATCC 3502-derived σ factor mutants (II)

sigF and *sigE* mutants

Gene expression in early-stage sporulation is regulated by σ^F and σ^E in *B. subtilis*. The genes encoding σ^F and σ^E are comparatively well conserved between *B. subtilis* and *C. botulinum* at the *spoIIA* and *spoIIG* loci, suggesting their regulation is similar to that of the *B. subtilis* model (Losick *et al*., 1986; Duncan and Losick, 1992; Min *et al*., 1993). The *sigF* and *sigE* genes of *C. botulinum* ATCC 3502 were insertionally disrupted using the ClosTron tool and relative expression levels of *spo0A*, *sigF*, *sigE*, *sigG*, and *sigK* were analysed by RT-qPCR. The phenotype of each mutant was assessed by light microscopy and TEM.

Transcriptional analysis of the *sigF* and *sigE* mutants suggested sporulation halted at an early stage. Expression levels of *spo0A* were slightly lower than those observed in the parent strain during exponential growth and decreased earlier. The regulation of *spo0A* in the clostridia is unknown and

may possibly be affected by the disruption of ostensibly downstream elements of the sporulation pathway, such as *sigF* and *sigE*, through an unknown mechanism. Expression of *sigG* and *sigK* were not significantly induced in either the *sigF* or *sigE* mutant, suggesting both σ^F and σ^E are required for transcribing the late-sporulation genes. Similarly, downstream expression of *sigG* and *sigK* was disrupted in *C. perfringens* and *C. acetobutylicum sigF* and *sigE* mutants (Harry *et al*., 2009; Li and McClane, 2010; Jones *et al*., 2011; Tracy *et al*., 2011). While late-stage expression of *sigK* was affected in our mutants, expression of *sigK* during exponential growth followed a similar trend to the parent strain. As σ^K was suggested to be involved in *spo0A* regulation in *C. acetobutylicum* (Al-Hinai *et al*., 2014), the slightly lower *sigK* expression levels in the *sigF* mutant may have affected *spo0A* expression, however this does not explain low *spo0A* levels observed in the *sigE* mutant. How *spo0A* is regulated in *C. botulinum* remains a question for future research. These findings suggest the σ cascades of our *C. botulinum sigF* and *sigE* mutants were disrupted similar to each other in early sporulation.

TEM images of our *C. botulinum sigF* and *sigE* mutants confirmed that sporulation was disrupted in the early stages. Both mutants halted sporulation during asymmetric cell division, corresponding to stage II of sporulation. This is consistent with the roles of these σ factors in the *B. subtilis* model and other clostridia. Mutants of *sigF* and *sigE* in *B. subtilis*, *C. perfringens*, *C. acetobutylicum*, and *C. difficile* exhibited disruption of sporulation in the early stages; (Piggot and Coote, 1976; Harry *et al*., 2009; Li and McClane, 2010; Jones *et al*., 2011; Tracy *et al*., 2011; Fimlaid *et al*., 2013; Pereira *et al*., 2013). The disruption of the σ cascade and disruption of sporulation observed in our *sigF* and *sigE* mutant strains suggests that σ^F and σ^E are essential for progression of sporulation beyond stage II in *C. botulinum* ATCC 3502.

sigG and *sigK* mutants

Late-sporulation gene expression is regulated by σ^G and σ^K in *B. subtilis*. In *C. botulinum*, the gene encoding σ^G is located downstream of *sigE*, a feature conserved in *B. subtilis*. However, the *sigK* of *C. botulinum* does not possess an intervening *skin* element like that of *B. subtilis* or *C. difficile*, suggesting it may be regulated differently. The ClosTron tool was used to insertionally disrupt *sigG* and *sigK* of *C. botulinum* ATCC 3502. Relative expression levels of *spo0A*, *sigF*, *sigE*, *sigG*, and *sigK* were analysed by RT-qPCR throughout growth and the sporulation phenotype of each mutant was assessed.

In the *sigG* mutant, expression of *spo0A* was affected similar to the *sigF* and *sigE* mutants and the sporulation pathway appeared delayed overall. This may have been due to the initial low levels of *spo0A* observed. Expression of *sigE* was not induced during late-exponential growth as in the parent strain, but occurred during stationary growth. Biphasic expression of *sigK* appeared similar to the parent strain, though *sigK* was not expressed to parent-strain levels during stationary growth. This demonstrates that *sigG* is not essential for *sigK* expression. TEM images showed that the *sigG* mutant had reached the point of engulfment (stage III of sporulation) and a spore coat (stage V) but no peptidoglycan cortex (stage IV) were present. This suggests that σ^G is essential for cortex formation. These observations are similar to other *sigG* mutants of clostridia (Tracy *et al*., 2011;

Fimlaid *et al*., 2013; Pereira *et al*., 2013), but markedly different to *B. subtilis sigG* mutants. *B. subtilis sigG* mutants halt sporulation prior to cortex formation at stage III (Cutting *et al*., 1990). In the *B. subtilis* model, σ^G is required to activate σ^K in late-stage sporulation. This indicates that σ^G of *C. botulinum* performs a different function to that of *B. subtilis* σ^G. The presence of a spore coat suggests that σ^G of *C. botulinum* ATCC 3502 may not be required to activate σ^K, which primarily regulates spore coat formation. This is the case in *C. perfringens* and *C. difficile* (Li and McClane, 2010; Saujet *et al*., 2013), however this remains to be demonstrated for *C. botulinum*.

Disruption of *sigK* resulted in no induction of expression of the sporulation pathway, as low expression levels of *spo0A* were detected during exponential growth. Expression of *sigF* failed to be induced in the late-exponential phase. This suggested that Spo0A~P, the transcription factor behind *sigF* expression, was significantly disrupted in the *sigK* mutant and the sporulation pathway had halted during early-sporulation. This was confirmed by light microscopy and staining, which indicated that a spore coat was not present due to non-retention of dye. The *sigK* mutants formed an elongated cell phenotype that was deviant from normal cell division and similar to *spo0A* mutants created by Heap *et al*. (2007) and a *sigK* mutant of *C. perfringens* (Harry *et al*., 2009), indicating an essential role for σ^K in early-stage sporulation.

Complementation of *sigK* via plasmid was able to restore the normal phenotype and viable spore formation. Our observations of low *spo0A* levels and the elongated-cell phenotype of the *sigK* mutant suggest that σ^K plays an essential role in early sporulation in *C. botulinum*. This is in contrast to *B. subtilis*, where σ^K is exclusively involved in late-stage sporulation (Errington, 1993). The expression of *sigK* in both early- and late-stage sporulation, as observed in the *sigG* mutant and parent strains, therefore suggests that σ^K may play a dual role in sporulation, like the σ^K of *C. acetobutylicum* (Al-Hinai *et al*., 2014).

Sporulation is the means by which *C. botulinum* spreads in the environment and contaminates food. Understanding the means by which sporulation is regulated in this organism allows us to better study its behaviour in foods and the environment. Further study of the $\sigma^{F, E, G, K}$ regulons in *C. botulinum* may yield alternative methods for preventing spore formation, leaving cells susceptible to pasteurisation in food processing, and uncover potential molecular targets for preventing spore germination in foods.

6.3 Role of *sigK* under stress (IV)

C. botulinum ATCC 3502 lacks a general stress response regulator, such as σ^B in other Gram-positive organisms (Boylan *et al*., 1993; Becker *et al*., 1998; Chan *et al*., 1998; Sebaihia *et al*., 2007). Recent evidence has suggested that sporulation and stress response is linked through *sigB* in *B. subtilis* (Reder *et al*., 2012b, 2012c). Given the potential activity of the traditionally sporulation-related σ^K during exponential growth in our previous studies (6.2; I-III), we investigated the role of σ^K under stressful conditions. Expression of *sigK* was evaluated in *C. botulinum* ATCC 3502 after cold, osmotic (NaCl), and acid stress conditions. Furthermore, the *C. botulinum* parent and *sigK*

mutant strains were grown under low temperature, high NaCl, or low pH stress conditions in order to determine if σ^K plays a role in stress response.

Expression of *sigK* in *C. botulinum* ATCC 3502 was induced immediately after cold shock, and expressed again at high levels in the hours following shock. Cold shock in *B. subtilis* induces a similar pattern of *sigB* expression (Méndez *et al*., 2004). σ^B also plays a role in cold adaptation and tolerance in *L. monocytogenes* (Chan *et al*., 2008). Under cold stress, the growth of *sigK* mutant strains was negatively affected compared to the parent strain, suggesting a role for σ^K in low temperature adaptation and tolerance. It should be noted that the ClosTron insertion is unlikely to affect growth at cold temperature in *C. botulinum* (Söderholm *et al*., 2011). This suggests that σ^K may play a comparable role in the cold-stress response of *C. botulinum* ATCC 3502 to that of σ^B in other Gram-positive bacteria.

Upon osmotic shock, expression of *sigK* was induced immediately and, following several hours of low expression, re-induced. The *sigK* mutant strains exposed to NaCl showed an extended lag phase compared to the parent strain, indicating that the mutants required a prolonged adaptation period to the high-NaCl conditions. This suggests that σ^K may play a role in *C. botulinum* ATCC 3502 adaptation to high-NaCl concentrations. Expression of *sigK* was not significantly induced in the parent strain following acid shock. In addition, no difference in growth was observed between the parent and *sigK* mutant strains under low-pH conditions. This indicates that σ^K does not play a role in response to acidic stress in *C. botulinum* ATCC 3502.

Food processing-induced stresses are designed to prevent bacterial growth. Understanding the ability of a neurotoxic organism like *C. botulinum* to respond to and overcome these stresses is of vital importance to the food industry. The suggested interconnection between sporulation and certain stress responses via σ^K in *C. botulinum* ATCC 3502 reveals an interesting new area of research focus. The concept of stress and sporulation being linked is not new, however the known mechanism of action in *B. subtilis* is not conserved in *C. botulinum*. Thus, exploration of these molecular mechanisms in *C. botulinum* may lead to novel strategies in preventing outgrowth from spores and neurotoxin production in foods.

7 Conclusions

1. 16S *rrn* was a suitable normalisation reference gene for RT-qPCR study in *C. botulinum* ATCC 3502. A stable normalisation reference in both exponential and stationary growth phases was required to calibrate relative expression levels of the sporulation regulators *spo0A*, *sigF*, *sigE*, *sigG*, and *sigK* at all time points.

2. The sporulation σ factors σ^F, σ^E, σ^G, and σ^K are essential for viable spore formation in *C. botulinum* ATCC 3502. The mutants of *sigF* and *sigE* halted sporulation at stage II. The *sigG* mutant halted sporulation after stage III, and also showed some spore coat formation. The *sigK* mutant exhibited impaired cell division and halted sporulation prior to initiating the sporulation pathway. Expression of the sporulation regulatory genes was disrupted in all mutant strains. Thus, we surmised that the order of transcription therefore begins with *sigK*, followed by *spo0A*, then by *sigF*, *sigE*, and *sigG* together, and finally ending with *sigK* expression again.

3. σ^K appears to play a role in stress response to cold and NaCl stresses, but not acidic stress, in *C. botulinum* ATCC 3502. Mutants of *sigK* displayed inhibited growth in cold temperatures and at high NaCl concentrations compared to the parent strain. *C. botulinum* cultures, upon cold or NaCl shock, showed increased expression of *sigK* compared to the pre-shocked culture. This indicated a regulatory role for σ^K in stress response and linking the sporulation and stress response pathways.

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