



Spore Germination Apparatus in *Clostridium botulinum* Group I and II

Hannah Pye

MSc by Research

University of East Anglia

Quadram Institute Bioscience

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ABSTRACT

Spore germination is a significant step in the transformation of dormant spores into exponentially dividing vegetative cells, and in the case of *Clostridium botulinum* in the formation of the deadly botulinum neurotoxin. The ability of nutrient and non-nutrient germinants to induce germination of strains Af84 (*C. botulinum* Group I) and Eklund 17B (*C. botulinum* Group II) was established by measuring the change in optical density and by microscopic observation. Germination of strains Af84 and Eklund 17B was initiated by the nutrient germinants L-alanine and L-cysteine and the non-nutrient germinants dodecylamine (DDA) and lysozyme, but not by calcium dipicolinic acid (CaDPA). Heating spores of strain Af84 for 4 hours at 95°C delivered a 3-log reduction in viability. Heat damaged spores of strain Af84 could not be recovered using lysozyme, L-alanine or DDA. Thermal death of spores of strain Eklund 17B occurred within 2 minutes of heating at 85°C, and resulted in a 5-log reduction in viability. The presence of lysozyme (10µg/ml) increased the recovery of heat damaged spores of strain Eklund 17B, however, the presence of L-alanine and DDA had no effect. The timing of DPA release in relation to cortex hydrolysis is still unknown in *C. botulinum*, however this study found that DPA is released within 18 hours of germinant addition in strain Af84 and within 50 minutes for strain Eklund 17B. In addition, the average DPA concentration per spore of strains Af84 and Eklund 17B was shown to be 1.9pg and 2.1pg, respectively. Results obtained indicate that part of the germination apparatus in Group II strain Eklund 17B is damaged by heating, rather than the spore DNA. Understanding the mechanisms involved in spore germination can improve the control of botulinum neurotoxin forming clostridia, prevent foodborne botulism, and thereby contribute to the microbiological safety of foods.

CONTENTS

ABSTRACT	ii
LIST OF FIGURES AND TABLES.....	vi
ABBREVIATIONS	viii
ACKNOWLEDGEMENTS	ix
1. Introduction	1
1.1 Botulinum Neurotoxins	2
1.2 Botulism	5
1.2.1 History of Botulism	5
1.2.2 Foodborne Botulism	6
1.2.3 Wound Botulism	8
1.2.4 Infant Botulism	9
1.3 Aetiology	10
1.3.1 Regular neurotransmission at a synapse	10
1.3.2 Mode of action of botulinum toxin	11
1.4 Uses of Botulinum Toxin	11
1.4.1 Therapeutic	11
1.4.2 Biological Weapon	13
1.5 Endospores	14
1.5.1 Sporulation	14
1.5.2 Spore Structure and Function	15
1.5.3 Germination	18
1.6 Experimental Rationale	22
1.7 Aims	24
2. Materials & Methods.....	25
2.1 Preparation of spores	25

2.2 Assessing the inhibitory effect of lysozyme on growth from spores of <i>C. botulinum</i> Group I and II	26
2.3 Preparation of germinant solutions.....	26
2.4 Germination assay measuring change in optical density.....	27
2.5 Germination assay monitoring percentage of phase-dark spores	28
2.6 Removal of spore coats using dithiothreitol	28
2.7 Removal of spore coats using sodium thioglycolate.....	28
2.8 Thermal death of spores of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B	29
2.9 Heat Inactivation and decoating of spores.....	29
2.10 Recovery of heat inactivated spores with lysozyme	30
2.11 Recovery of heat inactivated spores with L-alanine	30
2.12 Recovery of heat inactivated spores with dodecylamine	31
2.13 Monitoring CaDPA release using Terbium Fluorescence	31
2.14 Quantifying the DPA content per spore of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B.....	32
3. Results	33
3.1 Thermal death of spores of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B	33
3.2 L-alanine and L-cysteine germinate spores of <i>C. botulinum</i> Group I and II	35
3.3 Exogenous CaDPA does not induce germination in <i>C. botulinum</i> Group I strain Af84 or Group II strain Eklund 17B at 30°C	36
3.4 Exogenous CaDPA did not induce germination of coated or decoated spores of <i>C. botulinum</i> Group I strain Af84 or Group II strain Eklund 17B at 45°C	37
3.5 Germination assay using microscopic analyses of spores of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B gave similar results to those using the Bioscreen	38
3.6 DDA induced germination in spores of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B.....	39

3.7 Lysozyme does not have an inhibitory effect on growth from spores of <i>C. botulinum</i> Group I strain Af84 or Group II strain Eklund 17B	40
3.8 Lysozyme induces germination of decoated spores of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B	41
3.9 Lysozyme recovers heat-damaged spores of <i>C. botulinum</i> Group II strain Eklund 17B but not spores of Group I strain Af84	43
3.10 Spores of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B cannot be recovered with DDA after heat inactivation	45
3.11 Spores of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B cannot be recovered with L-alanine after heat-inactivation	47
3.12 Timing of DPA release during germination of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B with L-alanine	48
3.13 The average DPA content per spore of <i>C. botulinum</i> Group II strain Eklund 17B is higher than Group I strain Af84	53
3.14 Summary of Results	57
4. Discussion	58
4.1 Spore thermal death	58
4.2 Spore germination in response to nutrient germinants	59
4.3 Spore germination in response to CaDPA	61
4.4 Spore germination in response to DDA	62
4.5 Spore germination in response to lysozyme	64
4.6 Determination of which part of spore germination apparatus is damaged by heat	65
4.7 DPA content of spores	68
5. Conclusion and Future Research	70
6. References	72

LIST OF FIGURES AND TABLES

Figure 1. Gene arrangement in the botulinum neurotoxin gene clusters <i>orfX+</i> (a) for serotype A, E and F and <i>ha+</i> (b) for serotype A, B, C, D and G.	4
Table 1. Adverse effects resulting from the administration of botulinum toxin to treat a range of medical conditions.....	13
Figure 2. Schematic diagram of the spore structure in Bacillus and Clostridia.	16
Figure 3. Proposed germination pathways in <i>C. botulinum</i> Group I and II.	22
Figure 4. Proposed germination pathways in <i>C. botulinum</i> Group I and II, showing the input of nutrient and non-nutrient germinants and the position they act upon to induce germination.....	24
Figure 5. Number of surviving spores of <i>C. botulinum</i> Group I strain Af84 plated on PYGS medium after heat inactivation at 95°C, at various time intervals.....	33
Figure 6. Number of surviving spores of <i>C. botulinum</i> Group II strain Eklund 17B plated on PYGS medium after heat inactivation at 85°C, at various time points.	34
Figure 7. Anaerobic germination of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B with the nutrient germinants, L-alanine (50mM) and L-cysteine (50mM) in Tris-HCl buffer (20mM, pH 7.4) at 30°C.....	35
Figure 8. Anaerobic germination of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B with the non-nutrient germinant CaDPA (54mM) at 30°C.	36
Figure 9. Anaerobic germination of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B with the non-nutrient germinant CaDPA (54mM) at 45°C.....	37
Figure 10. Percentage of phase dark spores observed over time using phase-contrast microscopy with <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B. Spores were incubated with L-alanine (50mM) and L-cysteine (50mM) in Tris-HCl buffer (20mM, pH 7.4) at 30°C.	38
Figure 11. Anaerobic germination of <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B with the non-nutrient germinant DDA (1.08mM) at 45°C.	39
Figure 12. The effect of lysozyme concentration ($\mu\text{g/ml}$) on anaerobic growth of Group I strain Af84 and Group II strain Eklund 17B at 37°C and 30°C, respectively	40
Figure 13. Anaerobic germination of <i>C. botulinum</i> Group I strain Af84 with lysozyme (10 $\mu\text{g/ml}$) at 37°C.....	41
Figure 14. Anaerobic germination of <i>C. botulinum</i> Group II strain Eklund 17B with lysozyme (10 $\mu\text{g/ml}$) at 37°C.....	42

Figure 15. Number of surviving spores (\pm SD) after heat inactivation (Eklund 17B, 85°C for 2 minutes; Af84, 95°C for 4 hours) and recovery on PYGS agar supplemented with lysozyme (10 μ g/ml)	44
Figure 16. Number of surviving spores (\pm SD) after heat inactivation (Eklund 17B, 85°C for 2 minutes; Af84, 95°C for 4 hours) and recovery on PYGS agar after incubation with DDA (1.08mM) overnight at 45°C.	46
Figure 17. Number of surviving spores (\pm SD) after heat inactivation (Eklund 17B, 85°C for 2 minutes; Af84, 95°C for 4 hours) and recovery on PYGS agar with L-alanine (50mM)	47
Figure 18. Effect of DPA concentration on the relative fluorescence (550nm) following binding to TbCl ₃	48
Figure 19. Timing of DPA release in spores of <i>C. botulinum</i> Group I strain Af84 compared to the change in OD ₆₀₀ observed following the addition of the germinant L-alanine (50mM)..	49
Figure 20. Lack of DPA release and change in OD ₆₀₀ for spores of Group I strain Af84 without the addition of L-alanine (50mM).	50
Figure 21. Timing of DPA release in spores of <i>C. botulinum</i> Group II strain Eklund 17B compared to the change in OD ₆₀₀ observed following the addition of the germinant L-alanine (50mM).....	51
Figure 22. Lack of DPA release and change in OD ₆₀₀ for spores of Group II strain Eklund 17B without the addition of L-alanine (50mM).	52
Figure 23. Calibration curve for autoclaved spores of <i>C. botulinum</i> Group I strain Af84.....	53
Figure 24. Fluorescence reading of DPA released from autoclaved spores of <i>C. botulinum</i> Group I strain Af84 at different dilutions.....	54
Figure 25. Calibration curve for autoclaved spores of <i>C. botulinum</i> Group II strain Eklund 17B	55
Figure 26. Fluorescence reading of DPA released from autoclaved spores of <i>C. botulinum</i> Group II strain Eklund 17B at different dilutions.	56
Figure 27. Revised germination pathway diagram in <i>C. botulinum</i> Group I strain Af84 and Group II strain Eklund 17B, showing whether the nutrient and non-nutrient inputs successfully germinated strains of <i>C. botulinum</i>	71

ABBREVIATIONS

ATP Adenosine Triphosphate

Baby-BIG Baby Botulinum Immune Globulin

Bont Botulinum Neurotoxin

CaDPA Calcium Dipicolinic acid

CDC Centers for Disease Control and Prevention

CFU Colony Forming Units

CLR Collagen-like Repeat

DDA Dodecylamine

DPA Dipicolinic acid/ 2,6-Pyridinedicarboxylic acid

DTT Dithiothreitol

FDA Food and Drug Administration

H Heavy Chain

L Light Chain

MHRA Medicine and Healthcare Product Regulatory Agency

NAG N-acetylglucosamine

NAM N-acetylmuramic

ntnh nontoxin- non haemagglutinin

OD Optical Density

PYGS Peptone-Yeast-Glucose-Starch

RCM Reinforced Clostridial Medium

rDNA Ribosomal Deoxyribonucleic acid

SASPs Small acid-soluble proteins

SDS Sodium Dodecyl Sulphate

SNAP Synaptosome

SNARE Soluble N-ethylmaleimide-Sensitive Factor Attachment Protein Receptors

VAMP Vesicle-Associated Membrane Protein

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1. Introduction

Clostridium is a phylogenetically diverse genus of the phylum *Firmicutes*, encompassing approximately 110 heterogeneous bacteria of both medical and environmental importance (Kalia *et al.*, 2011, Kunisawa, 2015). Members of the order *Clostridiales* are Gram-positive, spore forming, obligately anaerobic bacteria, which include major pathogenic organisms such as *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani* and *Clostridium sordellii* (Swick *et al.*, 2016). Comparison of the genomes of sequenced Clostridia shows wide genetic variability in the genus and even substantial variability amongst organisms of the same species (Sebahia *et al.*, 2007). *C. botulinum* is defined by the ability to form the botulinum neurotoxin and thus the capability to cause botulism in humans and/or animals, rather than by close phylogeny (Peck, 2009). *C. botulinum* can be divided into four distinct groups (I-IV) based on their genetic and physiological properties, and the botulinum neurotoxin can be subdivided into seven serotypes (A to G) (Peck, 2009).

C. botulinum Group I strains are highly proteolytic and can degrade certain carbohydrates, such as glucose. These strains form botulinum neurotoxin of types A, B and/or F. *C. botulinum* Group I can be described as mesophilic with an optimum growth temperature of 37°C and a minimum growth temperature of 10-12°C (Lund and Peck, 2000, Peck *et al.*, 2011). However, in a recent study it was established that *C. botulinum* Group I type B strains grew considerably faster at 42°C than at 37°C (Hinderink *et al.*, 2009). Highly heat resistant spores are produced by *C. botulinum* Group I and the “botulinum cook” (121°C/ 3 min) is given to inactivate spores and thereby prevent subsequent growth in low acid canned foods (Carter and Peck, 2015). Growth and toxin formation by *C. botulinum* Group I is prevented at a pH of ≤ 4.6 or 10% NaCl. The minimum water activity [a_w] for growth of proteolytic *C. botulinum* is 0.96 for NaCl and 0.93 with glycerol (Lund and Peck, 2000). *C. botulinum* Group I strains are closely related to *Clostridium sporogenes* and therefore, it is often used as a surrogate organism for thermal processing experiments in food due to its similar spore heat resistance (Bradbury *et al.*, 2012, McClung, 1937).

C. botulinum Group II (non-proteolytic) are highly saccharolytic bacteria capable of metabolising a wide range of carbohydrates, including glucose, maltose, fructose, sucrose and mannose. *C. botulinum* Group II strains form a single neurotoxin of type B, E or F and have an optimum growth temperature of 25°C-30°C (Lund and Peck, 2000, Peck, 2009). *C. botulinum* Group II grows at a minimum temperature of 2.5-3.0°C and is a concern for

minimally heated chilled foods, such as chilled ready meals. The safety of these foods relies on a combination of mild heat treatment, chilled storage, a limited shelf-life and occasionally preservatives. Growth of *C. botulinum* Group II is prevented by a pH < 5 or a NaCl concentration ≥ 5%. The minimum water activity for growth of *C. botulinum* Group II is slightly higher than the water activity for *C. botulinum* Group I (0.97 and 0.94 for NaCl and glycerol, respectively). Spores of *C. botulinum* Group II are less heat resistant than spores of *C. botulinum* Group I (Peck, 2014).

C. botulinum Group III is mainly associated with avian and non-human mammalian botulism (Skarin *et al.*, 2011). *C. botulinum* Group III is a mesophilic organism with the ability to metabolise sugars, and a minimum and optimum growth temperature of 15°C and 40°C, respectively. Inhibition of growth of *C. botulinum* Group III occurs at a pH of < 5.1 (Lund and Peck, 2000). Strains of *C. botulinum* Group III produce neurotoxin genes of type C or D, however, genetic analysis of isolates from avian species revealed that the neurotoxin gene commonly comprises of two thirds of a gene of one toxin type and one third of a gene of the other toxin type and therefore is often referred to as a type C/D or D/C mosaic neurotoxin (Moriishi *et al.*, 1996, Takeda *et al.*, 2005). Analysis of 16S rDNA sequences indicate that strains of *C. botulinum* Group III are more similar genetically to *Clostridium novyi* and *Clostridium haemolyticum* than *C. botulinum* Groups I and II (Sasaki *et al.*, 2001).

Clostridium argentinense, also called *C. botulinum* Group IV, is a mesophilic bacterium with an optimal growth temperature of 37°C. *C. argentinense* produces toxin type G, however the bacterium has not been associated with human, avian or animal botulism outside of a laboratory (Lund and Peck, 2000, Suen *et al.*, 1988).

1.1 Botulinum Neurotoxins

Seven distinct botulinum neurotoxin serotypes (A-G) have been identified, differing in their amino acid sequence by 37-70% (Hill *et al.*, 2015). An eighth potential neurotoxin type has been identified within the last four years and has been referred to as a variety of names in recent papers, such as serotype H, FA or HA (Dover *et al.*, 2014, Maslanka *et al.*, 2016, Peck *et al.*, 2017). Heterogeneity in these serotypes has led to the identification of approximately 40 subtypes, which have variability of up to 36% in amino acid sequence within each toxin type (Hill and Smith, 2012, Peck *et al.*, 2017). Serotypes A and F have the greatest difference within their subtypes at 16% and 36% respectively, whereas serotype B (7%) and serotype E (6%) show less variance at the amino acid level (Dorner *et al.*, 2012,

Peck *et al.*, 2017). Botulinum neurotoxin of all types consist of a 100-kDa heavy chain (H) connected via a disulphide bond to a 50-kDa light chain (L) (Hatheway, 1990). The heavy chain consists of two functional domains, the C-domain and N-terminal domain, which are involved in neurotoxin binding to the nerve cell and movement of the light chain into the nerve cell cytoplasm (Carter and Peck, 2015). Light chains are zinc-endopeptidases which are active in the nerve cell and function by selectively cleaving proteins involved in the neurotransmitter-vesicle docking and fusion complex, preventing neurotransmitter release which ultimately results in flaccid paralysis, typical of botulism (Poulain *et al.*, 2008, Rossetto *et al.*, 2014).

C. botulinum Group I strains can produce one, two or three neurotoxin of types, A, B and F, and can possess up to three neurotoxin genes located on a large plasmid or chromosome. Whereas, *C. botulinum* Group II strains contain one toxin gene and form a single toxin of type B, E or F (Hill *et al.*, 2009). In *C. botulinum* Group II, the neurotoxin gene for serotype B is situated on a small plasmid, whereas the type F neurotoxin gene is located on the chromosome. The type E botulinum neurotoxin gene can be located on either the chromosome, most commonly, or on a plasmid; however only 6% of Group II strains analysed in a recent study by Carter *et al.* (2016) exhibited neurotoxin-encoding genes located on the plasmid. Some strains producing neurotoxin type F have fragments of both type B and E neurotoxin genes (Carter *et al.*, 2013). In *C. botulinum* Group III, neurotoxin genes are located on bacteriophages, and in *C. botulinum* Group IV the neurotoxin gene is situated on a plasmid (Carter *et al.*, 2016, Zhang *et al.*, 2013). The botulinum toxins that most commonly cause human botulism are serotypes A, B, E and F. Where strains produce two type of toxins, the uppercase letter denotes the dominant toxin (e.g. Ab) (Carter and Peck, 2015).

The botulinum neurotoxin forms progenitor protein complexes via covalent bonds with non-toxicogenic proteins. There are two types of botulinum neurotoxin gene cluster (*orfX+* and *ha+*) (Chen *et al.*, 2008, Connan *et al.*, 2013). The botulinum neurotoxin gene clusters are in the region of 11-14kb, encoding between three and seven proteins to give the neurotoxin complex (Smith *et al.*, 2007). Both clusters, *orfX+* and *ha+*, contain a nontoxin-nonhaemagglutinin encoding gene (*ntnh*) adjacent to the botulinum neurotoxin gene (*bont*) (Smith *et al.*, 2007). The *orfX+* gene cluster (Figure 1(a)) contains a neurotoxin gene (*bont*), a nontoxin-nonhaemagglutinin (*ntnh*) gene, a regulatory gene (*botR*) and a gene (*p47*) of unknown function, and three other genes of unknown function (*orfX1*, *X2*, *X3*). Neurotoxins of serotypes A, E and F are formed by the *orfX+* gene cluster. The second protein complex

found with neurotoxin of serotypes A, B, C, D and G is encoded by the haemagglutinin gene cluster (*ha+*) (Figure 1(b)), in which the *orfX1-X3* genes are replaced with three haemagglutinin encoding genes (*ha70*, *ha17*, *ha33*) (Chen *et al.*, 2008, Hill and Smith, 2012). The *botR* gene is situated at the 5' end of the botulinum locus in toxin serotypes C and D, whereas in *C. botulinum* types A, B and G, the *botR* gene is located between the *ntnh-bont* and *ha* genes (Raffestin *et al.*, 2004). Botulinum neurotoxin type A is the only toxin that can be encoded by either gene cluster (*orfX+* or *ha+*). New subtypes have arisen through recombination events (Hill *et al.*, 2015).

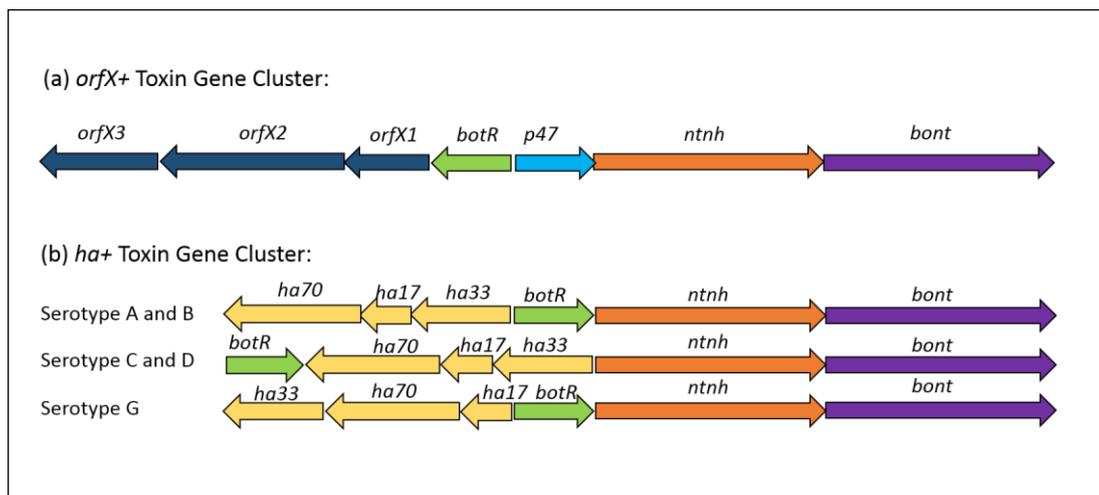


Figure 1. Gene arrangement in the botulinum neurotoxin gene clusters *orfX+* (a) for serotype A, E and F and *ha+* (b) for serotype A, B, C, D and G. *C. botulinum* Group II strains of serotype E and F do not contain the *botR* encoding gene. The botulinum toxin is depicted as *bont* (Adapted from Hill *et al.* (2015)).

A recent study has identified 41 botulinum subtypes which all display inter- and less frequently intra-subtype differences (Peck and van Vliet, 2016). The term subtype can be used to describe variation amongst neurotoxin serotype based on amino acid sequence (Peck *et al.*, 2017). Only strains of *C. botulinum* Group I produce toxin serotype A, and this serotype can be categorised into eight toxin subtypes (A1-A8). Although subtypes exist, type B and E toxins display less than 2.6% variability in their amino acid sequence. The most significant difference in neurotoxin variability occurs in serotype F, where subtypes F5 and F7 show the greatest dissimilarity (Peck and van Vliet, 2016). *C. botulinum* Group II type F carries the F6 neurotoxin gene exclusively, whereas Group I *C. botulinum* type F can carry neurotoxin genes of F1-F5 (Raphael *et al.*, 2010). A novel neurotoxin has recently been proposed in the literature containing regions of type A and F toxin types and therefore is

referred to as type FA (or H/HA) (Dover *et al.*, 2014, Fan *et al.*, 2016, Kalb *et al.*, 2015). As mentioned previously, hybrid neurotoxins composed of both type C and D toxins (often referred to as mosaic neurotoxins) can occur and are formed more frequently than single neurotoxins of type C or D (Woudstra *et al.*, 2012). Hybrid subtypes, composed of two other subtypes, have been identified in the literature, for example, botulinum neurotoxin subtype A2 is an amalgam of subtypes A1 and A3. Likewise, botulinum neurotoxin F6 is a hybrid of subtypes F1 and F2 (Hill and Smith, 2013).

1.2 Botulism

1.2.1 History of Botulism

The first strain of *C. botulinum* was described in 1897 when Emile van Ermengem isolated an anaerobic, spore-forming, toxin producing *Bacillus* from the remnants of a salted ham which had been eaten by 34 musicians in a Belgium restaurant in 1895, resulting in severe neuromuscular disease and 3 fatalities (Collins and East, 1998). However, symptoms of botulism had been reported almost 100 years previously in Germany and a large outbreak of botulism associated with the consumption of a locally produced blood sausage in 1802 resulted in the acquisition of the term 'botulism' from the latin '*botulus*', meaning sausage (Peck, 2014). Historically, *C. botulinum* was named *Bacillus botulinus* by van Ermengem until the separation of the *Bacillus* genus from the *Clostridium* genus due to differences in their aerobic and anaerobic growth requirements (Winslow *et al.*, 1917). An investigation by Landmann in 1904 reported a large outbreak of botulism associated with canned white beans in Darmstadt, Germany. An anaerobic bacterium, similar to the one isolated by van Ermengem, was found to be the causative agent, however, further analysis revealed that the neurotoxin produced was different to the one found previously (Peck, 2014). Heterologous toxins produced by different strains of *C. botulinum* were first recognised by Leuchs in 1910 on production of an antitoxin for the van Ermengem and Landmann strains. During this investigation, Leuchs discovered that the antitoxin produced for one toxin did not protect against the toxin formed by the other strain (Burke, 1919).

Serological methods were introduced shortly after the isolation of the Landmann strain of *C. botulinum* to classify the bacteria by the toxin produced. Antiserum produced by one toxin was used to neutralise toxin or agglutinate bacteria of different isolates, initially resulting in the identification of two toxin serotypes, A and B (Burke, 1919). Agglutination methods separated the *C. botulinum* toxins into different agglutination groups (Schoenholz

and Meyer, 1925). *C. botulinum* continues to be classified on the ability to produce the botulinum neurotoxin, however, repeated sub-culturing of a single strain of *C. botulinum* in a laboratory can result in a loss of toxigenicity and therefore it is difficult to rely on the agglutination method to differentiate between strains of *C. botulinum* (Gunnison and Meyer, 1929, Smith *et al.*, 2015).

In 1947, a simple method using egg yolk agar was introduced to detect botulinum neurotoxin producing strains in mixed cultures (McClung and Toabe, 1947). Strains of *C. botulinum* are distinguished from other organisms in a mixed culture by their interaction with lipase and lecithinase on egg yolk agar. A positive lipase reaction results in colony growth with an oily sheen, whereas a positive lecithinase reaction causes a white opaque ring to appear below the surface of the colony in the agar. *C. botulinum* can be identified on egg yolk agar due to its positive and negative reaction with lipase and lecithinase, respectively. However, closely related organisms, such as *C. sporogenes*, also react with lipase and lecithinase and therefore it may be difficult to differentiate between clostridia (McClung and Toabe, 1947). Some strains of *C. botulinum* produced variable results with lipase and lecithinase and therefore it has been concluded that this method for identifying *C. botulinum* cannot be used as the sole technique (Smith *et al.*, 2015).

1.2.2 Foodborne Botulism

Foodborne botulism is caused by ingesting food contaminated with toxin formed by a strain of *C. botulinum* Group I or Group II. The incubation period for the onset of symptoms of foodborne botulism usually occurs between 2 hours and 8 days after ingestion of contaminated food, depending on the toxin serotype and dose of toxin consumed. The main clinical manifestation of foodborne botulism is a descending, symmetrical flaccid paralysis (Wenham and Cohen, 2008). Patients often present with a combination of symptoms, therefore respiratory failure may occur before botulism is diagnosed (Wictome and Shone, 1998). Suspected botulism cases can often be confused with other diseases with similar symptoms due to the rarity of botulism in the UK (Wenham and Cohen, 2008). The botulinum neurotoxin is the most potent toxin known. Extrapolation from foodborne botulinum outbreaks and primate studies have enabled the lethal dose of botulinum neurotoxin in humans to be estimated as 30-100ng. Outbreaks of foodborne botulism are often associated with improper canning and bottling procedures which are used to enhance a product's shelf life (Peck, 2009). In the USA between 1918 and 1924 there were

230 fatal cases of foodborne botulism reported (Meyer and Eddie, 1965). In August 1922, the first outbreak of foodborne botulism, resulting in eight fatalities, in the UK was associated with the consumption of under-processed wild duck paste (Leighton, 1923). The 'botulinum cook' was developed after research on *C. botulinum* Group I spore heat resistance was conducted and concluded that a standard minimum heat treatment should be given to all low-acid canned foods to prevent *C. botulinum* spore germination, growth and toxin formation (Esty and Meyer, 1922). The number of foodborne botulism cases in Europe exceeded 2500 in 1999/2000 and the majority of cases were reported from Russia (887 cases), Belarus (344 cases) and Azerbaijan (181 cases) (Peck, 2006).

Inadequate processing of canned or bottled foods is not the only source of foodborne botulism. In recent years, outbreaks have been associated with poor refrigeration of foods allowing neurotoxin formation (Peck, 2009). Foodborne botulism caused by *C. botulinum* Group I usually involves neurotoxin types A and B, with outbreaks caused by *C. botulinum* Group II most frequently associated with type B or E toxins (Peck, 2009). One of the largest economically devastating outbreaks of foodborne botulism occurred in Augusta, Georgia (USA) in June 2007 when four cases of botulism were reported and linked to the consumption of contaminated hot dog chilli sauce, which led to more than one hundred million cans being recalled and closure of the manufacturing plant (CDC, 2007). The largest UK outbreak of foodborne botulism occurred in June 1989 and was associated with contaminated hazelnut yoghurt resulting in 27 symptomatic patients and 1 fatality. Hazelnut conserve that had been improperly heat treated was contaminated with spores of *C. botulinum* Group I type B, which germinated leading to growth and neurotoxin formation in the conserve. The conserve, containing the toxin, was then combined with natural yoghurt and sold to the public (O'Mahony *et al.*, 1990). A severe case of botulism was also associated with improper storage of commercial chilled carrot juice in Canada and the USA in 2006. Large amounts of toxin were present in the carrot juice, and it was estimated that consuming approximately 5µl of the contaminated carrot juice would constitute a lethal dose and consequently all six patients, one of which died, required medical ventilation (CDC, 2006, Sheth *et al.*, 2008).

The largest outbreak of botulism since 1978 in the USA was reported in June 2016 and was caused by the consumption of an illicit alcohol drink, termed "hooch", in a medium security federal correctional facility in Mississippi. In total, 31 inmates showed symptoms of botulism after consuming "hooch" and 24 patients required hospitalisation, including 15 patients that were admitted to an intensive care unit and nine that required ventilation

(McCrickard *et al.*, 2017). The botulinum antitoxin was administered to 20 of the 31 patients, and botulinum neurotoxin type A was detected in serum and stool samples. The “hooch” was reportedly prepared by combining honey, potatoes, apples and tomato paste in a plastic bag and allowing it to ferment for 3-5 days at room temperature, however the source of *C. botulinum* contamination remains unknown (McCrickard *et al.*, 2017).

The majority of outbreaks involving *C. botulinum* Group II are associated with home-cured meat and sausages, salted, dried or vacuum-packed fish, and home-made products prepared by the indigenous inhabitants of Alaska and Northern Canada, such as fermented seal flipper, salmon roe, beaver tail and muktuk (Peck, 2009). During the fermentation process of these homemade foods, botulinum neurotoxin (most often type E) can form. Toxin production can occur at a pH >5.0 or if fermentation takes place at temperatures greater than 3°C, i.e. during the soaking or early fermentation stage (Peck, 2014).

1.2.3 Wound Botulism

Wound botulism occurs when a wound is contaminated with spores of *C. botulinum*, which germinate to produce vegetative cells that form the botulinum neurotoxin. Infection at a wound site can lead to abscess formation, which is often caused by a variety of *Clostridium* species (Brett *et al.*, 2004). The milieu of an abscess provides the ideal anaerobic environment to allow *C. botulinum* to thrive and as a result, wound botulism has become increasingly more common in drug abusers (Sobel, 2005). Black tar heroin is the most common form of heroin in Western United States due to the ease of which it can be sourced and as a result there is a strong positive correlation with wound botulism cases in California (Passaro *et al.*, 1998). Injection of black tar heroin subcutaneously or intramuscularly, rather than directly into the veins, is referred to as “skin-popping” and results in abscess formation and skin devitalisation (Werner *et al.*, 2000). Heroin is insoluble in water and thus, it is often dissolved in citric acid to make it suitable for injection, which damages the tissue surrounding the injection site and creates a favourable condition for spore germination and growth of *C. botulinum* (Brett *et al.*, 2004, Sieradzan, 2005). In the UK, there were 170 cases of wound botulism outlined in a Public Health England (PHE) report between 2000 and 2014, and all of these cases were attributed to injecting drug users (Public Health England, 2016). The majority of reported wound botulism cases that have been identified have been caused by *C. botulinum* Group I type A and B (Sieradzan, 2005). Clinically, wound botulism is indistinguishable from foodborne

botulism, apart from the absence of gastrointestinal symptoms. The incubation period for wound botulism is unknown as drug users often inject multiple times during a day (Sobel, 2005). However, the incubation period for the onset of neurological symptoms of wound botulism unrelated to drug abuse has been reported to be between 4-13 days, compared to 6 hours-8 days in foodborne botulism (Hughes *et al.*, 1981).

1.2.4 Infant Botulism

Infant botulism occurs when spores of *C. botulinum* are ingested by infants, usually less than 1 year old. The spores germinate and produce vegetative cells that are capable of forming the botulinum neurotoxin and temporarily colonise the infants' colon due to their poorly developed gut microflora and lack of gastric acid (Arnon, 1980). Infant botulism is the most prevalent form of botulism encountered in the United States with approximately 80-100 cases reported annually (Shapiro *et al.*, 1998). A large epidemiological study in Japan and California revealed that there were 31 cases reported from 1986-2011 and 978 cases reported from 1986-2010 in the two locations, respectively (Dabritz *et al.*, 2014, Kenri *et al.*, 2014). The main clinical manifestations of infant botulism are constipation, lethargy, muscle weakness, abnormal eye movements and poor feeding, progressing to flaccid paralysis and respiratory arrest if untreated (Long, 2007). The majority of infant botulism cases have been associated with botulinum neurotoxin serotype A and B, with type A occurring most commonly in Western United States and type B more prevalent in Eastern United States (Paisley *et al.*, 1995). *C. botulinum* spores are ubiquitous in the environment, however honey and dust have been found to be the main transmitters of the spores leading to infant botulism (Midura, 1996). The number of infant botulism cases related to the consumption of honey has been decreasing in the United States, due to a successful public awareness campaign communicating the potential risk of botulism. As a result, only approximately 20% of infant botulism cases are now associated with honey in the United States, compared to 59% of cases in Europe (Aureli *et al.*, 2002). Infant botulism is rarer in the UK than in the USA and up until 2012, only 12 cases of infant botulism had been reported (Abdulla *et al.*, 2012). Although the infant minimum infective dose of *C. botulinum* spores is not known, it has been hypothesised that as few as 10-100 spores in honey is sufficient to cause infant botulism (Arnon *et al.*, 1979).

In October 2003, the Food and Drug Administration approved the use of human botulism immune globulin (Baby-BIG) in cases of infant botulism. Plasma from adults that have been

immunised with pentavalent botulinum toxoid and that show a high concentration of antibodies able to neutralise botulinum neurotoxin type A and B are used in the development of Baby-BIG (Fox *et al.*, 2005). A 5-year study by Arnon *et al.* (2006) reported a significant reduction in the duration of hospital stays when cases of infant botulism were treated with Baby-BIG (2.6 weeks compared to 5.7 weeks), and also a reduction in the length of time mechanical ventilation and tube feeding was required by 3.2 and 6.4 weeks, respectively. Intravenous Baby-BIG has a half-life of approximately 28 days and a single dose is able to neutralise all botulinum toxin present in the bloodstream for at least 6 months. The ability of the antitoxin to remain in the infant body for up to 6 months eradicates the potential issues of increased botulinum toxin absorption due to vegetative *C. botulinum* cell lyses, arising from antibiotics being administered to treat secondary bacterial infections (Johnson *et al.*, 1979).

1.3 Aetiology

1.3.1 Regular neurotransmission at a synapse

Neurotransmitters are endogenous chemicals capable of sending signals across a synapse from a neuron to a postsynaptic cell. Acetylcholine is a neurotransmitter that is synthesised from acetate and choline (Rossetto *et al.*, 2014). Under resting conditions, neurotransmitters are synthesised in the neuronal cytosol and stored in the cytoplasm of a presynaptic nerve terminal (Jahn and Fasshauer, 2012). On arrival of an action potential at the distal nerve terminal, voltage-gated calcium channels open causing an influx of calcium ions. There are three proteins within the soluble group *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins; synaptobrevin (a vesicle-associated membrane protein (VAMP)), syntaxin and synaptosome-associated protein SNAP-25, that assist with attaching vesicles containing acetylcholine to the cell membrane (Rossetto *et al.*, 2014). Consequently, membrane-associated synaptotagins detect the increased concentration of calcium ions and become activated, leading to the association of three SNARE proteins responsible for docking, fusion and the release of vesicle contents into the synaptic cleft (Heidelberger, 2007). Degraded vesicle and membrane parts are recycled and reused to form new vesicles in the nerve terminal (Fagerlund and Eriksson, 2009). When an action potential arrives at the distal nerve terminal, many vesicles are released in synchrony creating a number of miniature endplate potentials. If the endplate potentials are large

enough to depolarise the muscle cell, then calcium will be released activating the actin-myosin interaction, resulting in muscle contraction (Cohen-Cory, 2002).

1.3.2 Mode of action of botulinum toxin

C. botulinum neurotoxin serotypes A-G all interfere with the transmission of acetylcholine across the neuromuscular junction. The inhibition of acetylcholine release from the presynaptic nerve results in muscle paralysis, the main clinical manifestation of botulism (Burgen *et al.*, 1949). The botulinum neurotoxin can act at different parts of the body that release acetylcholine, including the neuromuscular junction, autonomic ganglia (cluster of ganglion in the autonomic nervous system), postganglionic parasympathetic nerve endings and postganglionic sympathetic nerve endings (Sellin, 1985). Botulinum toxin binds to different gangliosides, such as synaptic vesicle-2 and synaptotagmin I/II, which then cleave the SNARE complex present in motor neurons. SNARE molecules normally function to mediate the fusion of the presynaptic plasma membrane with synaptic vesicles, initiating neurotransmitter release (Risselada and Grubmüller, 2012). The heavy chain (H) of the toxin binds irreversibly to receptors in the presynaptic neuron creating a toxin-receptor complex which is absorbed into the cell by endocytosis. Cleavage of the disulphide bonds between the heavy (H) and light (L) chains causes toxin to be released into the cytoplasm (Sellin, 1985, Stanley and Drachman, 1983). The light chain (L) of botulinum serotype A and E cleave SNAP-25 (a synaptosome-associated protein of 25kDa), serotype B, D, F and G cleave synaptobrevin (VAMP) and botulinum toxin type C cleaves both SNAP-25 and syntaxin (a plasma membrane anchored SNARE), preventing neurotransmitter release (Rossetto and Montecucco, 2008, Rummel *et al.*, 2004).

1.4 Uses of Botulinum Toxin

1.4.1 Therapeutic

The botulinum neurotoxin has been approved for use in treating a range of neurological and non-neurological disorders, mainly due its ability to inhibit neurotransmission at the neuromuscular junction (Gooriah and Ahmed, 2015). The Food and Drug Administration (FDA) has approved the use of botulinum serotypes A and B in the USA. The approved formulations of botulinum toxin type A are onabotulinumtoxinA (A/Ona; Allergan), abobotulinumtoxinA (A/Abo; Ipsen) and incobotulinumtoxinA (A/Inco; Merz Pharmaceuticals), or more commonly referred to as their brand names Botox®, Dysport®

and Xeomin[®], respectively. The only botulinum B toxin that has been permitted for use in the USA is rimabotulinumtoxinB (B/Rima; Solstice Neurosciences), which is more commonly known as Myobloc[®]/ Neurobloc[®] (Hallett *et al.*, 2013). In the late 1970s, botulinum toxin was first used in the treatment of strabismus (misalignment of the eyes) and since then, the FDA has approved its use for this condition and many others (Scott, 2004). Cervical dystonia, characterised by involuntary contractions of cervical muscles, can be treated with botulinum toxin A after it was approved by the FDA in 2000, however treatment failure is reported in approximately 20% of patients due to optimal dosage and dosage interval discrepancies (Comella and Thompson, 2006). Use of botulinum toxin type B can also be administered to treat cervical dystonia; however, it is usually only used if the patient shows resistance to botulinum toxin type A (Albanese *et al.*, 2011).

Abnormal contractions of the surrounding eye areas (blepharospasm) is a neurological condition often treated with botulinum toxin. In 1989, the FDA approved the administration of botulinum toxin type A as an injection into the orbicularis oculi muscle that functions to close the eyelids in order to treat blepharospasm (Hallett *et al.*, 2013). Muscle stiffness (spasticity) resulting from a spinal cord injury, multiple sclerosis, cerebral palsy or a stroke can be treated with botulinum toxin A and B. A randomised-double blind study showed that patients improved by 92.3% after receiving botulinum toxin treatment in their upper arm after suffering from a stroke, compared to 50% in the placebo group (Childers *et al.*, 2004). Approximately 1-2% of the population is affected by chronic migraines, which can be defined as a headache lasting ≥ 15 days per month for ≥ 3 months (Jackson *et al.*, 2012). The use of botulinum toxin A for the treatment of chronic migraines was discovered unintentionally through patients receiving cosmetic Botox[®]. The FDA and Medicine and Healthcare Product Regulatory Agency (MHRA) approved the use of botulinum toxin A for chronic migraines in 2010 in the USA and UK (Gooriah and Ahmed, 2015). Hyperhidrosis is defined as the production of excess sweat from the axilla, palms, face and soles of the feet and has been shown to have serious social implications (Naumann *et al.*, 2013). Intradermal or subdermal injection of botulinum toxin into the area of skin that is producing excess sweat can reduce axillary hyperhidrosis (Dressler, 2012). Adverse effects have been reported with the use of botulinum neurotoxins (Table 1).

MEDICAL CONDITION	TREATMENT	ADVERSE EFFECTS	REFERENCE
CERVICAL DYSTONIA	Botulinum Toxin A and B	Dysphagia, dry mouth, neck weakness	(Comella and Thompson, 2006)
BLEPHAROSPASM	Botulinum Toxin A	Ptosis, aggravated dry eyes, diplopia, lagophthalmos	(Truong <i>et al.</i> , 2008)
CHRONIC MIGRAINE	Botulinum Toxin A	Ptosis, muscle weakness, neck pain, neck stiffness	(Jackson <i>et al.</i> , 2012)
HYPERHIDROSIS	Botulinum Toxin A	Cold and flu-like symptoms, nonaxillary sweating	(Naumann <i>et al.</i> , 2003)

Table 1. Adverse effects resulting from the administration of botulinum toxin to treat a range of medical conditions.

1.4.2 Biological Weapon

Bioterrorism can be described as the intentional use of biological agents, such as bacteria, viruses or fungi, to cause deliberate harm to humans, animals or the environment (Greenfield *et al.*, 2002). This can result in numerous victims in a short period and therefore, it is imperative that healthcare professionals are aware of the potential uses of biological agents as weapons (Keim and Kaufmann, 1999). The Centers for Disease Control and Prevention (CDC) has classified microbial agents capable of being used as bioterror weapons into three groups; A, B and C. Multiple factors, such as mortality rate and dissemination ease, have enabled organisms and their toxins to be grouped into these three categories and category A agents pose the most threat, due to their fatality. Category A agents include variolar major (smallpox), *Bacillus anthracis* (anthrax), *Yersinia pestis* (plague), *Francisella tularensis* (tularemia), filoviruses including Ebola and Marburg haemorrhagic fever, arenaviruses including Lassa (Lassa Fever) and Junin (Argentine haemorrhagic fever) and *C. botulinum* neurotoxin (CDC, 2000). Botulinum neurotoxin was utilised during World War II by the Japanese and thereafter synthesised by the USA, USSR and Iraq for potential use as a biological weapon (Berger *et al.*, 2016). The Japanese cult, Aum Shinrikyō, released aerosolised botulinum toxin at three sites in Japan between 1990 and 1995, however all attempts failed reportedly due to erroneous microbial technique, internal sabotage or issues with aerosol-generating equipment (Tucker, 2000). Botulinum toxin still poses a threat to be used as a biological weapon and hence, the CDC has a well-established surveillance system in place to detect possible bioterrorism events (Shapiro *et al.*, 1997).

1.5 Endospores

1.5.1 Sporulation

Endospore-forming aerobic and anaerobic bacteria, including *C. botulinum*, form endospores as a virulence mechanism in order to survive unfavourable conditions. Initially, the model organism for studying spore development was *Bacillus subtilis*, and this organism was one of the first Gram-positive bacteria to have its genome sequenced, however spore development follows a similar process in most endospore-forming bacteria (Henriques and Moran, 2007, Kunst *et al.*, 1997). The initiation of sporulation due to nutrient depletion is evident in most *Bacillus* species, however there is some debate as to whether the same occurs in all *Clostridium* species (Dürre, 2005). Some *Clostridium* species initiate sporulation in response to an accumulation of end products of metabolism, e.g. *Clostridium acetobutylicum*, where the accumulation of acetone and butanol and a reduction in pH prompts sporulation (Dürre, 2005, Sauer *et al.*, 1995).

Sporulation of *B. subtilis* is initiated at the start of the stationary phase when nutrients are depleted. Histidine sensor kinases (KinA, KinB and KinC) are then activated resulting in the indirect phosphorylation of the transcription factor, Spo0A (a DNA-binding protein) via a multiple component phosphorelay (Stephenson and Hoch, 2002). Asymmetrical division of a cell entering sporulation is initiated by the activation of Spo0A and σ^H (a sporulation specific sigma factor) and results in the production of two daughter cells in a structure termed a sporangium; a mother cell and a forespore (McKenney *et al.*, 2013, Piggot and Hilbert, 2004). The mother cell then engulfs the forespore during a membrane fusion and migration process, similar to phagocytosis, during which the cortex, coat and other internal structures are developed. The forespore advances into the mature endospore and the mother cell lyses to release the endospore after completion (Henriques and Moran, 2007).

Sporulation in *C. botulinum* follows a similar process to *B. subtilis*; the endospore is formed due to asymmetric cell division, followed by engulfment of the forespore by the mother cell. The mother cell provides the forespore with the necessary structural properties to aid development of the mature spore and to maximise its survival (Errington, 2003). The spores are released into the environment when the mother cell lyses and remain metabolically inactive (Setlow, 2014). Spores can remain dormant in the environment for many years and can resist germicidal agents that usually kill non-endospore forming bacteria, such as chemical solvents, ionising radiation, high temperatures, detergents and hydrolytic enzymes (Nicholson *et al.*, 2000).

1.5.2 Spore Structure and Function

Spores of Bacillales and Clostridiales contain multiple similar concentric layers that form a spherical spore (Swick *et al.*, 2016). The outermost layer of the spore is termed the exosporium (Figure 2a), which prevents large molecules from entering the spore. Although the exact function of the exosporium is unknown, it may be involved in attachment and colonisation, because it is the surface layer of the spore that is exposed to the environment containing major spore surface antigens (Ball *et al.*, 2008). It had been argued that some species of *Bacillus*, specifically *B. subtilis*, do not have an exosporium and instead have an exosporium-like layer which is a version of the outermost coat layer (Setlow, 2006). In contrast, spores of *Bacillus megaterium* and *B. cereus* contain an exosporium which is noticeably different to the spore coat in morphology (Manetsberger *et al.*, 2015). The exosporium gene proteins in *B. megaterium* are encoded on a plasmid, which demonstrates that there is an association between plasmid-encoded genes and the formation of the exosporium in *Bacillus* species (Manetsberger *et al.*, 2015). This work by Manetsberger *et al.* (2015) on *B. megaterium* was the first study to demonstrate this relationship in *Bacillus* species. Research on *B. anthracis* has established that the exosporium plays a major role in concealing epitopes in the spore coat, which would otherwise be recognised by macrophages in order to provide protection (Basu *et al.*, 2007). The exosporium is comprised of specific proteins, carbohydrates and lipids, however the exact composition of the exosporium varies between different species and strains of endospore-forming bacteria (Henriques and Moran, 2007, Matz *et al.*, 1970).

The exosporium consists of a paracrystalline basal layer and external hair-like appendages, often termed the 'hairy nap', situated approximately 10-20nm from the coat surface in *Bacillus cereus* and *B. anthracis* (Beaman *et al.*, 1972). Exosporium specific glycoproteins, such as the BclA protein in *B. anthracis*, contain a collagen-like repeat domain (CLR) which contributes to the formation of the hairy nap layer of the exosporium and is involved in the attachment and entry into the host, which influences spore surface proteins (Lequette *et al.*, 2011, Sylvestre *et al.*, 2003, Xue *et al.*, 2011). In *B. anthracis*, the ExsY protein is essential for synthesising the exosporium, whereas in Clostridia the analogous proteins, BclA1 and CdeC, are required for the assembly of the coat and exosporium (Boydston *et al.*, 2006, Paredes-Sabja *et al.*, 2014). The exosporium in *C. sporogenes* differs from the exosporium described in *C. difficile*, which lacks an inner coat space (Brunt *et al.*, 2015, Paredes-Sabja *et al.*, 2014). Limited information is available about the exosporium structure and function in *C. botulinum*, however researchers have found that in a *C. botulinum* Group

I type A strain the exosporium was arranged hexagonally and showed resistance to urea, dithiothreitol, sodium dodecyl sulfate and proteolytic enzymes (Masuda *et al.*, 1980, Takumi *et al.*, 1979).

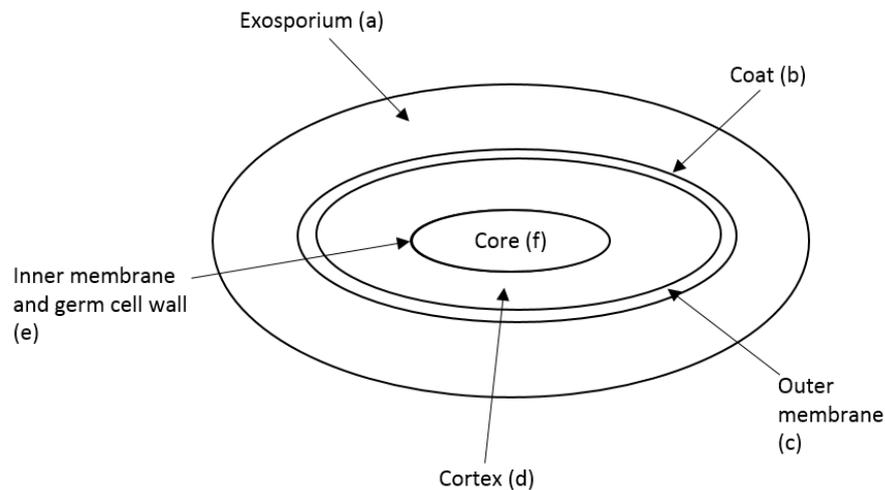


Figure 2. Schematic diagram of the spore structure in *Bacillus* and *Clostridia* (not drawn to scale) (Adapted from Setlow (2006)). N.B. only some species of *Bacillus* and *Clostridia* contain an exosporium.

The spore coat (Figure 2b) is situated within the exosporium and is formed from a varying number of concentric layers, depending on the species of bacteria. *B. subtilis* features two coat layers, an inner and outer, and also a basement layer which forms between the inner coat and the cortex and the outer coat and the spore surface (Aronson *et al.*, 1992). The inner layer of the coat is formed from between three and six lamellae and is approximately 75nm wide in *B. subtilis*, whereas the outer layer consists of electron-dense striations which makes the structure thicker and it can measure up to 200nm wide in some species (Driks, 1999). The majority of the spore coat is made from soluble and insoluble protein, which constitutes approximately 50-80% of the total spore protein (Aronson and Fitz-James, 1976, Pandey and Aronson, 1979). A hydrophilic protein, CotG, is abundant throughout the soluble portion of the spore coat and is a crucial protein in the development of the coat once another protein, CotB, has been incorporated into the mature spore (Sacco *et al.*, 1995). A study by Donovan *et al.* (1987) revealed additional coat proteins, CotA, CotB, CotC and CotD, in the soluble fraction of *B. subtilis* coat which can be deleted without any dysfunction to the formation of the spore coat, however spores

lacking the CotD protein have been found to germinate less efficiently (Leggett *et al.*, 2012). The spore coat functions as an internal barrier to prevent large molecules, such as lysozyme, from accessing the spore cortex (Driks, 1999). Although a fraction of *C. botulinum* Group II spores are permeable to lysozyme following heat treatment (Peck *et al.*, 1993). Molecules that are approximately 8kDa and 2kDa in size are able to pass through the spore coat in *B. cereus* and *B. megaterium*, respectively, which allows the passage of small molecular germinants to reach the germinant receptors in the spore inner membrane (Driks, 1999, Nishihara *et al.*, 1989, Scherrer *et al.*, 1971).

The outer membrane (Figure 2c) is situated under the spore coat and is involved in spore formation (Piggot and Hilbert, 2004). The exact role of the outer membrane during sporulation is unclear, however studies have shown that it does not contribute to the spore's heat, chemical or radiation resistance (Nicholson *et al.*, 2000). The spore cortex (Figure 2d) is situated between the spore coat and germ cell wall, and is composed of peptidoglycan (Atrih *et al.*, 1996). Spore peptidoglycan differs from the peptidoglycan in vegetative cells due to the substitution of approximately 50% of the disaccharides with muramic δ -lactam residues, at alternating positions (Atrih *et al.*, 1996, Warth and Strominger, 1969, Warth and Strominger, 1972). In *B. subtilis*, vegetative cell wall peptidoglycan contains *N*-acetylglucosamine (NAG) and *N*-acetylmuramic (NAM) residues, which are cross-linked via peptide side chains to other glycan strands (Ghuysen, 1968, Warth and Strominger, 1971). However, in *B. subtilis* spore cortex peptidoglycan, approximately half of the NAM residues do not form peptide side chains with other glycan strands and as a result a cyclic muramic δ -lactam is formed (Warth and Strominger, 1972). The muramic δ -lactam structure is exclusive to spore cortex peptidoglycan, and therefore it may contribute to the spore's dormancy and resistance properties (Popham, 2002).

The germ cell wall (or primordial cell wall) (Figure 2e) is also composed of peptidoglycan, however as it transforms into the vegetative cell wall during outgrowth, the components differ from the peptidoglycan found in the spore cortex (Leggett *et al.*, 2012). Degradation of the spore cortex during spore germination but not the germ cell wall results from the structural differences in cortex peptidoglycan and the germ cell wall. The muramic δ -lactam in cortex peptidoglycan, which are not present in germ cell wall peptidoglycan, acts as a substrate for specific cortex-lytic enzymes (autolysins), which degrade the cortex of the spore (Atrih *et al.*, 1998). The inner membrane (Figure 2e) provides the spore core with protection and is the final barrier that prevents harmful molecules from reaching the spore core (Setlow, 2006, Zheng *et al.*, 2016). After germination, the inner membrane develops

into the plasma membrane of the vegetative cell during outgrowth (Zheng *et al.*, 2016). The lipid composition of the inner membrane of a *B. megaterium* spore is similar to the vegetative plasma membrane; however, the protein configuration is remarkably different (Griffiths and Setlow, 2009). The spore's inner membrane contains germinant receptors and SpoVA protein channels, which are absent from vegetative cells (Setlow, 2003).

The spore core (Figure 2f) retains the spore's DNA and metabolic components of the cell (Atrih and Foster, 2002). The spore core is dehydrated, which contributes to spore dormancy. Dependent on the species, ~27-55% of the total wet weight of the spore core is made up of water, compared to 75-80% in a growing cell (Gerhardt and Marquis, 1989). As a result, the amount of free water available in the spore core is minimised, which restricts the movement of large molecules for enzymatic action (Cowan *et al.*, 2003). The spore core also contains dipicolinic acid (pyridine-2,6-dicarboxylic acid, DPA), which is synthesised in the mother cell compartment during sporulation (Setlow, 2006). In *Bacillus* and *Clostridium* species, approximately 5-15% of the total dry weight of the spore contains DPA, which can combine with divalent cations, such as Ca²⁺, to form a 1:1 chelate (CaDPA) (Gerhardt and Marquis, 1989).

1.5.3 Germination

Germination is the process by which dormant spores advance to vegetative cells, via the process of outgrowth (Setlow, 2003). Germination in the model organism, *B. subtilis*, is well documented; however, less is understood about the germination mechanisms involved in *Clostridium* species, including *C. botulinum* (Setlow, 2014). Recently, the germination pathways in *C. botulinum* Group I and II have been proposed by Brunt *et al.* (2016), and this analysis provided the first insight into the differences in germination between these two groups. Germination is activated by the presence of strain and species-specific nutrient germinants that have a low molecular weight, such as amino acids and some sugars (Paredes-Sabja *et al.*, 2011). Spores can also germinate in response to non-nutrient germinants, such as lysozyme, CaDPA and dodecylamine (a cationic surfactant) (Gould, 1969). The germinant receptors in *B. subtilis* are encoded by *gerA*, *gerB* and *gerK* homolog operons, composed of three protein subunits (A, B and C) located in the inner membrane of the spore in a tricistronic operon (termed *gerA* operon homologs) (Brunt *et al.*, 2014, Igarashi *et al.*, 2004). Homologous GerA proteins have been found in other *Bacillus* species and in some *Clostridium* species (Brunt *et al.*, 2014, Moir *et al.*, 2002, Paidhungat and

Setlow, 2002). Proteins A and B are integral membrane proteins, whereas protein C is a peripheral lipoprotein anchored to the membrane via a diacylglycerol chain (Igarashi *et al.*, 2004).

The germinant receptor GerA requires all proteins to be intact for germination to proceed, whereas GerB and GerK can function with only GerBA and GerBC, and GerKC present, respectively. In *B. subtilis*, the germinant L-alanine is recognised by the GerA receptor (Paidhungat and Setlow, 2000). As described earlier the spore coat functions as a sieve to allow small molecular germinants to penetrate through the coat and gain access to the inner membrane (Moir and Cooper, 2015). A recent study on *B. anthracis* revealed that the absence of GerP in the spore coat reduces the efficiency of nutrient germinant stimulated germination and suggested that the proteins encoded by the *gerP* operon affect spore coat permeability (Carr *et al.*, 2010). *B. megaterium*, a bacterium often found in soil and named due to its large size, is relatively distinct from other members of the *Bacillus* genus, especially *B. subtilis* and *B. cereus* (Eppinger *et al.*, 2011, Ramirez-Peralta *et al.*, 2013). There are six germinant receptor operons within the *B. megaterium* genome and *gerU* (a plasmid-borne operon) has been identified as the tricistronic operon that triggers germination in response to glucose, leucine, proline and some inorganic salts (Christie and Lowe, 2007, Christie *et al.*, 2010a). A recent study by Brunt *et al.* (2014) has indicated germinant receptors in *C. botulinum* and *C. sporogenes* are composed of three protein subunits (A, B and C), according to the GerX subtype. *C. botulinum* Group I possess three germinant receptor types (GerX1, GerX2 and GerX3), whereas, *C. botulinum* Group II only has one class of germinant receptor (GerX3) (Brunt *et al.*, 2016).

The mechanism by which germinant receptors are activated by nutrient/ non-nutrient germinants is not fully understood, however it has been proposed that a conformational change happens to the protein when a germinant binds (Moir and Cooper, 2015). In *C. botulinum* Group I and its closely related surrogate, *C. sporogenes*, germinant receptors are activated by a combination of amino acids with L-lactate/ NaHCO₃, however some studies have reported variable effects of using L-lactate/ NaHCO₃ (Broussolle *et al.*, 2002). In contrast, the addition of L-lactate as a germinant is essential for germination of *C. botulinum* Group II strains (Plowman and Peck, 2002). One study in particular has reported that *C. botulinum* Group I and *C. sporogenes* germinated in the presence of L-alanine, suggesting that these species may have a GerA germinant receptor, or another homologous receptor that is activated by L-alanine, similar to the germinant receptor that binds L-alanine in *B. subtilis* (Broussolle *et al.*, 2002). Amino acid stimulated germination in *C.*

botulinum Group I strain ATCC3502 requires two type 1 GerXA germinant receptors (Brunt *et al.*, 2014). On receipt of a germinant and the initiation of a signal transduction, the spore is committed to germinate and cannot revert to dormancy, even after the removal of the germinant (Stewart *et al.*, 1981).

During stage I of germination in *B. subtilis*, cations (H^+ and Zn^{2+}) are excreted from the spore core, resulting in an increase in pH from ~6.5- 7.7 which aids 3-phosphoglycerate metabolism, generating ATP (Nerandzic and Donskey, 2010). Then, a large portion of a 1:1 chelate of CaDPA in the spore core is released via SpoVA channels, which in turn causes an influx of water to the spore core contributing to the loss in heat resistance (Li *et al.*, 2012). The SpoVA proteins, specifically SpoVAD, function to package CaDPA during sporulation and create a mechanosensitive channel in which this large store of CaDPA can be expelled from the spore core during germination (Li *et al.*, 2012). Three SpoVA proteins have been identified, however, the mechanism by which these proteins interact with each other and their surrounding proteins is still poorly understood (Brunt *et al.*, 2016). During the second stage of germination in *B. subtilis*, cortex lytic enzymes, CwlJ and SleB, initiate peptidoglycan degradation in the spore cortex (Fukushima *et al.*, 2002, Popham *et al.*, 1996). Cortex lytic enzymes are located in different parts of the spore; CwlJ is synthesised in the mother cell and confined in the spore by YwdL (GerQ) (spore coat protein), whereas, SleB is synthesised in the forespore during sporulation and is situated in both the inner and outer membrane in association with the protein YpeB (Chirakkal *et al.*, 2002, Ragkousi *et al.*, 2003). There is also evidence in *B. anthracis*, that another cortex lytic enzyme SleL is involved in further degrading large fragments of peptidoglycan produced by CwlJ and SleB, so that they are small enough to pass through the spore coat (Lambert and Popham, 2008). The main cortex lytic enzyme involved in germination of *B. megaterium* strain QM B1551 is the outcome of transglycosylation relating to SleB lytic activity (Christie *et al.*, 2010b). However, in the absence of the cortex lytic enzymes CwlJ and SleB, germination in *B. megaterium* can still proceed due to the presence of SleL (an N-acetylglucosaminidase), which initiates degradation of the spore cortex during the second stage of germination (Üstok *et al.*, 2014, Üstok *et al.*, 2015). Spores of some *Clostridium* species also contain the cortex lytic enzymes CwlJ and SleB, whereas spores of *C. difficile*, *C. perfringens* and *C. botulinum* Group II contain the cortex lytic enzyme SleC, which hydrolyses peptidoglycan and is required for germination (Miyata *et al.*, 1995, Setlow, 2003). The breakdown of the cortex results in the complete rehydration and swelling of the spore core, which stimulates the inner membrane proteins and lipids to become mobile, terminating spore dormancy

(Moir and Cooper, 2015). Many other spore formers, including *C. perfringens* follow a similar germination pathway to *B. subtilis*, in which CaDPA release is followed by cortex hydrolysis, however, this process is reversed in *C. difficile* as cortex hydrolysis precedes CaDPA release (Francis *et al.*, 2015, Paidhungat *et al.*, 2001). In *C. botulinum* Groups I and II, it is still unknown whether CaDPA release precedes or follows cortex hydrolysis (Figure 3) (Brunt *et al.*, 2016).

The germination mechanisms in *C. botulinum* are still poorly understood, however, by gathering information from a variety of sources relating to *B. subtilis* and *C. perfringens*, and from experimental data, the germination pathways in *C. botulinum* Group I and II have recently been proposed for the first time (Figure 3) (Brunt *et al.*, 2016). Strains of *C. botulinum* Group I follow a similar germination pathway to *B. subtilis*; germinant receptors recognise nutrient germinants, CaDPA is released through SpoVA channels, cortex lytic enzymes (CwlJ and SleB) are activated, the cortex is hydrolysed via peptidoglycan cleavage, the coat and membrane is degraded, resulting in the initiation of lipid and protein metabolism and finally cell outgrowth occurs. Spores of *C. botulinum* Group II follow a germination pathway more similar to *C. perfringens*; nutrient germinants are recognised by their corresponding germinant receptors, CaDPA is released via SpoVA channels and the cortex lytic enzyme (SleC) is activated by the protein CspB. Afterwards, the cortex is hydrolysed and then membrane and coat degradation occurs, resulting in macromolecule metabolism and cell outgrowth (Figure 3) (Brunt *et al.*, 2016). The genes required for cell outgrowth are equivalent to those required in vegetative cell growth (Murray *et al.*, 1998, Nessi *et al.*, 1995).

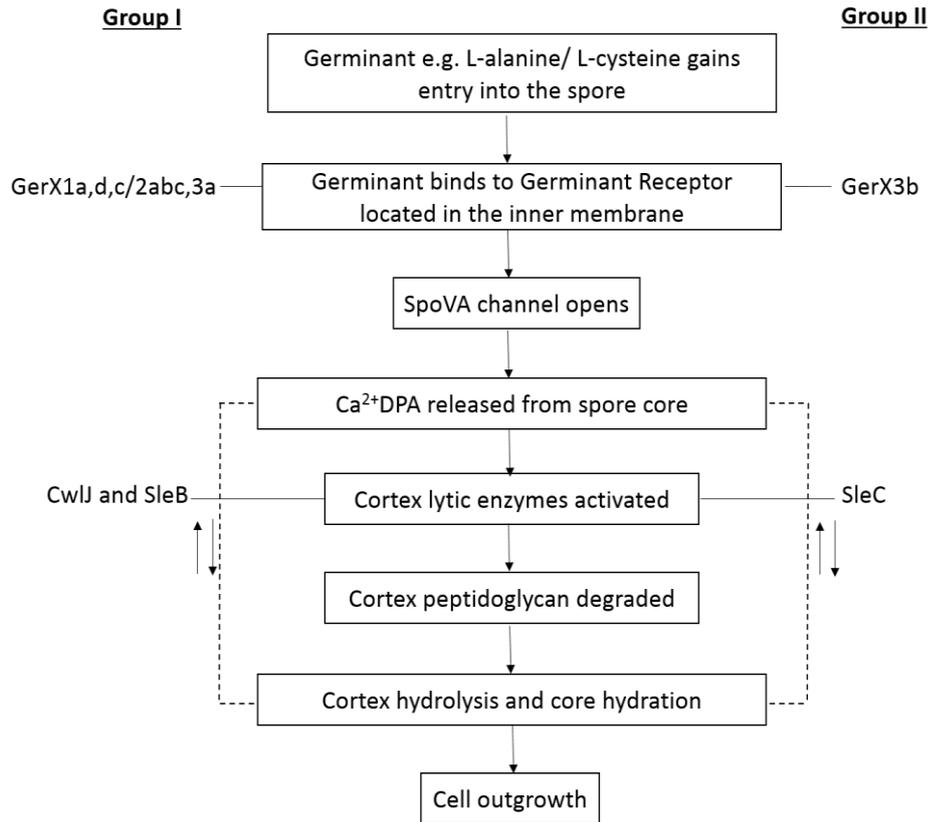


Figure 3. Proposed germination pathways in *C. botulinum* Group I and II. It is presently unknown whether cortex hydrolysis and core hydration precedes or follows CaDPA release (Adapted from Brunt *et al.* (2016)).

1.6 Experimental Rationale

To further understand the proposed germination pathways in *C. botulinum* Group I and II, a selection of nutrient and non-nutrient germinants have been used that act on different parts of the spore. Nutrient germinants, such as L-alanine, activate the germinant receptors located in the spore's inner membrane (Brunt *et al.*, 2014). A range of amino acid germinants, including L-alanine and L-cysteine have been shown to germinate *C. botulinum* Group II (Plowman and Peck, 2002). Using this combination of germinants, germination occurred within six hours and resulted in $\geq 90\%$ phase dark spores at 20°C (Plowman and Peck, 2002). Spores of *C. botulinum* Group I can be triggered to germinate with L-alanine/L-lactate or L-cysteine/L-lactate in the presence of a neutral buffer, such as Tris-HCl (pH 7.0) containing NaHCO_3 (Rowley and Feeherry, 1970). The study by Plowman and Peck (2002) demonstrated that L-cysteine and L-alanine were the germinants that induced the most rapid germination in *C. botulinum* Group II, and therefore these amino acids have been selected as the nutrient germinants for both strains of *C. botulinum* in the present study.

DDA induces CaDPA release and cortex degradation in spores of *B. subtilis*. In a previous study, spores lacking CwlJ, SleB or both cortex lytic enzymes germinated successfully with DDA, indicating that DDA does not act directly on the cortex lytic enzymes to initiate germination (Paidhungat and Setlow, 2002). DDA may germinate spores by altering the spore's inner membrane properties, suggesting that DDA acts on SpoVA channels responsible for packaging (during sporulation) and release (during germination) of CaDPA (Setlow *et al.*, 2003). Exogenous CaDPA has been shown to germinate spores of *B. subtilis* by activating the CwlJ protein (Paidhungat *et al.*, 2001). Germination with lysozyme bypasses germinant receptors and cortex lytic enzymes, and directly hydrolyses the spore cortex initiating spore germination (Vepachedu and Setlow, 2007). A study by Peck *et al.* (1992a) demonstrated that spores of *C. botulinum* Group II strain Eklund 17B are damaged by heating at 85°C for 1 minute and can be recovered by lysozyme. Using the germinants mentioned, it is possible to dissect the proposed germination pathway in *C. botulinum* Groups I and II to determine which part of the spore is damaged by heat (Figure 4). Understanding the mechanisms involved in spore germination can improve the control of botulinum neurotoxin forming Clostridia. Knowledge of how spore germination can affect food safety will be beneficial to industry and may contribute to a greater effort to enable consumer demands for novel foods (e.g. more fresh-like minimally processed) to be realised.

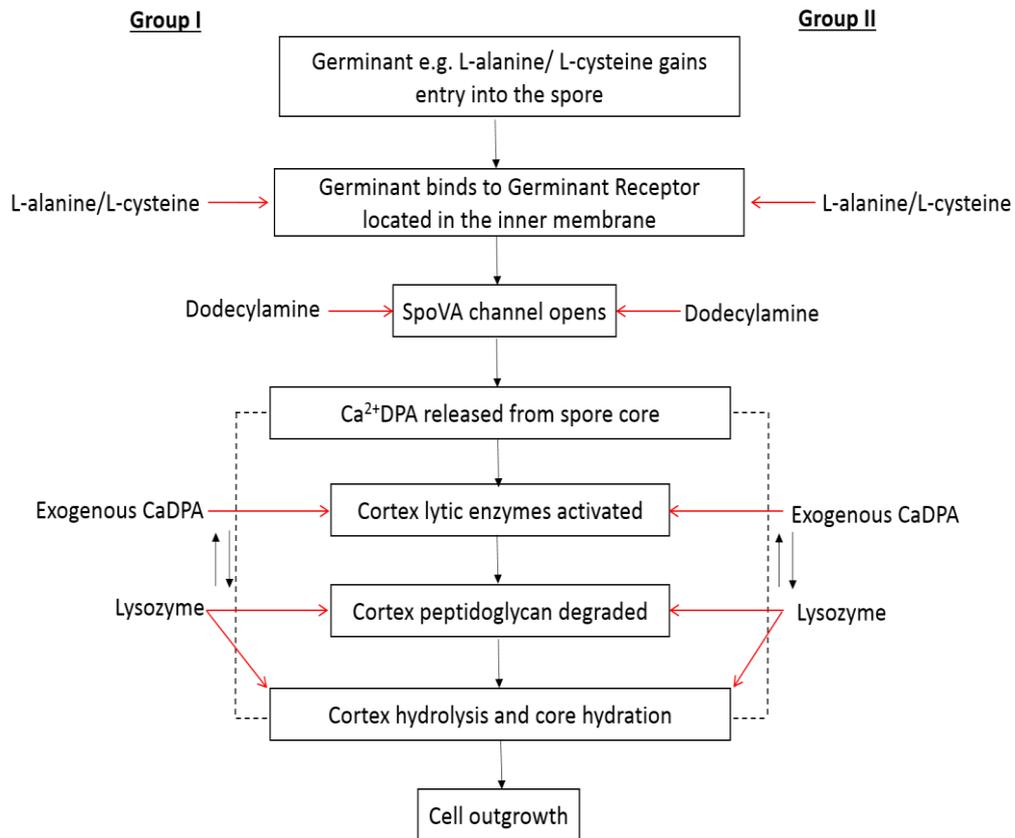


Figure 4. Proposed germination pathways in *C. botulinum* Group I and II, showing the input of nutrient and non-nutrient germinants and the position they act upon to induce germination. It is unknown whether cortex hydrolysis and core hydration precedes or follows CaDPA release (Adapted from Brunt *et al.* (2016)).

1.7 Aims

The formation of highly resistant spores is one reason that *C. botulinum* Groups I and II are dangerous pathogens, with the other major reason being the ability to form the highly potent botulinum neurotoxin during growth of vegetative cells. Spore germination is the key step connecting spores to neurotoxin-forming vegetative cells. The aim of this study is to evaluate the proposed germination pathways in *C. botulinum* Group I and II as described by Brunt *et al.* (2016), by using a range of physiological techniques. The effect of heat on the germination apparatus of *C. botulinum* Group I and II will be evaluated by heating to 95°C and 85°C, respectively. The timing of CaDPA release from the spore core is presently unknown in *C. botulinum*, and techniques outlined in a recent paper by Francis *et al.* (2015) will be used to determine whether CaDPA release precedes or follows cortex hydrolysis.

2. Materials & Methods

2.1 Preparation of spores

C. botulinum Group I strain Af84 was isolated from soil in Argentina in 1966 and forms three botulinum neurotoxins (subtypes A2, F4, F5). *C. botulinum* Group II strain Eklund 17B was isolated from Pacific sediments in 1965 and forms one botulinum neurotoxin (subtype B4). *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B were grown on PYGS (peptone-yeast-glucose-starch) (Oxoid) agar at 30°C overnight in an anaerobic cabinet (Don Whitley) containing CO₂:H₂:N₂ (5:10:85). Colonies formed on PYGS agar were used to make a Microbank™ (Pro-Lab Diagnostics) bead stock, and stored at -80°C until required. To grow *C. botulinum* from the bead stock, one Microbank™ bead of Group I strain Af84 and Group II strain Eklund 17B was transferred to a 20ml Robertson's Cooked Meat broth (Southern Group Laboratories) that had been reduced by heating for 15 minutes at 100°C prior to bead inoculation and subsequent incubation at 30°C overnight in an anaerobic cabinet.

Spores of Group I Strain Af84 and Group II strain Eklund 17B were produced in tissue culture flasks (Helena Biosciences) containing 40ml Cooked Meat Medium. Cooked Meat Medium was prepared by macerating Robertson's Cooked Meat broth, using an Omni 2000 tissue homogeniser (Camlab), and adding 15g agar per litre (Oxoid) and 1g glucose per litre (Fisher Chemical). Tissue culture flasks were inoculated with 3ml of an overnight culture of each strain grown in Robertson's Cooked Meat broth. Inoculated tissue culture flasks were incubated at 30°C in an anaerobic cabinet for one week. Spores were harvested with 20ml sterile ultra-pure water and approximately eight glass beads (per flask) and transferred to sterile 50ml centrifuge tubes. The spore suspension was centrifuged (Sorvall™) (12000g, 15 minutes, 4°C) and the supernatant discarded, leaving a pellet. The pellet was resuspended in 1ml sterile ultra-pure water, vortexed and washed (12000g, 15 minutes, 4°C) three times with 20ml sterile ultra-pure water. After the final wash, the pellet was resuspended in 2ml sterile ultra-pure water. Spores were purified using 50% concentration Gastrografin (Bayer) by centrifugation (8000g, 60 minutes, 4°C). The resultant spore pellet was washed three times with 20ml water (10000g, 15 minutes, 4°C). Clean spore suspensions consisted of >95% phase-bright spores, as determined by visual assessment of approximately ten fields of view using phase contrast microscopy at 40x magnification (Zeiss). Spores were diluted with sterile ultra-pure water to an optical density (OD₆₀₀) of ~0.5 and stored at 4°C until required. Purity checks were performed by inoculating spores crops onto PYGS agar plates and Reinforced Clostridial Medium containing 5% (w/v) skimmed milk and incubated at 30°C overnight in an anaerobic cabinet.

2.2 Assessing the inhibitory effect of lysozyme on growth from spores of *C. botulinum* Group I and II

PYGS agar was prepared in individual Duran bottles and sterilised by autoclaving (121°C/15 minutes). A 10mg/ml stock solution of Chicken Egg White Lysozyme (Sigma-Aldrich) was prepared in ultra-pure water and filter sterilised using a 0.22µm filter (Sartorius Stedim). In a Laminar flow cabinet (Wolf Laboratories), differing amounts of the stock solution of lysozyme were added to the liquid PYGS to a final concentration of 0, 1, 3, 10, 30 and 100µg/ml of lysozyme. Each PYGS/lysozyme solution was poured into individual triple vent petri dishes (Thermo-Fisher) and transferred to separate anaerobic cabinets set to a temperature of 30°C and 37°C to reduce overnight. Spores of Group I strain Af84 and Group II strain Eklund 17B were serially diluted (10^0 - 10^{-5}) in saline (0.85%) and 100µl of the final dilution of each strain was spread onto each agar plate at each lysozyme concentration using a sterile L-shaped spreader (Thermo-Fisher), in triplicate. Inoculated plates were incubated at 30°C for strain Eklund 17B and 37°C for strain Af84 for 2 days. Colonies were enumerated after 2 days, and the viable count calculated for each concentration of lysozyme.

2.3 Preparation of germinant solutions

Tris-HCl (20mM, pH 7.4) (Sigma-Aldrich) buffer was prepared and supplemented with 100mM NaHCO₃ (Fisher Scientific) and 100mM Sodium L-lactate (Melford). A control buffer was prepared containing only Tris-HCl (20mM, pH 7.4) buffer. The nutrient germinants L-alanine (50mM) and L-cysteine (50mM) were prepared in Tris-HCl (20mM, pH 7.4) buffer, supplemented with NaHCO₃ (100mM) and Sodium L-lactate (100mM). A 1.2mM solution of dodecylamine (DDA) (Sigma- Aldrich) was prepared by adding 10mg of crystalline solute to 50ml sterile ultra-pure water and heating at 55°C in a hybridisation oven overnight to allow DDA to dissolve. DDA was stored at 55°C and used within one week. For germination with CaDPA (60mM), a 120mM stock of 2,6-Pyridinedicarboxylic acid (DPA) (Sigma-Aldrich) was prepared and adjusted to pH 8.0-8.3 using Tris Base (Sigma-Aldrich) and a 120mM solution of CaCl₂ (Sigma-Aldrich) was prepared in 30ml water. A 10µg/ml stock of chicken egg white lysozyme (Sigma-Aldrich) was prepared in Tris-HCl buffer (20mM, pH 7.4). Each germinant solution was filter sterilised (0.22µm filter) and freshly prepared for each experiment.

2.4 Germination assay measuring change in optical density

Spores of strain Af84 and strain Eklund 17B were heat activated in a water bath for 15 minutes at 80°C and 60°C, respectively, and held on ice before use within one hour. The effect of various nutrient and non-nutrient germinants on spore germination was measured using a Bioscreen C analyser system (Labsystems) to measure the fall in OD₆₀₀. Amino acid germinant (50µl), L-alanine or L-cysteine, was dispensed into a well of a honeycomb plate (Steri) and then 50µl of spore suspension added (OD₆₀₀ ~0.5). Each test was performed in triplicate. Controls containing Tris-HCl (20mM, pH 7.4) buffer and spores of each strain were also added in the same volumes. The Bioscreen was positioned inside an anaerobic cabinet and the temperature set to 30°C. For germination of spores with DDA, 1ml of 1.2mM DDA was combined with 30µl potassium phosphate buffer (1M, pH 7.6) and stored at 45°C, DDA/potassium phosphate solution (100µl) was added to wells containing 10µl spores (OD₆₀₀ ~ 0.5). Each test was performed in triplicate and the temperature set to 45°C. CaDPA (90µl, 60mM) was added to wells before the addition of a 10µl spore suspension (OD₆₀₀ ~ 0.5) of each strain and the temperature was set to 30°C or to 45°C. Controls containing Tris-HCl (20mM, pH 7.4) buffer and spores of each strain were also added in the same volumes. Germination of spores with lysozyme (10µg/ml) was performed using 50µl of lysozyme (10µg/ml) with 50µl (OD₆₀₀ ~ 0.5) spores of each strain, in triplicate. Controls containing Tris-HCl (20mM, pH 7.4) buffer and spores of each strain were also added in the same volumes. The Bioscreen was used inside an anaerobic cabinet and the temperature was set to 37°C. Spore germination with lysozyme (10µg/ml) was also determined using a haemocytometer (C-Chip™, Digital Bios). The number of spores present in 20 squares was enumerated at the beginning of the experiment and after 24 hours of incubation with lysozyme (10µg/ml).

At the end of all germination experiments, a 5µl sample from each well was examined by phase-contrast microscopy (Zeiss, 40x magnification) to estimate the extent of germination.

2.5 Germination assay monitoring percentage of phase-dark spores

A separate experiment was performed with L-alanine, L-cysteine and CaDPA using screw-cap tubes (2ml) in the same ratio of germinant to spores as used in Bioscreen experiments, and the conditions reflected those used during germination experiments. Samples (5 μ l) were taken every 10 minutes during the first hour for *C. botulinum* Group II and then hourly thereafter, and every hour for *C. botulinum* Group I. The percentage of phase-dark spores was estimated by visual representation of 10-fields of view using phase-contrast microscopy (40x magnification).

2.6 Removal of spore coats using dithiothreitol

Spore coats were removed prior to incubation with lysozyme, CaDPA or DDA for germination experiments. For removal of spore coats, spore crops were harvested by centrifugation (12000g, 15 minutes, 4°C), resuspended in 0.2ml sterile ultra-pure water and added to 1.6ml of decoating solution. The decoating solution was prepared by combining 1% SDS (Sigma-Aldrich), 0.1M 1,4-dithiothreitol (Sigma-Aldrich), 0.1M NaCl (Sigma-Aldrich), pH 10 (adjusted with 2M NaOH) (Sigma-Aldrich). The spore/decoating solution was then placed in a 37°C incubator and shaken at 30 minute intervals for two hours. The spores were harvested by centrifugation (12000g, 15 minutes, 4°C), washed three times in 20ml sterile ultra-pure water (12000g, 15 minutes, 4°C) and diluted with sterile ultra-pure water to an OD₆₀₀ ~0.5. Decoated spore suspensions were stored at 4°C and used within 48 hours of preparation.

2.7 Removal of spore coats using sodium thioglycolate

A 2M solution of sodium thioglycolate (Sigma-Aldrich) was prepared in ultra-pure water and adjusted to pH 10.0 with 6N NaOH (Sigma-Aldrich). The decoating solution was filter sterilised (0.22 μ m filter) and prepared fresh for each experiment. To decoat spores of Group I strain Af84 and Group II strain Eklund 17B, 5ml of sodium thioglycolate solution was added to the spore pellet (OD₆₀₀ ~0.5) and incubated at 45°C for 30 minutes. The decoated spores were then harvested and washed three times with 20ml sterile cold ultra-pure water using a centrifuge (15000g, 15 minutes, 4°C) and the spore pellet was resuspended in 100 μ l sterile ultra-pure water. Sodium thioglycolate decoated spores were

used in heat inactivation and recovery experiments with lysozyme, DDA and L-alanine. Spores were decoated prior to each experiment and used within one hour.

2.8 Thermal death of spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B

The submerged tube method was used to determine the thermal death of Group I strain Af84 and Group II strain Eklund 17B, essentially according to Peck *et al.* (1992). Sorenson's phosphate buffer (9.9ml, pH 7.0, 67mM) was dispensed into HACH tubes and sterilised by autoclaving (121°C, 15 minutes). The HACH tubes containing Sorenson's buffer solution were submerged in a water bath at 95°C and 85°C for Group I and II, respectively. When the temperature in the water bath reached 95°C (Group I) and 85°C (Group II), 100µl of spores (OD₆₀₀ ~ 0.5) of Group I strain Af84 or Group II strain Eklund 17B was added to each tube using a Gas Tight Hamilton syringe and 21Gx 1.5" needle (Terumo).

For Group I strain Af84 the tubes were removed at 1, 2, 3, 4, and 6 hours after spore inoculation and plunged into ice. Controls contained 100µl unheated spores and 9.9ml Sorenson's phosphate buffer (pH 7.0, 67mM). After each time point, the heated spore suspension was serially diluted (10⁰-10⁻⁵) in saline (0.85%), and 100µl of each dilution was spread onto PYGS plates (in triplicate). The plates were incubated for 1 week at 30°C in an anaerobic cabinet and the number of colonies formed was counted daily. The experiment was repeated at 85°C with Group II strain Eklund 17B at 0, 1, 2, 5, 10 and 20 minutes.

2.9 Heat Inactivation and decoating of spores

Spores were heat inactivated by inoculating two HACH tubes containing 9.9ml Sorenson's phosphate buffer (pH 7.0, 67mM) with 100µl spores (OD₆₀₀ ~0.5), following the submerged tube method as above. Each set of tubes were heated for 4 hours at 95°C for *C. botulinum* Group I strain Af84 and 2 minutes at 85°C for *C. botulinum* Group II strain Eklund 17B. Two unheated HACH tubes containing 9.9ml Sorenson's phosphate buffer (pH 7.0, 67mM) were also injected with 100µl spores of each strain (OD₆₀₀ ~0.5) as a control. Heat treated and control samples were transferred to a 15ml Falcon tube and centrifuged (1558g, 15 minutes, 4°C).

To decoat spores using dithiothreitol, spore pellets were resuspended in 200µl ultra-pure water and 1.6ml dithiothreitol decoating solution was added. The spore suspension was

incubated at 37°C for 2 hours, shaking every 30 minutes to remove spore coats. Following incubation, spores were harvested by centrifugation (1558g, 15 minutes, 4°C) and the resulting pellet was washed three times with 1ml ultra-pure water (1558g, 15 minutes, 4°C). The spore pellet was resuspended in 100µl ultra-pure water to an OD₆₀₀ ~0.5 and re-inoculated into a fresh HACH tube containing 9.9ml Sorenson's phosphate buffer (pH 7.0, 67mM).

To decoat spores using sodium thioglycolate, the heat inactivation process was the same and 5ml decoating solution was added to the resulting spore pellet after harvesting (15000g, 15 minutes, 4°C). Tubes were incubated at 45°C for 30 minutes and washed three times with 20ml cold ultra-pure water (15000g, 15 minutes, 4°C). The spore pellet was resuspended in 100µl ultra-pure water and inoculated into new HACH tube containing Sorenson's phosphate buffer (pH 7.0, 67mM).

2.10 Recovery of heat inactivated spores with lysozyme

Heat inactivated and unheated spore suspensions (coated or decoated) of both strains were serially diluted (10^0 - 10^{-5}) in saline (0.85%) and spread onto PYGS plates and PYGS plates containing lysozyme (10µg/ml), in triplicate. Plates of Group II Eklund 17B strain were incubated at 30°C and plates of Group I strain Af84 were incubated at 37°C in an anaerobic cabinet. Colonies were enumerated after 2 day incubation and the viable count (CFU/ml) determined.

2.11 Recovery of heat inactivated spores with L-alanine

To determine whether a nutrient germinant could recover heat inactivated spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B, spores were plated on PYGS plates, supplemented with 50mM L-alanine. Spores were heat inactivated as above, and coated and decoated spores were plated onto PYGS plates and PYGS plates containing 50mM L-alanine. Plates of Group II Eklund 17B strain were incubated at 30°C and plates of Group I strain Af84 were incubated at 37°C in an anaerobic cabinet. Colonies were enumerated after 2 day incubation and the viable count (CFU/ml) determined.

2.12 Recovery of heat inactivated spores with dodecylamine

After heat inactivation and decoating of spores by either dithiothreitol or sodium thioglycolate, an aliquot (50µl) of spores (decoated and coated) was incubated with 1.03ml of 1.17mM DDA and 30mM of potassium phosphate solution overnight at 45°C. The DDA treated samples were washed three times with 1ml sterile ultra-pure water in a centrifuge (12000g, 4°C, 10 minutes) and resuspended in the original volume (50µl) with sterile ultra-pure water. All 50µl treatment samples were diluted (one in ten) with Sorenson's phosphate buffer (pH 7.0, 67mM) and serially diluted in saline (0.85%) (10^0 - 10^{-5}). An aliquot (100µl) of each sample was spread onto PYGS plates and incubated for two days at 37°C or 30°C for Group I and Group II, respectively. After two days of incubation, the plates were enumerated and the viable count (CFU/ml) determined. The PYGS plates remained in the anaerobic cabinet for a maximum of seven days and were checked periodically to measure any increase in number of colonies formed.

A minimum detection limit for calculating the viable count was set at less than five colonies per plate for all experiments.

2.13 Monitoring CaDPA release using Terbium Fluorescence

To produce a calibration curve for DPA fluorescence, a 20µM and 200µM stock of DPA was prepared (see above). A 1600µM stock of Terbium (III) Chloride ($TbCl_3$) (Sigma-Aldrich) was also prepared in sterile ultra-pure water. Wells of an opaque 96-well plate (Thermo Scientific) were filled with 100µl of the following concentrations of DPA (µM); 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 (diluted with Tris-HCl, 20mM, pH 7.4). To each well, 100µl $TbCl_3$ (1600µM) was added to give a final concentration in each well of 800µM. Fluorescence was measured using a FLUOstar Omega plate reader (BMG Labtech) (excitation, 230nm; emission, 550nm; measurement start time, 0.5; flashes per well, 20; positioning delay, 0.5s; top optic used).

CaDPA release from spores of *C. botulinum* was measured using terbium fluorescence, essentially according to Francis *et al.* (2015). A 50mM germinant solution of L-alanine was prepared in Tris-HCl buffer (20mM, pH 7.4), supplemented with 100mM $NaHCO_3$ and 100mM Sodium L-lactate, as described above. A control solution was prepared which contained only Tris-HCl buffer (20mM, pH 7.4). Spores were adjusted to an OD_{600} of ~60 and heat activated for 15 minutes at 80°C and 60°C, for Group I and II respectively, then cooled on ice. Wells of an opaque 96-well plate (Thermo Scientific) contained 115µl 50mM

L-alanine germinant solution and 10µl 800µM Terbium (III) Chloride. To each well, 5µl of heat activated spores were added and mixed by aspiration. Controls included 800µM TbCl₃ with spores (no germinant), and L-alanine with TbCl₃ only (no spores). CaDPA release was monitored at 25°C for 24 hours using a FLUOstar Omega fluorescence plate reader (BMG Labtech) (excitation, 230nm; emission, 550nm; measurement start time, 0.5; flashes per well, 20; positioning delay, 0.5s; top optic used). At the end of the experiment, a 5µl sample was taken from each set of three wells and observed under phase-contrast microscopy for presence of phase bright or phase dark spores as outlined above.

2.14 Quantifying the DPA content per spore of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B

The concentration of spores of strain Af84 and strain Eklund 17B was determined, using a haemocytometer, to estimate the number of spores present in a 10µl sample. A concentrated suspension of spores of each strain was serially diluted (1/10) from the original concentration to 10⁰ using sterile ultra-pure water. Dilutions were autoclaved for 15 minutes at 121°C, and cooled on ice. Wells of an opaque 96-well plate were filled with 1600µM TbCl₃ and 100µl of autoclaved spores of each strain to give a final TbCl₃ concentration of 800µM. Wells containing various concentrations of DPA and TbCl₃ were also included in the same plate as a calibration curve for quantifying DPA concentration. Fluorescence intensity was read using a FLUOstar Omega fluorescence plate reader (BMG Labtech) (excitation, 230nm; emission, 550nm; measurement start time, 0.5; flashes per well, 20; positioning delay, 0.5s; top optic used).

3. Results

3.1 Thermal death of spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B

To determine the thermal death of spores of Group I strain Af84, spores were heated at 95°C for 1, 2, 3, 4, 5 and 6 hours in Sorenson's phosphate buffer, cooled and inoculated onto PYGS medium, incubated at 37°C for 2 days and then the number of colonies formed enumerated (Figure 5). Spores were heated for a total of 6 hours, however after 6 hours of heating, no colonies were observed on PYGS medium. The $D_{95^{\circ}\text{C}}$ -value, which is the time required to reduce spore viability by a factor of ten at 95°C, for Group I strain Af84 was 69 minutes (Figure 5). Thus, heating Group I strain Af84 for 4 hours at 95°C was sufficient to deliver a 3-log reduction in spore viability.

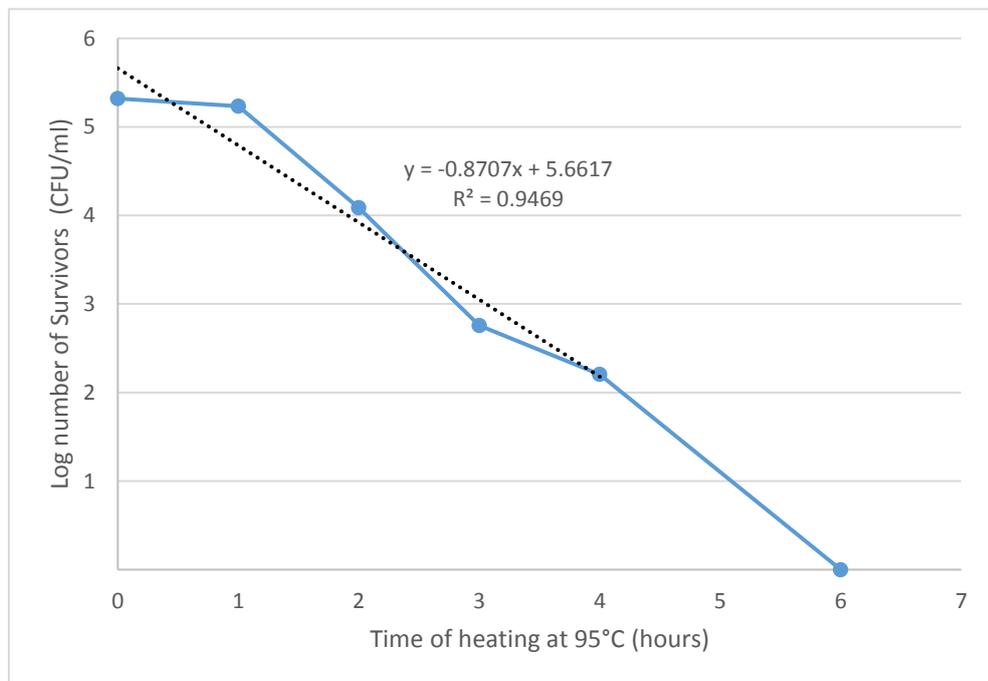


Figure 5. Number of surviving spores of *C. botulinum* Group I strain Af84 plated on PYGS medium after heat inactivation at 95°C, at various time intervals. No spores were recovered after 6 hours of heating on PYGS medium.

Heat inactivation of *C. botulinum* Group II strain Eklund 17B was measured at 85°C (Figure 6). Spores were heated for 1, 2, 5, 10 and 20 minutes in Sorenson's phosphate buffer, cooled on ice, spread onto PYGS medium and incubated at 30°C for 2 days. After 2 minutes at 85°C, no colonies were observed on PYGS medium. The $D_{85^{\circ}\text{C}}$ -value for *C. botulinum* Group II strain Eklund 17B was 0.19 minutes, which was calculated using the equation of the dashed line in figure 6. Heating Eklund 17B spores for 1 minute at 85°C delivered a 5-log reduction in spore viability.

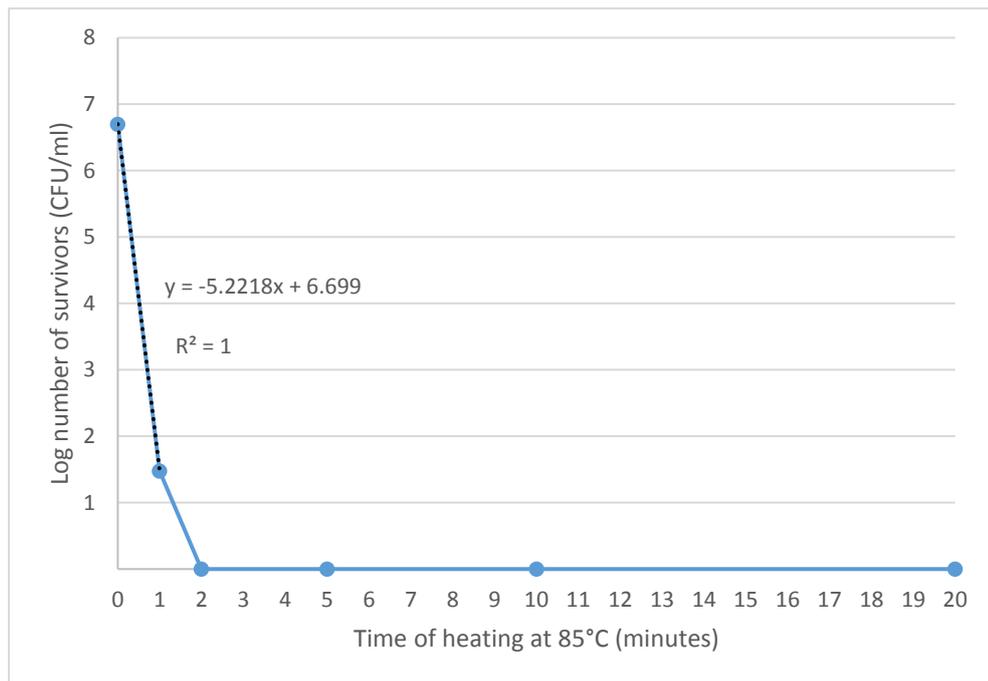


Figure 6. Number of surviving spores of *C. botulinum* Group II strain Eklund 17B plated on PYGS medium after heat inactivation at 85°C, at various time points. After 2 minutes of heating at 85°C (and longer), no spores were recovered on PYGS plates.

3.2 L-alanine and L-cysteine germinate spores of *C. botulinum* Group I and II

Germination of Group I strain Af84 and Group II strain Eklund 17B with the nutrient germinants L-alanine and L-cysteine was evaluated by measuring the fall in OD₆₀₀ over time using a Bioscreen C analyser. Germination of Af84 with L-cysteine occurred at a faster rate than with L-alanine, and L-cysteine germination was completed within 10 hours (Figure 7). For L-cysteine, a 65% drop in OD₆₀₀ was observed representing ~99% spore germination of Group I strain Af84, as determined by phase-contrast microscopy. Germination of Group I Strain Af84 with L-alanine was complete within 18 hours and a fall in OD₆₀₀ of ~50% resulted in ~99% phase dark spores. Group II strain Eklund 17B germinated with both L-alanine and L-cysteine at a similar rate and germination was completed within the first hour of the experiment for both germinants. An 80% fall in OD₆₀₀ with L-alanine resulted in ~99% spores germinating. In comparison, a 75% fall in OD₆₀₀ with L-cysteine resulted in ~99% spores germinating, as determined by phase-contrast microscopy. There was no fall in OD₆₀₀ in the absence of L-alanine or L-cysteine (controls in Figure 7). L-alanine and L-cysteine were both efficient germinants for Eklund 17B, however L-cysteine germinated Af84 at a faster rate when compared to the germinant L-alanine.

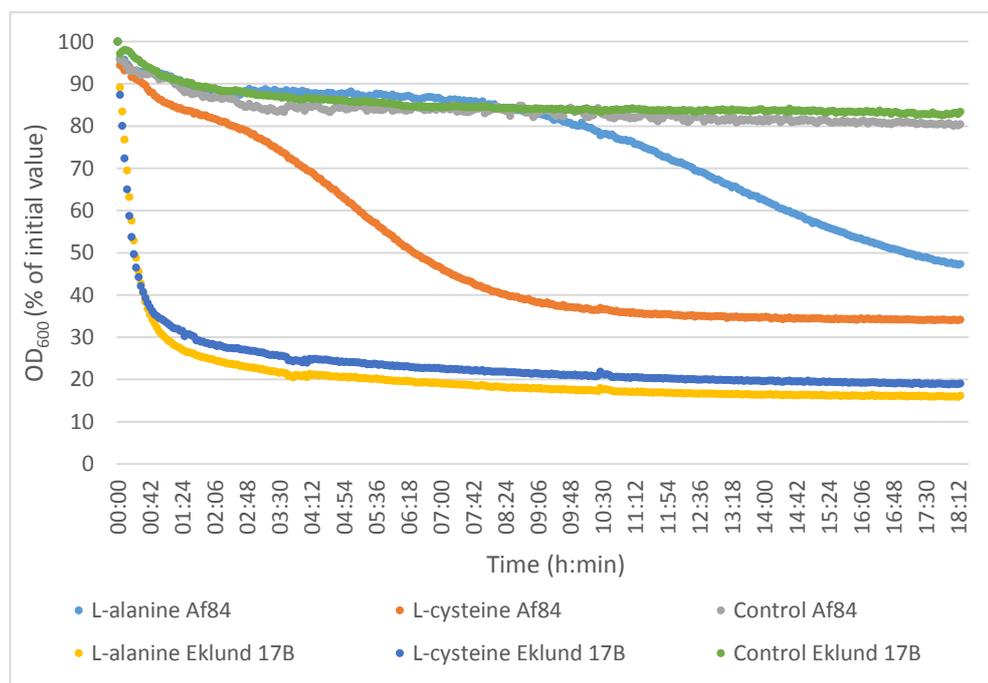


Figure 7. Anaerobic germination of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B with the nutrient germinants, L-alanine (50mM) and L-cysteine (50mM) in Tris-HCl buffer (20mM, pH 7.4) at 30°C, using a Bioscreen C analyser system to measure OD₆₀₀.

3.3 Exogenous CaDPA does not induce germination in *C. botulinum* Group I strain Af84 or Group II strain Eklund 17B at 30°C

The effect of exogenous CaDPA on spore germination was examined by measuring the fall in OD₆₀₀, using a Bioscreen C Analyser (Figure 8). Exogenous CaDPA did not induce germination in *C. botulinum* Group I strain Af84 or Group II strain Eklund 17B. However, small decreases in OD₆₀₀, ~10% for Group I strain Af84 and 18% for Group II strain Eklund 17B, were observed in the experiment. Microscopic analysis of spores incubated with CaDPA for 24 hours confirmed that no germination had occurred, with all spores remaining phase bright.

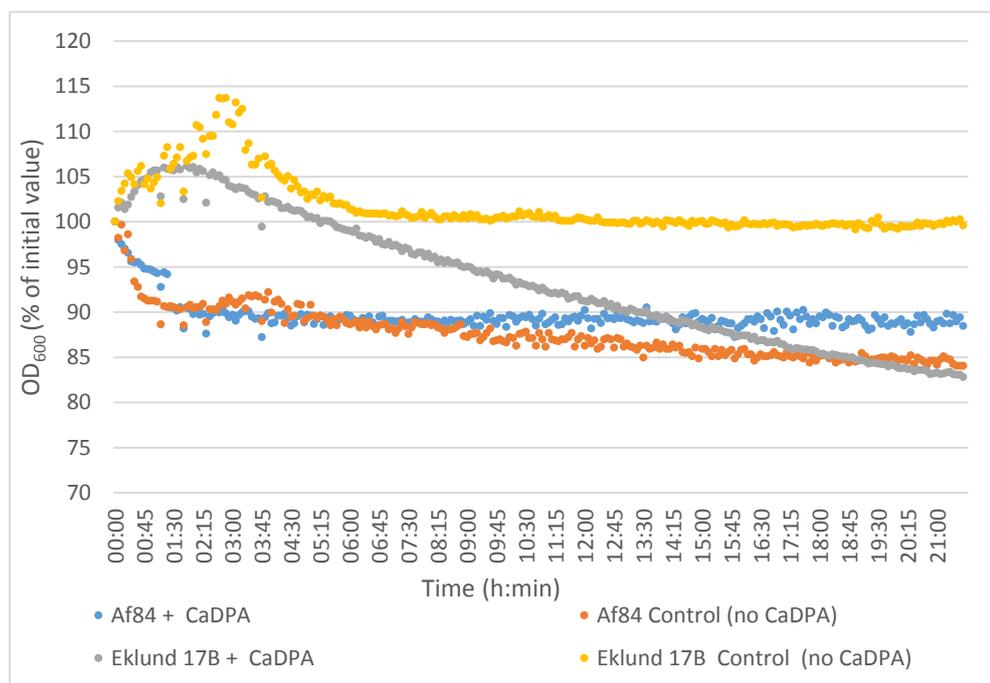


Figure 8. Anaerobic germination of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B with the non-nutrient germinant CaDPA (54mM) at 30°C, using a Bioscreen C analyser system to measure OD₆₀₀.

3.4 Exogenous CaDPA did not induce germination of coated or decoated spores of *C. botulinum* Group I strain Af84 or Group II strain Eklund 17B at 45°C

The ability of the non-nutrient germinant CaDPA to germinate spores of Group I strain Af84 and Group II strain Eklund 17B was further assessed at 45°C, as some previous work had indicated a greater effect of CaDPA on spore germination at 45°C (Riemann and Ordal, 1961). Spores of Group I strain Af84 with intact coats and coats removed showed a decrease in OD₆₀₀ of ~17% and 30%, respectively (Figure 9). However, the control spores (no CaDPA) with intact coats and control spores (no CaDPA) with absent coats also showed a drop in OD₆₀₀ of approximately 25%. Visualisation of all samples under phase-contrast microscopy revealed no germination had occurred as all of the spores remained phase bright. Although initial results showed a drop in OD₆₀₀ by approximately 17% for spores of Group II strain Eklund 17B with intact coats, microscopic analyses revealed that all of the spores appeared phase bright. Decoated spores of Eklund 17B also showed signs of germination, as the OD₆₀₀ had fallen by ~13%. However, all the spores appeared phase bright when viewed under phase-contrast microscopy. Both controls showed a slight drop in OD₆₀₀, 20% and 5% for coated and decoated controls, respectively, but remained phase bright (controls not shown).

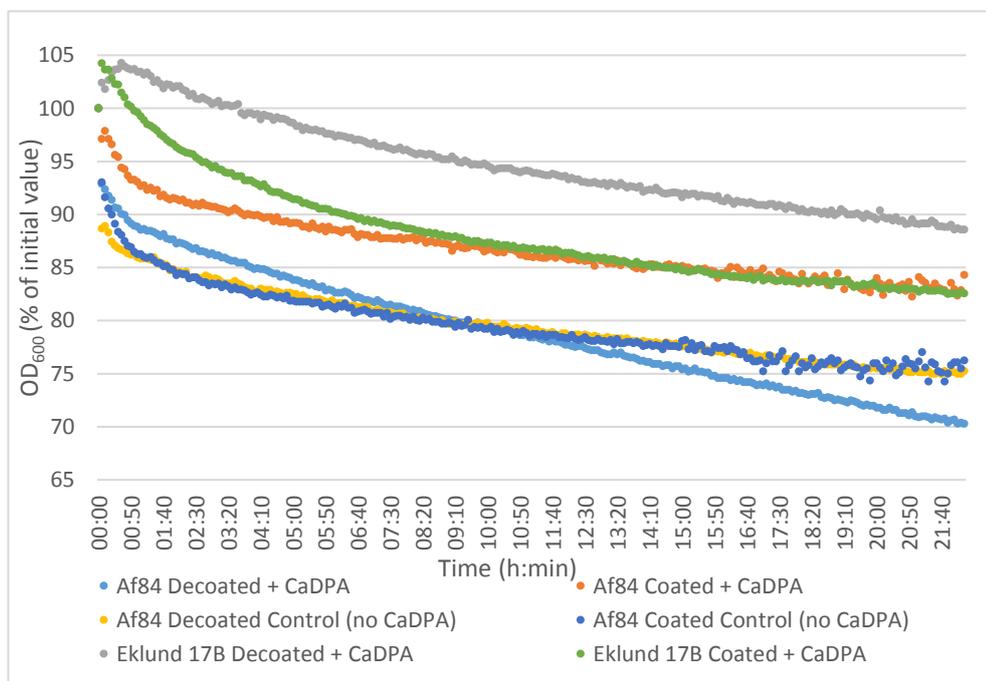


Figure 9. Anaerobic germination of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B with the non-nutrient germinant CaDPA (54mM) at 45°C, using a Bioscreen C analyser system to measure OD₆₀₀. Spores were decoated using dithiothreitol.

3.5 Germination assay using microscopic analyses of spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B gave similar results to those using the Bioscreen

Spores were examined for germination with L-alanine, L-cysteine and CaDPA by measuring (using a microscope) the percentage of phase-dark spores at varying time intervals at 30°C (Figure 10). Results were consistent with those obtained using the Bioscreen. Approximately 95% of spores of strain Af84 incubated with L-alanine appeared phase dark after 20 hours, with only ~5% spores remaining phase bright. In the presence of L-cysteine, approximately 90% of spores of strain Af84 appeared phase-dark after 20 hours of incubation. Germination of strain Eklund 17B with L-alanine proceeded more rapidly than strain Af84 with approximately ~50% of spores appearing phase bright after 10 minutes of incubation with the germinant. After 20 hours, >99% spores of Eklund 17B appeared phase dark under the microscope. Germination of strain Eklund 17B with L-cysteine appeared to proceed at a similar rate to L-alanine, as found by measuring the fall in OD₆₀₀ using the Bioscreen (Figure 7). No phase dark spores were observed for controls (no germinant) or spores incubated with CaDPA for the entirety of the experiment (data not shown).

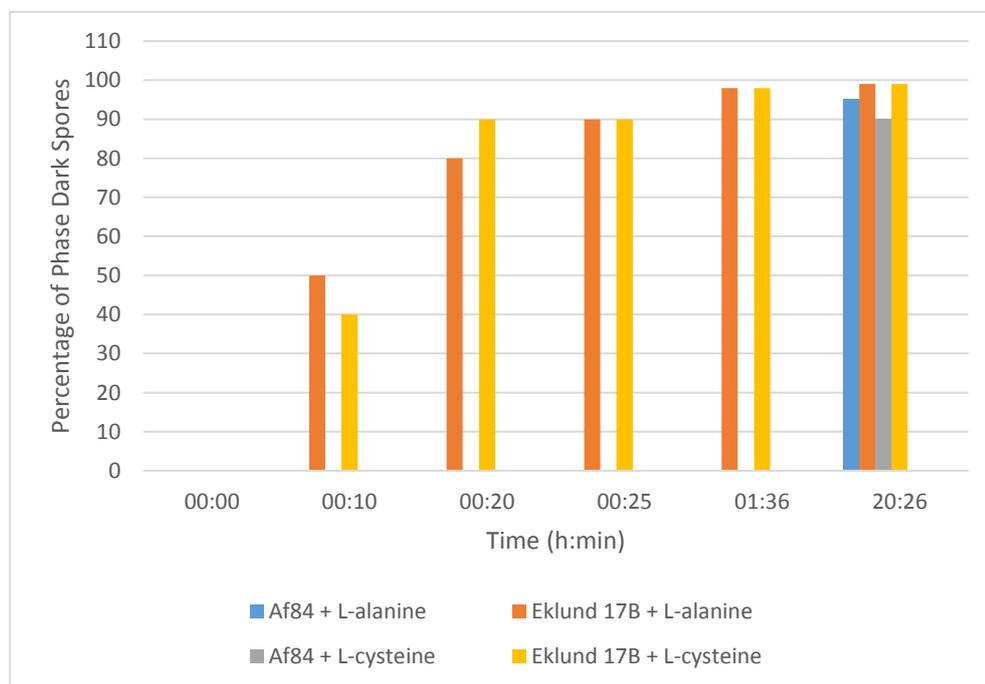


Figure 10. Percentage of phase dark spores observed over time using phase-contrast microscopy with *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B. Spores were incubated with L-alanine (50mM) and L-cysteine (50mM) in Tris-HCl buffer (20mM, pH 7.4) at 30°C.

3.6 DDA induced germination in spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B

Germination of Group I strain Af84 and Group II strain Eklund 17B with 1.08mM DDA was measured at 45°C (Figure 11). Spores of both strains germinated in the presence of DDA, showing a fall in OD₆₀₀ by 35% and 40% for Af84 and Eklund 17B, respectively. The control (no DDA addition) for strain Af84 showed a small drop in OD over the 24-hour period. The Eklund 17B control (no DDA addition) increased in OD₆₀₀ by approximately 15% in the first hour of the study, however the OD₆₀₀ subsequently returned to its original OD₆₀₀ and remained relatively constant for the remainder of the study. Following 24 hours incubation with DDA, spores were examined under phase contrast microscopy. Spores of Group I strain Af84 had ~50% phase bright spores remaining. Spores of Group II strain Eklund 17B appeared ~40% phase bright under the microscope. All controls were observed under the microscope and >99% spores appeared phase bright. Finally, DDA germination was also analysed using decoated Eklund 17B spores (data not shown). Interestingly, removal of the spore coats prevented any spore germination with DDA.

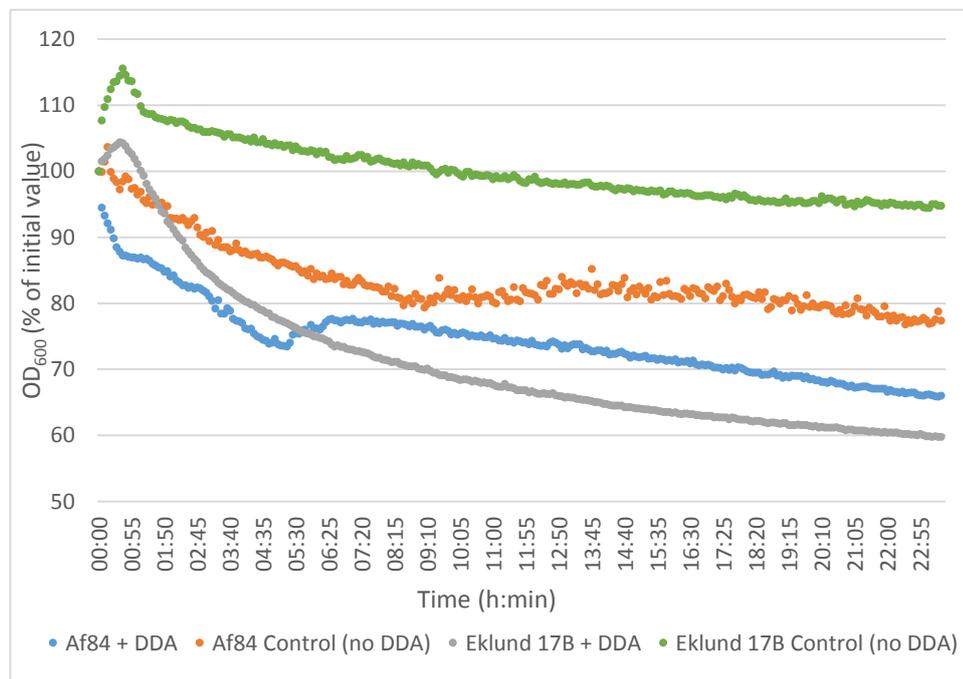


Figure 11. Anaerobic germination of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B with the non-nutrient germinant DDA (1.08mM) at 45°C, using a Bioscreen C analyser system to measure OD₆₀₀.

3.7 Lysozyme does not have an inhibitory effect on growth from spores of *C. botulinum* Group I strain Af84 or Group II strain Eklund 17B

The effect of lysozyme concentration on the growth of *C. botulinum* Group I and II strains was evaluated by inoculating unheated spores ($OD_{600} \sim 0.5$) of each strain onto PYGS agar, supplemented with different concentrations of lysozyme (0, 1, 3, 10, 30 and 100 $\mu\text{g/ml}$) at 37°C and 30°C, respectively (Figure 12). There was no effect of lysozyme concentration on the viable count of Group II strain Eklund 17B. The viable count for Group I strain Af84 was also unaffected by the lysozyme concentration and was approximately 1-log lower than for Group II strain Eklund 17B, due to a lower initial spore concentration.

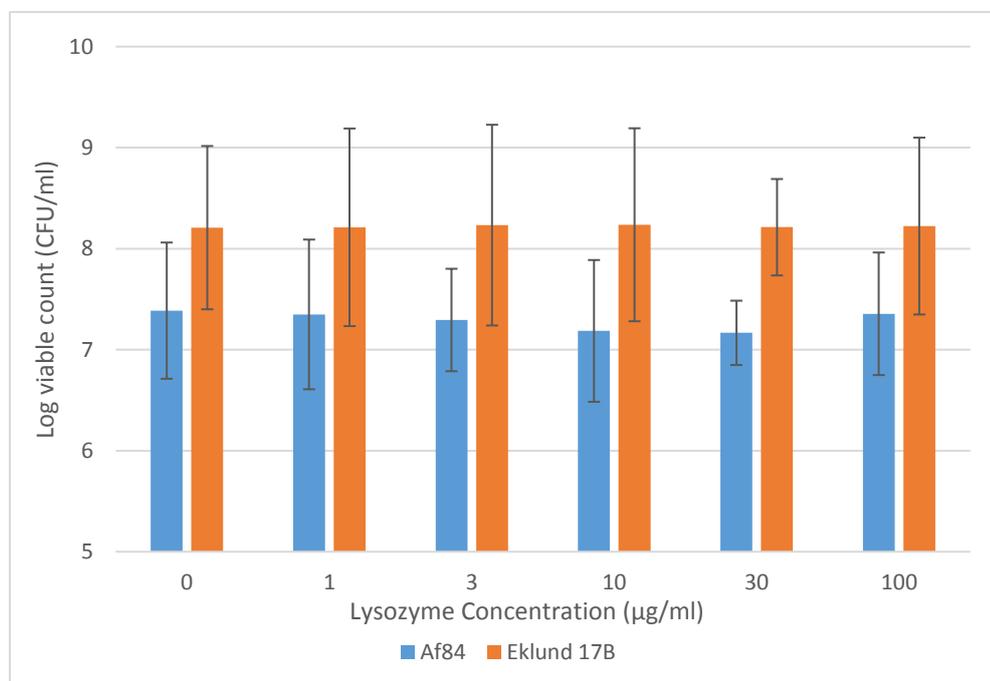


Figure 12. The effect of lysozyme concentration ($\mu\text{g/ml}$) on anaerobic growth of Group I strain Af84 and Group II strain Eklund 17B at 37°C and 30°C, respectively. The standard deviation is shown.

3.8 Lysozyme induces germination of decoated spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B

The ability of lysozyme (10µg/ml) to germinate spores of Group I strain Af84 was determined at 37°C (Figure 13). Spores of strain Af84 that had been previously decoated using sodium thioglycolate showed a slight decrease in OD₆₀₀ of 25% within 9 hours (Figure 13). Microscopic visualisation, after 24 hours, revealed very low spore titres, compared to that observed prior to the addition of lysozyme. However, a small number of clumps of ~98% phase dark (germinated) spores were observed. A haemocytometer was used to enumerate, more accurately, the number of decoated spores of strain Af84 following germination in the presence of lysozyme. There was a 99% decrease in the total spore count after 24 hours incubation, suggesting that the spores had germinated and lysed. Coated spores of Af84 decreased in OD₆₀₀ by ~25% within 24 hours, however, these spores all appeared phase bright under the microscope (and there was no decrease in spore titre). The controls used in this experiment were spores of Af84 incubated with Tris-HCl buffer without the addition of lysozyme. Both controls (coated and decoated) remained 100% phase-bright when observed using phase-contrast microscopy. The buffer only control (Tris-HCl only) which contained no spores remained at a constant OD₆₀₀ for the entirety of the experiment.

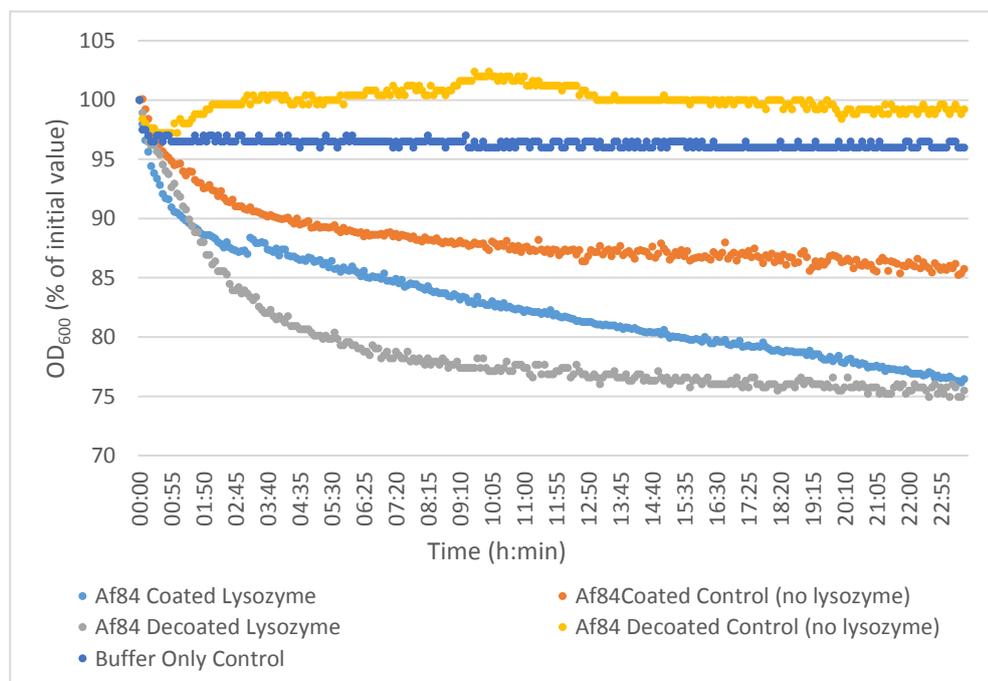


Figure 13. Anaerobic germination of *C. botulinum* Group I strain Af84 with lysozyme (10µg/ml) at 37°C using a Bioscreen C analyser to measure OD₆₀₀. Spores were decoated using sodium thioglycolate.

Lysozyme (10µg/ml) was also tested as a germinant for Group II strain Eklund 17B (Figure 14). Germination of decoated spores of Eklund 17B was complete within 2 hours of incubation with lysozyme, which corresponds with a 55% fall in OD₆₀₀ resulting in ~99% spores appearing phase dark under phase-contrast microscopy. Even though an ~18% fall in OD₆₀₀ was observed for coated spores of Eklund 17B that had been incubated with lysozyme, approximately 99% of these spores appeared phase bright when visualised under phase-contrast microscopy. Decoated total spore titres remained the same for Group II strain Eklund 17B after incubation in lysozyme. None of the controls germinated, as all spores appeared phase-bright. The buffer only control, which contained no spores, remained at a constant OD₆₀₀ throughout the experiment.

Germination of both strains with lysozyme was also tested using dithiothreitol as the decoating reagent rather than sodium thioglycolate, and similar results were obtained with both decoating reagents.

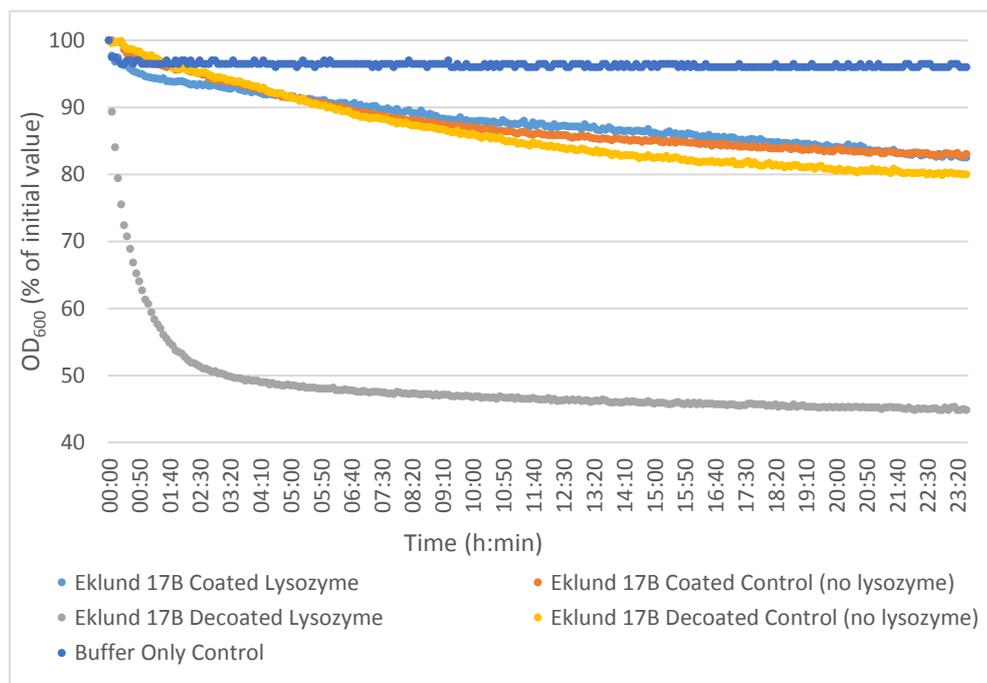


Figure 14. Anaerobic germination of *C. botulinum* Group II strain Eklund 17B with lysozyme (10µg/ml) at 37°C using a Bioscreen C analyser to measure OD₆₀₀. Spores were decoated using sodium thioglycolate.

3.9 Lysozyme recovers heat-damaged spores of *C. botulinum* Group II strain

Eklund 17B but not spores of Group I strain Af84

The ability of lysozyme (10µg/ml) to recover spores of Group I strain Af84 and Group II strain Eklund 17B was evaluated (Figure 15). Spores of Group I strain Af84 and Group II strain Eklund 17B were heat inactivated by submerged heating for 4 hours at 95°C and 2 minutes at 85°C, respectively. After heating, no spores of Group I strain Af84 were recovered on either PYGS alone or PYGS with lysozyme, regardless of whether the spore coat had been removed or remained intact. Viable counts for unheated Af84 spores (coated and decoated) were similar on PYGS medium and PYGS with lysozyme. There was a 1-log difference in the viable count between unheated coated spores and unheated decoated spores on both PYGS medium and PYGS supplemented with lysozyme (Figure 15).

Following heat treatment, coated spores of Group II strain Eklund 17B were not recovered on unsupplemented PYGS medium. In comparison, when heat treated spores were plated on PYGS medium supplemented with lysozyme (10µg/ml), a >4.4-log increase in recovery was observed. When spores of Eklund 17B had their coats chemically removed using sodium thioglycolate, there was a ~2-log increase in recovery compared to coated spores when spread onto PYGS medium supplemented with lysozyme. For heat-treated decoated spores of Group II strain Eklund 17B, there was a total increase in recovery of >5.9-log when plated onto PYGS medium containing lysozyme, compared to heat treated spores spread onto unsupplemented medium. Unheated spores of Eklund 17B with an intact coat formed colonies on both types of media, although when the spore coat was removed the recovery rate was reduced (Figure 15).

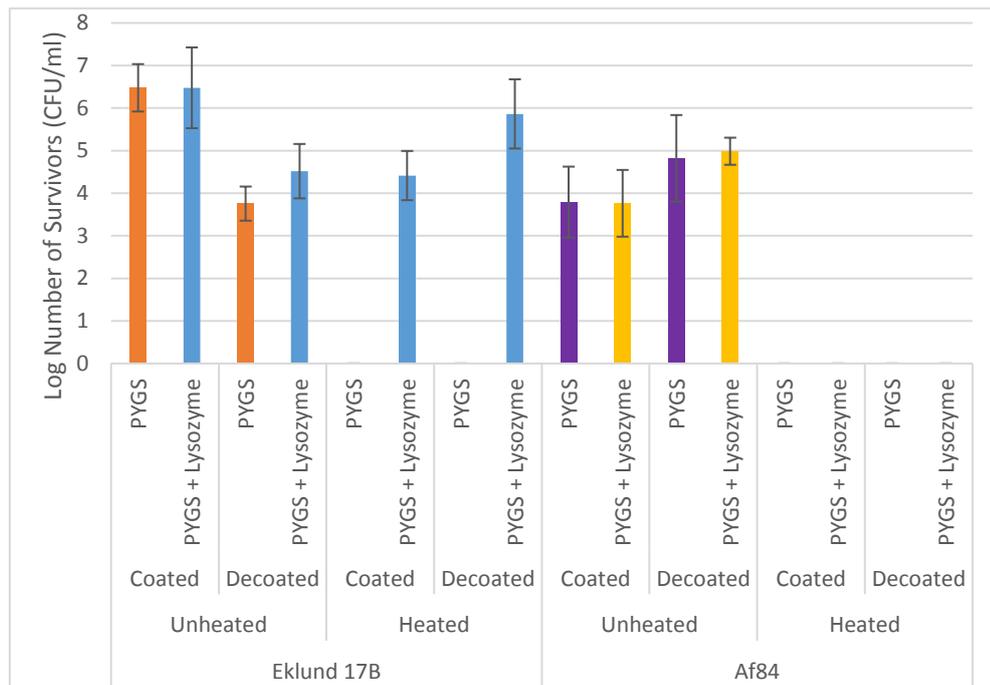


Figure 15. Number of surviving spores (\pm SD) after heat inactivation (Eklund 17B, 85°C for 2 minutes; Af84, 95°C for 4 hours) and recovery on PYGS agar supplemented with lysozyme (10 μ g/ml). Spores were decoated using sodium thioglycolate.

3.10 Spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B cannot be recovered with DDA after heat inactivation

It was previously shown (Figure 11) that unheated spores of strains Af84 and Eklund 17B with an intact coat are able to germinate in response to DDA (1.08mM). The recovery of heat-damaged spores with DDA was now tested (Figure 16). Spores were heated in a water bath at 95°C for 4-hours and at 85°C for 2 minutes for Group I strain Af84 and Group II strain Eklund 17B, respectively. A proportion of spores were pre-incubated with DDA (1.08mM) overnight (coated and decoated) and rinsed to minimise DDA toxicity. The subsequent formation of colonies from spores of Group I strain Af84 and Group II strain Eklund was now measured. Unheated spores of Group I strain Af84 formed colonies on PYGS agar with and without prior incubation with DDA. However, the viable count was marginally lower with DDA incubation than without for coated and decoated spores of Group I strain Af84 (Figure 16). After heat inactivation at 95°C for 4 hours, colonies of strain Af84 were not formed when incubated with DDA (coated nor decoated). There were also no colonies formed from heated spores of strain Af84 when spread onto PYGS without prior DDA incubation.

Unheated spores of strain Eklund 17B (coated and decoated) formed colonies on PYGS medium, both with and without prior DDA incubation. Decoating of the spores and incubation with DDA each brought about a reduction in the viable count from unheated spores (Figure 16). After heat inactivation of spores of Eklund 17B at 85°C for 2 minutes, no spores (coated nor decoated) were recovered on PYGS medium with or without prior DDA incubation.

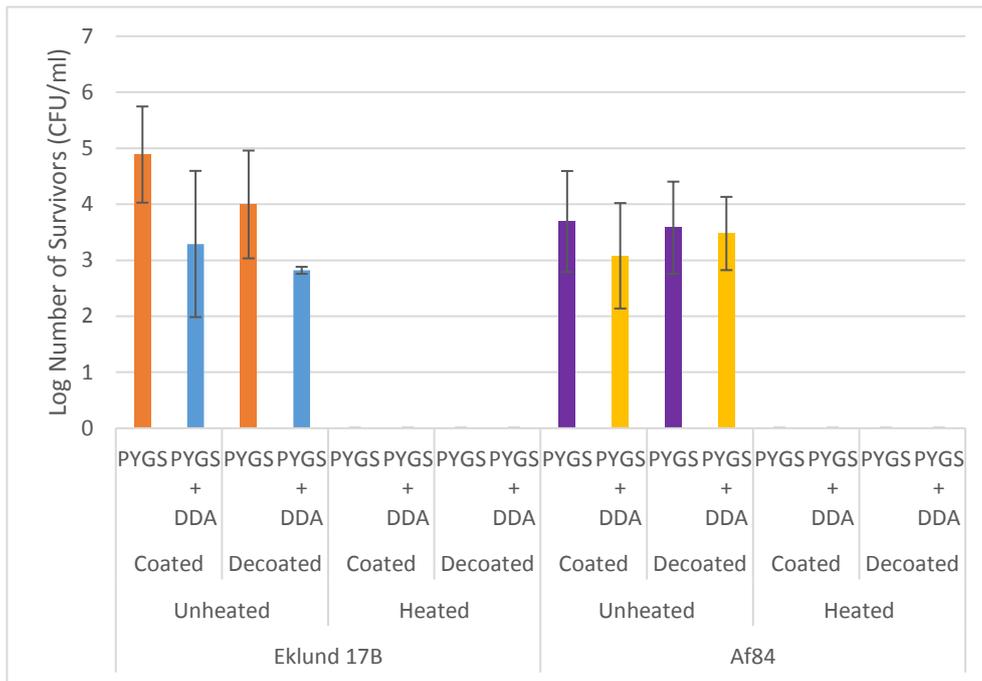


Figure 16. Number of surviving spores (\pm SD) after heat inactivation (Eklund 17B, 85°C for 2 minutes; Af84, 95°C for 4 hours) and recovery on PYGS agar after incubation with DDA (1.08mM) overnight at 45°C. Spores were decoated using sodium thioglycolate.

3.11 Spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B cannot be recovered with L-alanine after heat-inactivation

Spores of Group I strain Af84 and Group II strain Eklund 17B were previously shown to germinate in response to the nutrient germinant L-alanine (50mM) (Figure 7). The use of L-alanine to recover heat-damaged spores when inoculated onto PYGS agar supplemented with L-alanine (50mM) was evaluated (Figure 17). Unheated spores of Group I strain Af84 formed colonies on PYGS medium and PYGS supplemented with L-alanine and the viable count was similar for each media for coated and decoated spores. There was an approximately 1-log difference between the viable counts for coated and decoated spores. No colonies were formed from spores of Group I strain Af84 that had been heated for 4 hours at 95°C, even on the PYGS plates that had been supplemented with L-alanine. Unheated and coated spores of Eklund 17B gave similar viable counts on PYGS and PYGS supplemented with L-alanine. The number of colonies formed from unheated and decoated spores was similar on the two media, but lower than that for coated spores (Figure 17). Following a heat treatment at 85°C for 2 minutes, no colonies were formed on PYGS or PYGS with L-alanine supplementation.

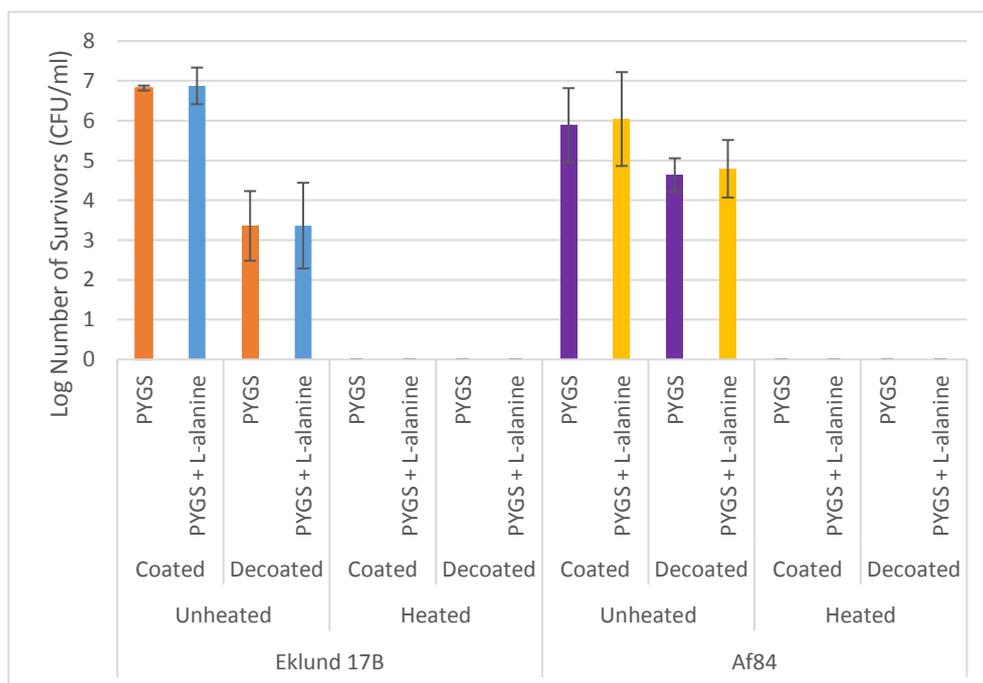


Figure 17. Number of surviving spores (\pm SD) after heat inactivation (Eklund 17B, 85°C for 2 minutes; Af84, 95°C for 4 hours) and recovery on PYGS agar with L-alanine (50mM). Spores were decoated using sodium thioglycolate.

3.12 Timing of DPA release during germination of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B with L-alanine

Various concentrations of DPA were incubated with TbCl_3 ($800\mu\text{M}$), to determine the relationship between relative fluorescence and DPA concentration (Figure 18). The highest concentration of DPA used, corresponded to a fluorescence reading of $\sim 240000\text{AU}$. The minimum concentration of DPA that was detectable during this assay was $1\mu\text{M}$, which corresponded to ~ 145000 fluorescence AU. The assay was not very sensitive for the lower concentrations of DPA ($0\text{-}10\mu\text{M}$), as represented by an R^2 of 0.293 .

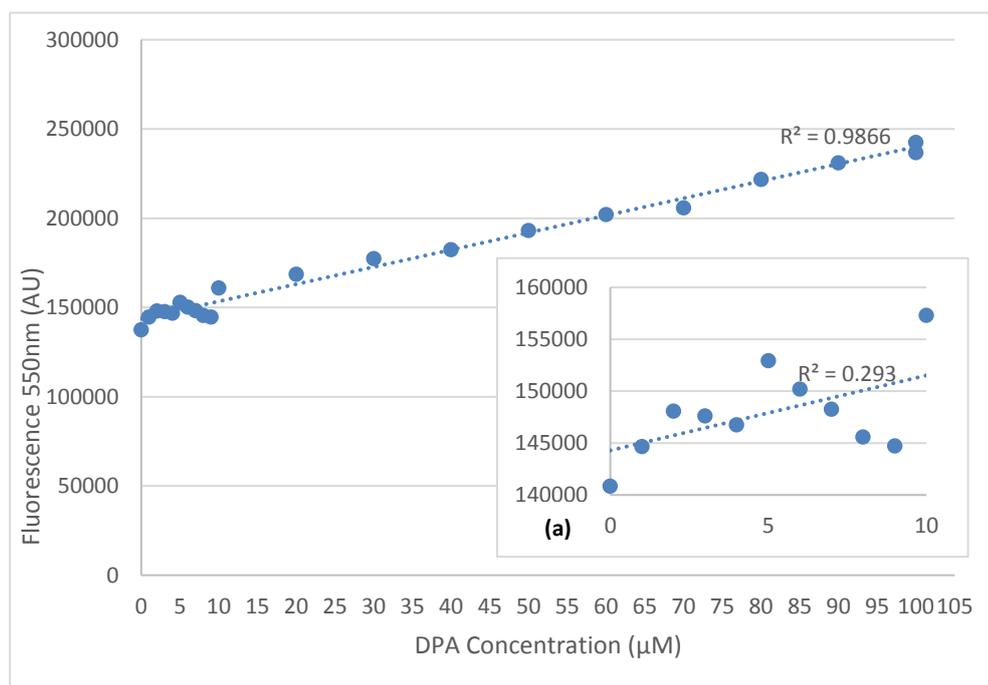


Figure 18. Effect of DPA concentration on the relative fluorescence (550nm) following binding to TbCl_3 . Insert (a) represents fluorescence readings for low concentrations of DPA ($0\text{-}10\mu\text{M}$).

In order to determine the timing of DPA release in spores of Group I strain Af84, spores were incubated with L-alanine (50mM) and $TbCl_3$ (800 μ M) and monitored over 18 hours at 25°C using a fluorescence plate reader (Figure 19). The fluorescence increased steadily from ~120000AU to ~160000AU over the 18 hour period, while the OD₆₀₀ decreased quickly and then slowly and then quickly again (Figure 19). Overall, the OD₆₀₀ fell to 55% of the initial value. Associating results from Figure 19 to the calibration curve in Figure 18, it shows that a total fluorescence reading of 160000AU corresponds to a final DPA concentration of 15 μ M. This is 0.005pg of DPA released per spore of strain Af84 during germination with L-alanine. In the absence of L-alanine, there was no fall in OD₆₀₀ detected and only a marginal increase in fluorescence, which does not signify DPA release (Figure 20).

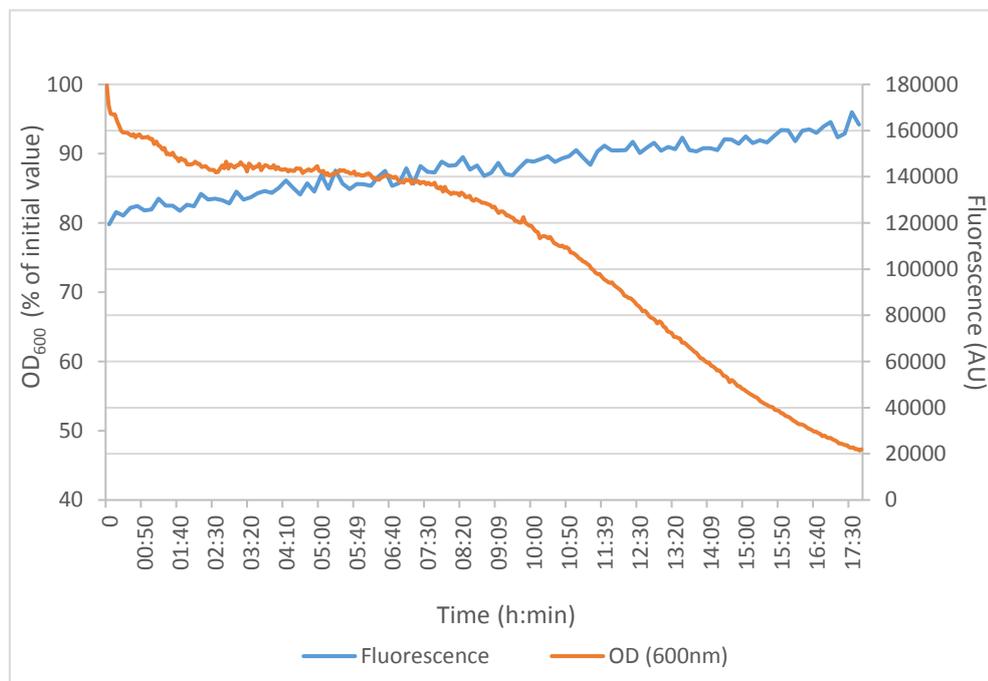


Figure 19. Timing of DPA release in spores of *C. botulinum* Group I strain Af84 compared to the change in OD₆₀₀ observed following the addition of the germinant L-alanine (50mM).

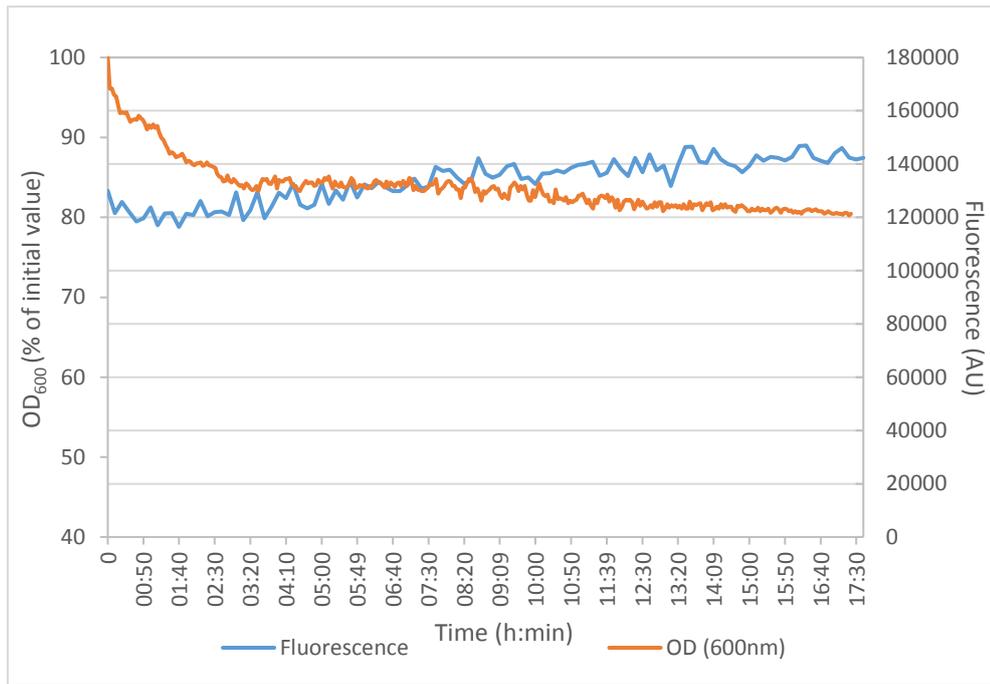


Figure 20. Lack of DPA release and change in OD₆₀₀ for spores of Group I strain Af84 without the addition of L-alanine (50mM).

Spore germination of Group II strain Eklund 17B in the presence of L-alanine (as measured by change in OD₆₀₀) and DPA release were both more rapid than observed with Group I strain Af84 (Figure 19). A total fluorescence reading of ~150000AU was detected within 50 minutes of incubation with the nutrient germinant L-alanine and the fluorescence reading remained relatively constant for the remainder of the incubation period. A fluorescence reading of ~150000AU correlates with a final DPA concentration of ~8μM (Figure 18) released from spores of Group II strain Eklund 17B, when germination is induced by L-alanine. The amount of DPA released per spore of strain Eklund 17B during germination with L-alanine is 0.003pg. In the absence of L-alanine, there was no increase in fluorescence or fall in OD₆₀₀ (Figure 22).

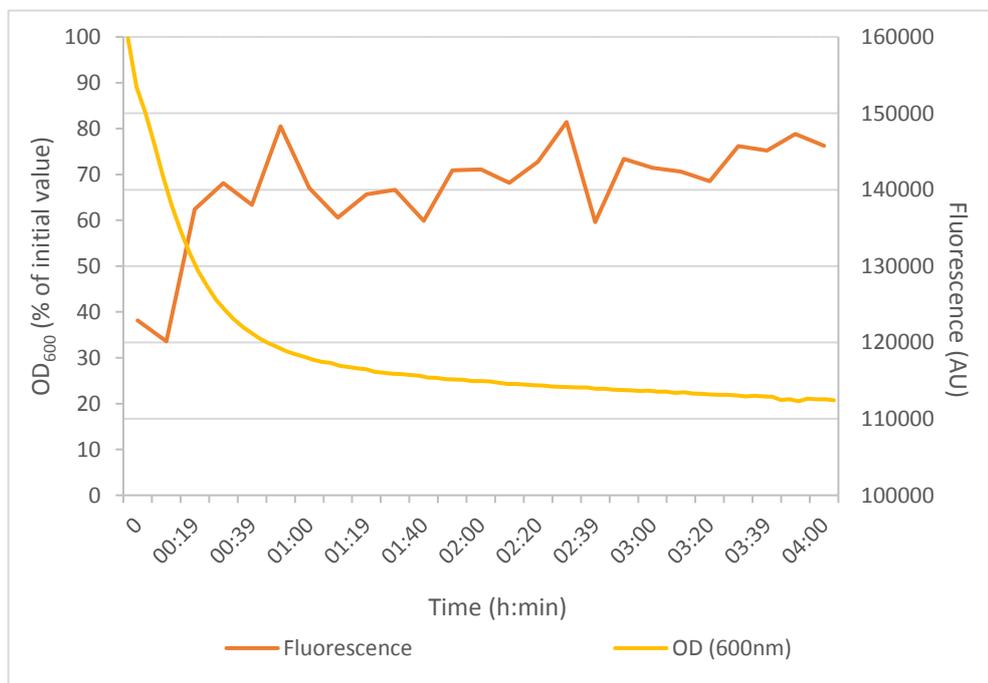


Figure 21. Timing of DPA release in spores of *C. botulinum* Group II strain Eklund 17B compared to the change in OD₆₀₀ observed following the addition of the germinant L-alanine (50mM).

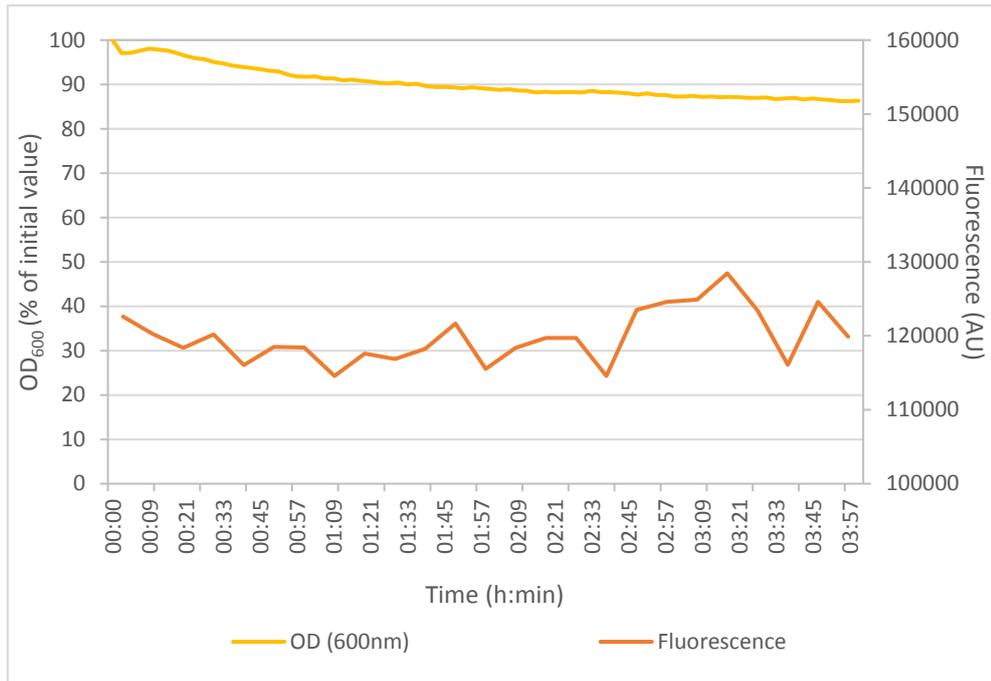


Figure 22. Lack of DPA release and change in OD₆₀₀ for spores of Group II strain Eklund 17B without the addition of L-alanine (50mM).

3.13 The average DPA content per spore of *C. botulinum* Group II strain Eklund 17B is higher than Group I strain Af84

A known concentration dilution series of spores of Group I strain Af84 was autoclaved at 121°C for 15 minutes. The most concentrated sample contained 10^7 spores/ml, measured using a haemocytometer. Diluted samples (10^5 - 10^1) gave fluorescence readings lower than that detectable on the calibration curve (Figure 23). A calibration curve was produced for each *C. botulinum* strain to ensure plate to plate variation did not interfere with results obtained during the assay. The most concentrated sample for strain Af84 (8.7×10^7 spores/ml) gave a fluorescence reading of 191706AU (Figure 24), which corresponds to a DPA concentration of $50 \mu\text{M}$ using the calibration curve in Figure 24 and the estimated resulting average DPA concentration of Group I strain Af84 is 1.9pg/spore.

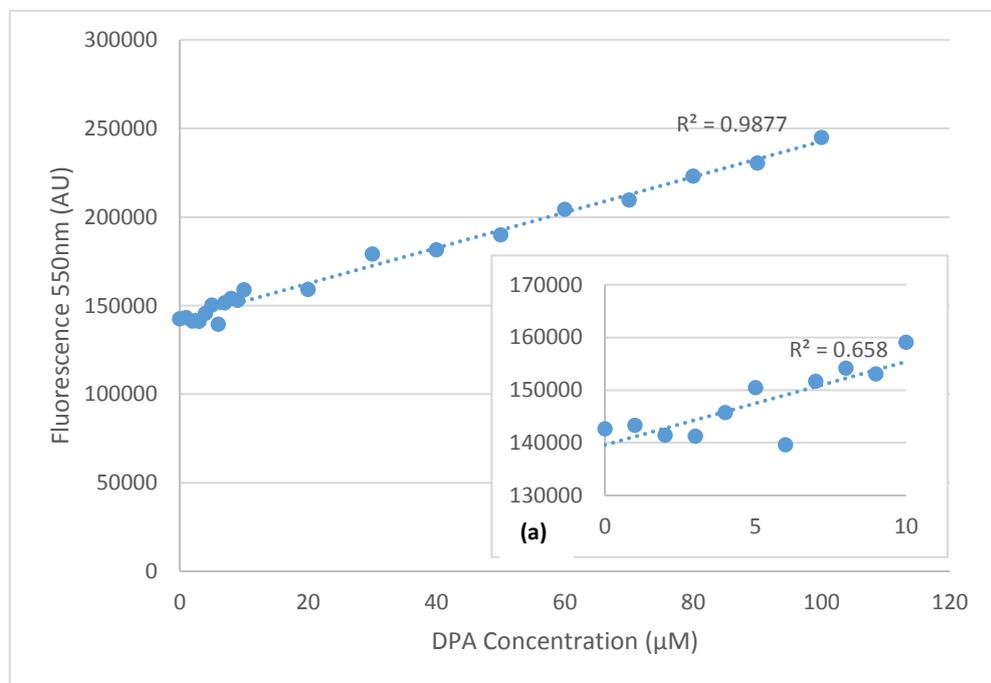


Figure 23. Calibration curve for autoclaved spores of *C. botulinum* Group I strain Af84. Insert (a) represents fluorescence readings for lowest concentrations of DPA used (0-10 μM).

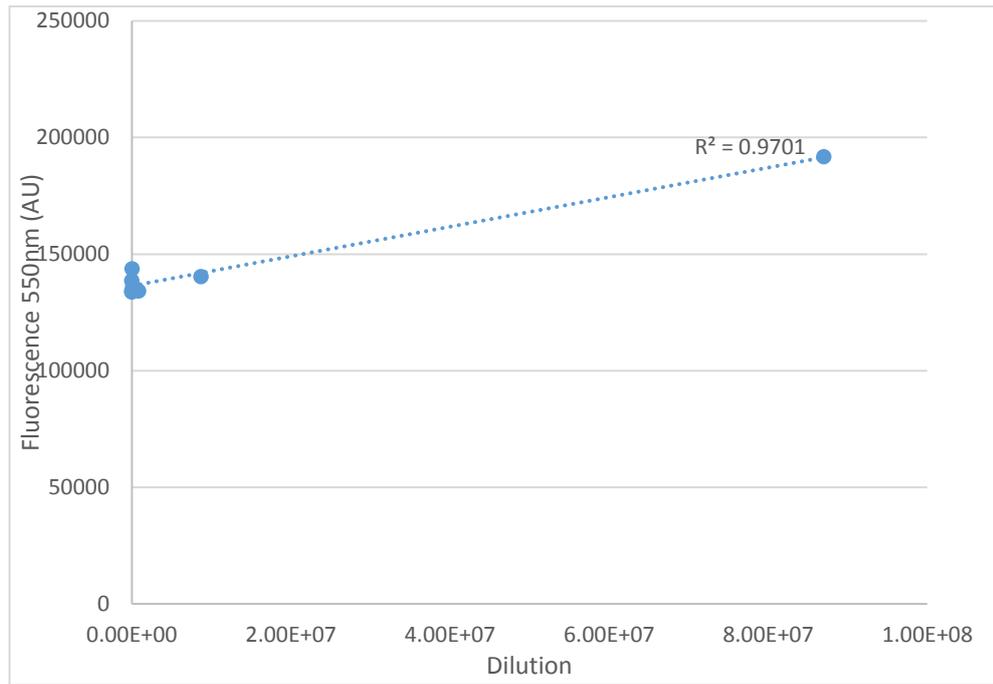


Figure 24. Fluorescence reading of DPA released from autoclaved spores of *C. botulinum* Group I strain Af84 at different dilutions.

Spores of Group II strain Eklund 17B were also enumerated and serially diluted, prior to autoclaving at 121°C for 15 minutes. DPA in the supernatant of autoclaved spores was detected using $TbCl_3$ and fluorescence was measured using a plate reader. There was a linear relationship between spore concentration and fluorescence (Figure 25). The DPA in the supernatant of spores in the lowest dilutions (10^6 - 10^1) was not readily quantifiable (fluorescent readings ranged from 130000-138000AU) (Figure 26). As mentioned above, a separate calibration curve was produced for each strain to reduce plate to plate variability. The highest concentration (1×10^8 spores/ml) of Eklund 17B spores yielded a fluorescence reading of ~ 210000 AU, which corresponds to $64 \mu M$ DPA using the calibration curve in Figure 25 and the resulting estimated DPA concentration is 2.1pg/spore.

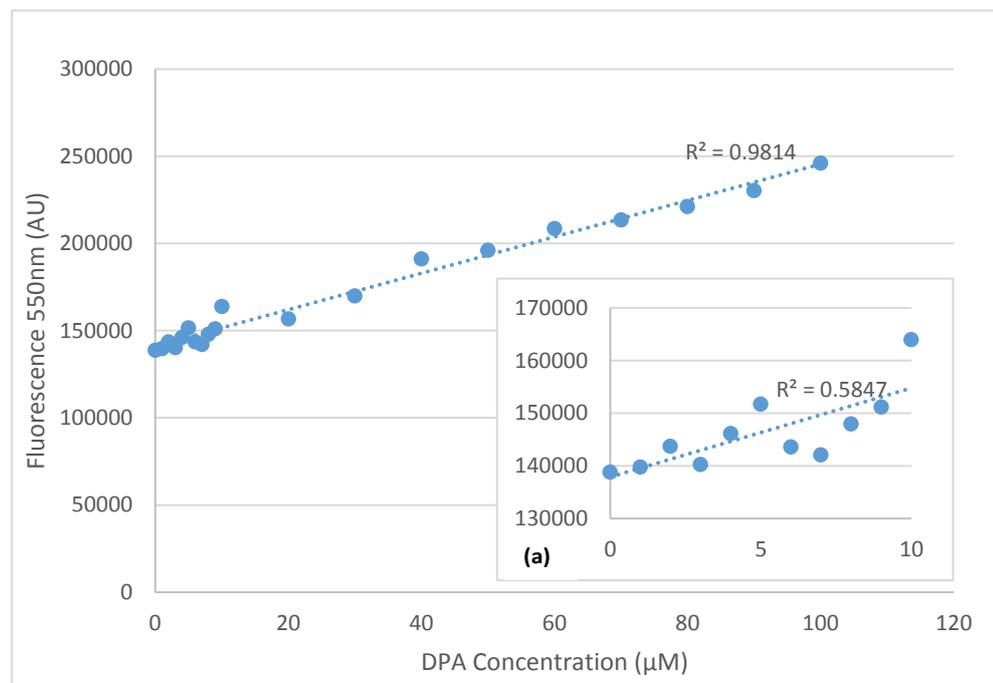


Figure 25. Calibration curve for autoclaved spores of *C. botulinum* Group II strain Eklund 17B. Insert (a) represents fluorescence readings for DPA concentrations 0-10 μM .

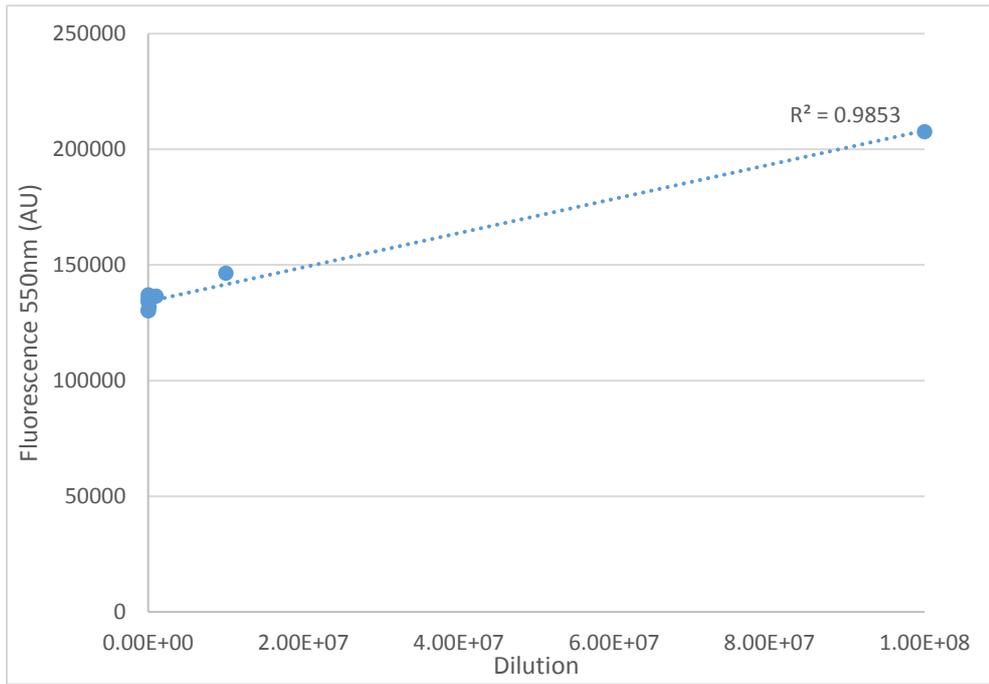


Figure 26. Fluorescence reading of DPA released from autoclaved spores of *C. botulinum* Group II strain Eklund 17B at different dilutions.

3.14 Summary of Results

- Heating *C. botulinum* Group II strain Eklund 17B for 1 minute at 85°C is enough to deliver a 5-log reduction in spore viability
- Heating *C. botulinum* Group I strain Af84 for 4 hours at 95°C is sufficient to deliver a 3-log reduction in spore viability
- Nutrient germination of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B occurs with both L-alanine and L-cysteine, at varying efficacy
- Spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B do not germinate in response to the addition of exogenous CaDPA
- Spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B with an intact coat germinate in response to DDA (but not spores with the coat removed)
- Decoated spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B both germinate with the addition of lysozyme
- Spores of *C. botulinum* Group II strain Eklund 17B were recovered with lysozyme (10µg/ml) after heating for 2 minutes at 85°C
- Decoated spores of Eklund 17B gave the highest spore recovery yield compared to coated spores when incubated with PYGS medium containing lysozyme (10µg/ml)
- Spores of *C. botulinum* Group I strain Af84 were not recovered with lysozyme (10µg/ml) after heating for 4 hours at 95°C
- Heated spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B were not recovered with DDA or the nutrient germinant L-alanine
- During germination, DPA is released within 18 hours for Group I strain Af84 and within 50 minutes for Group II strain Eklund 17B
- The average DPA concentration per spore (following autoclaving) of Group I strain Af84 and Group II strain Eklund 17B is 1.9pg and 2.1pg, respectively.

4. Discussion

4.1 Spore thermal death

Spores of *C. botulinum* Groups I and II germinate under favourable conditions and produce vegetative cells that form the botulinum neurotoxin (Peck, 2009). The aim of this study was to confirm the germination pathways of *C. botulinum* Group I and Group II outlined by Brunt *et al.* (2016) and to determine which part of the germination apparatus is affected by heat, preventing germination, and thereby subsequent outgrowth and toxin formation. Food manufacturers employ rigorous processes in order to prevent foodborne botulism, including for the safe production of chilled ready-to-eat meals and canned goods (Peck, 2006). The methods used to ensure the safety of food products include thermal processing and high-pressure treatment; there is also an increasing demand from consumers for minimally processed food (Bello *et al.*, 2014). Many studies state that these two treatment methods act synergistically to ensure spore death because high-pressure treatment alone is insufficient to inactivate spores (Ananta *et al.*, 2001, Okazaki *et al.*, 2000, San Martín *et al.*, 2002).

In the current work, thermal death experiments with Group I strain Af84, concluded that a 4-hour heating time at 95°C was enough to deliver a 3-log reduction in spore viability. The $D_{95^{\circ}\text{C}}$ -value was 69 minutes, and similar to that reported in the literature. There are limited recent publications establishing thermal death in Group I strains, however by extrapolation of results from Anderson *et al.* (1996) the $D_{95^{\circ}\text{C}}$ -value for spores of Group I strain 213B is approximately 80 minutes. Similarly, an extensive study by Esty and Meyer (1922) determined the heating time required to prevent growth from $\sim 6 \times 10^{10}$ spores of Group I spores as being 4 minutes at 120°C, 10 minutes at 115°C, 32 minutes at 110°C, 100 minutes at 105°C and 330 minutes at 100°C. From this work, the $D_{95^{\circ}\text{C}}$ -value can be estimated as 80 minutes. Spores of *C. botulinum* Group I pose a threat to low-acid canned food and have a $D_{121^{\circ}\text{C}}$ -value of 0.21 minutes, making them the most heat resistant group of *C. botulinum* (Peck, 2009). Spores of *C. botulinum* Group II are less heat-resistant than Group I, and in the present study, a 5-log reduction for spores of Group II strain Eklund 17B was achieved by heating at 85°C for 2 minutes, and the $D_{85^{\circ}\text{C}}$ -value was 0.19 minutes. A previous study by Peck *et al.* (1992a), demonstrated that heating Group II strain Eklund 17B for one minute at 85°C resulted in an approximate 5.5 log reduction in viable count. A review by Wachnicka *et al.* (2016) reported a similar spore resistance for other strains of *C. botulinum* Group II.

4.2 Spore germination in response to nutrient germinants

Various amino acids are capable of germinating spores of *Bacillus* or *Clostridium* species by binding to specific protein complexes termed germinant receptors (Setlow, 2014). Spores of *C. botulinum* Group I and II germinate in response to a combination of nutrient and non-nutrient germinants, particularly low-molecular weight biological molecules such as amino acids (Bhattacharjee *et al.*, 2016, Peck, 2009). In the current study, tests were carried out to determine whether spores of Group I strain Af84 and Group II strain Eklund 17B respond to amino acid germinants. Heat activated spores were incubated with L-alanine and L-cysteine and their resulting change in optical density at 600nm was measured using a Bioscreen. Optical density decreases during germination due to the loss of CaDPA from the spore core and the uptake of water, which causes a change in the refractive index of the spore (Ghosh and Setlow, 2009). In the present study, germination occurred with Group I strain Af84 and Group II strain Eklund 17B in the presence of L-alanine or L-cysteine, with L-lactate and NaHCO₃ in Tris-HCl buffer. The most effective amino acid germinant for Group I strain Af84 in the present study was L-cysteine (with L-lactate and NaHCO₃), and the most effective germinant for Group II strain Eklund 17B was L-alanine (with L-lactate and NaHCO₃), albeit, the difference between L-alanine and L-cysteine germination for Group II strain Eklund 17B was minimal. A 50% drop in OD₆₀₀ was observed for spores of Group I strain Af84 with L-alanine/L-lactate/NaHCO₃, whereas in a previous study, only a 30% fall in OD₅₈₀ was observed for Group I strain Beans (NCTC 7273) in response to this germinant (Broussolle *et al.*, 2002). These authors also reported that spores of Group I strain Beans do not decrease in OD₅₈₀ with L-alanine as much as spores of the closely related organism *C. sporogenes* strain NCIMB 701792 (Broussolle *et al.*, 2002). The difference in change in optical density observed for Group I strains in the current study compared to the study by Broussolle *et al.* (2002), could be due to the different strain of Group I used or the different method used to produce the spores. Similarly, in the study by Brunt *et al.* (2014), L-cysteine in combination with L-lactate/NaHCO₃ also initiated germination most effectively in strains of Group I and *C. sporogenes*, even though L-alanine had a lower minimum effective germination concentration than L-cysteine. Germination of Group I strain ATCC 3502 was most effective with L-phenylalanine rather than L-alanine, which may suggest that amino acid germinants are species and strain specific (Brunt *et al.*, 2014). Brunt *et al.* (2014) also stated that L-lactate was not an essential requirement for germination of Group I strain ATCC 3502 with L-alanine or L-cysteine. Interestingly, a paper by Meaney *et al.* (2015) suggested that

NaHCO₃ is required for spore germination in *C. botulinum* Group I strain ATCC 3502 rather than L-lactate.

The effect of numerous amino acids on the germination of spores of *C. botulinum* Group II strains Eklund 17B, Beluga and Craig 610 was studied by Plowman and Peck (2002). The amino acids L-alanine, L-serine and L-cysteine were the most effective germinants for spores of Group II strains, in combination with L-lactate/NaHCO₃ in phosphate buffer. The results obtained by Plowman and Peck (2002), revealed that L-cysteine supplemented with both L-lactate/NaHCO₃ produced the most rapid germination in the strains of *C. botulinum* Group II. However none of the amino acids germinated spores in the absence of L-lactate.

One hypothesis for the difference in which amino acid was the most effective between L-alanine and L-cysteine for spores of strain Eklund 17B in the present study and a previous study by Plowman and Peck (2002), may be due to the different sporulation conditions used. Recent publications have shown that the sporulation condition has a direct effect on the germinating capability of the spore (Brunt *et al.*, 2014, Meaney *et al.*, 2015). A positive correlation has been reported between spore yield and germination rate in *C. sporogenes*, a surrogate for *C. botulinum* Group I, whereas for *C. botulinum* Group II spore yield was affected by sporulation temperature, but not the germination or heat resistance properties of the spore (Brunt *et al.*, 2014, Peck *et al.*, 1995). In a study by Hornstra *et al.* (2006), the rate and efficiency of germination of spores of *B. cereus* were greatly decreased when spores were produced in suboptimal conditions, mimicking the sporulation environment that contaminating spores of *B. cereus* are formed in, as often the food environment is less than optimal in terms of temperature and composition of media (Hornstra *et al.*, 2006). Thus, challenge tests in foods may be more realistic if the spores used have been formed in conditions relevant to product contamination, and it may also provide a more realistic model for characterising spore germination (Ramirez-Peralta *et al.*, 2012).

Spores of Group I strain Af84 and Group II strain Eklund 17B respond most effectively with different amino acid stimuli (L-cysteine for Group I strain Af84 and L-alanine for Group II strain Eklund 17B), which may be due to the different germination pathways in the two groups of *C. botulinum*. The germinant receptors differ between *C. botulinum* Group I and Group II strains; there are three germinant receptors in Group I strains (GerX1, 2, 3) with one (GerX1) dominant, whereas there is only one class of germinant receptor in Group II strains, which is similar in orientation to GerX3 in Group I, and for simplicity is also termed GerX3 (Brunt *et al.*, 2014). Amino acids bind to inner membrane germinant receptors and

the differences in the number and types of germinant receptors in Group I and Group II may explain the varied germination that occurred with the amino acids tested in both strains during this study.

4.3 Spore germination in response to CaDPA

Non-nutrient germinants are able to germinate spores of *Bacillus* and *Clostridium* by bypassing the germinant receptors located in the inner membrane (Paidhungat and Setlow, 2000). The most studied non-nutrient germinants in *Bacillus* species include CaDPA and DDA, however these are only relevant in laboratory studies (Setlow, 2013). The mechanism by which exogenous CaDPA is able to germinate spores of *B. subtilis* was proposed by Paidhungat and Setlow (2000) and suggested that CaDPA may activate a downstream effector in *B. subtilis* because it does not require the GerA receptor family in order to germinate. More recently, CaDPA has been shown to activate the cortex lytic enzyme CwlJ in *B. subtilis*, without involving a germinant receptor (Setlow, 2013). Unlike germinant receptors, CwlJ is located within the cortex-coat boundary rather than in the inner membrane (Bagyan and Setlow, 2002). In the current study, exogenous CaDPA did not stimulate germination in Group I strain Af84 or Group II strain Eklund 17B. The reasons for this are not clear, however, germination with CaDPA may be species or strain dependent. In the literature, no data are available for exogenous CaDPA-induced germination of spores of *C. botulinum*; however, there have been previous studies, which show that some Clostridia, such as *C. perfringens*, are able to germinate effectively with CaDPA, such as the recent studies by Paredes-Sabja *et al.* (2009b) and Wang *et al.* (2012). These studies showed that *C. perfringens*, a foodborne pathogen capable of causing gastrointestinal disease in humans and animals and the causative agent of gas gangrene, is able to germinate in response to CaDPA (Myers *et al.*, 2006, Paredes-Sabja *et al.*, 2009b, Wang *et al.*, 2012). Notably, CaDPA seems to initiate germination in *C. perfringens* by activating the GerK germinant receptor, rather than by activating the CwlJ cortex lytic enzyme, which is the mechanism by which CaDPA induces germination in *B. subtilis* and *B. megaterium* (Paredes-Sabja *et al.*, 2008, Setlow *et al.*, 2009). Germinant receptors in similar configuration to GerK are present in *C. botulinum* Group I and II (Brunt *et al.*, 2016).

Spores of *C. difficile*, the leading cause of gastroenteritis and infectious nosocomial diarrhoea in the UK and US, did not germinate with exogenous CaDPA (Wang *et al.*, 2015). One hypothesis was that this was due to the lack of inner membrane germinant receptors

in *C. difficile* (Lessa *et al.*, 2015, Wang *et al.*, 2015). A later study by Kochan *et al.* (2017) showed that exogenous CaDPA is unable to germinate spores of *C. difficile*, unless supplemented with taurocholate. In the same study, it was also found that CaCl₂ with taurocholate induced germination of *C. difficile* in the absence of exogenous DPA. Contrastingly, *B. anthracis* was able to germinate in response to CaDPA but germination with CaCl₂ alone was unsuccessful. As a result, it has been hypothesised in the literature that calcium ions can function as co-germinants and initiate cortex hydrolysis by activating SleC, rather than CwlJ, in *C. difficile* (Kochan *et al.*, 2017).

Interestingly, spores of *C. sporogenes*, a surrogate for *C. botulinum* Group I, are capable of germinating with exogenous CaDPA (Brunt, personal communication). This may suggest that the germination mechanism in these species is subtly different and raises the question as to whether *C. sporogenes* should be used as a surrogate in germination studies, even though they are genetically closely related. A similar comment was made by Brunt *et al.* (2014) based on the characterisation of nutrient germinant receptors. The conditions in which spores are prepared could have had an effect on the ability of spores to germinate with CaDPA. However, this possibility is not supported by the recent findings by Ramirez-Peralta *et al.* (2012) who reported that germination rates in *B. subtilis* with exogenous CaDPA were relatively similar for spores produced in a poor-medium at 23°C compared to spores prepared in a rich-medium at 37°C. As mentioned earlier, CaDPA activates CwlJ in *B. subtilis*, SleC in *C. difficile* and GerK in *C. perfringens*, which suggests that something is preventing exogenous CaDPA-induced germination in *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B as CwlJ is present in the germination mechanism of *C. botulinum* Group I, SleC is involved in the germination mechanism of *C. botulinum* Group II and both *C. botulinum* Groups I and II contain a germinant receptor in a similar arrangement to GerK.

4.4 Spore germination in response to DDA

Dodecylamine (DDA) was first reported as a non-nutrient germinant for spores of *B. megaterium* in 1960 (Rode and Foster, 1960). Since then, further investigation into cationic surfactant-induced germination has been conducted with spores of *B. subtilis*, using genetic mutations to determine the germination mechanism utilised, in particular by the primary alkylamine DDA (Christie, 2012). One study in particular has shown that germination of spores of *B. subtilis* with DDA follows a similar mechanism to high-pressure stimulated germination, where the germinant receptors are bypassed at low concentrations, and DPA

release and cortex lytic enzyme activation occurs via another mechanism (Setlow *et al.*, 2003). Understanding the mechanism by which DDA induces germination could be beneficial to food industry because DDA germinated spores are less heat resistant and more prone to environmental stresses than dormant spores. Thus, inducing germination by using cationic surfactants in food products could reduce the requirement for thermal processing at high temperatures, improve taste, reduce energy consumption and reduce cost for the producer (Setlow, 2000, Setlow *et al.*, 2003). Results obtained during the current study show that DDA-induced germination occurs with Group I strain Af84 and Group II strain Eklund 17B, however prior removal of the spore coat prevented germination. In the study by Setlow *et al.* (2003), the effect of DDA on spores of *B. subtilis* was measured by monitoring DPA release using OD₂₇₀. This study suggested that spore clumping caused by the addition of DDA can affect OD₆₀₀ readings and therefore, a decrease in OD₆₀₀ may not necessarily correlate with phase dark spores, hence why OD₂₇₀ was used instead. Results from Setlow *et al.* (2003) showed that not only did DDA germinate spores of *B. subtilis*, it also killed germinated spores; a phenomena previously described by Rode and Foster (1961).

In the study by Setlow *et al.* (2003), DDA induced germination of *B. subtilis* spores at 37°C. Similarly, germination of *C. botulinum* in the current study occurred with DDA. Germination in *B. subtilis* occurred in spores with defective germinant receptors and spores missing the cortex lytic enzymes CwJ or SleB (Setlow *et al.*, 2003). Decoating spores of *C. botulinum* prior to incubation with DDA was carried out during the present study and it was evident that decoating spores prevented spore germination as the spores remained entirely phase-bright. This may suggest that germination with DDA requires an unknown protein, which is located in the spore coat, and hence, when the coat is removed germination with DDA ceases. In contrast, decoating spores of *B. subtilis* had little effect on germination with DDA (Setlow *et al.*, 2003). A study by Vepachedu and Setlow (2007) also observed *B. subtilis* germination with DDA and found that the rate of germination with DDA increased as SpoVA levels increased. Thus, DDA may induce germination in spores of *B. subtilis* via channels composed of SpoVA proteins (Vepachedu and Setlow, 2007). In the present study, DDA addition brought about a 35% reduction in OD₆₀₀ over a 24 hour period for spores of Group I strain Af84 and ~50% spores appeared phase dark using phase-contrast microscopy. Spores of Group II strain Eklund 17B germinated more efficiently in response to DDA, with a 40% fall in OD₆₀₀ observed, and ~60% phase dark spores. During end-point microscopic analysis, not all of the spores appeared phase dark, which is a similar finding to results

published by Setlow *et al.* (2003), where they reported that only 75% of *B. subtilis* became phase dark in response to DDA. Interestingly, Setlow *et al.* (2003) also found that spores prepared in solid medium did not germinate as effectively with DDA as spores prepared in liquid medium. The spores used in the present study were prepared on a solid medium, and although a high yield of spores was produced, this may partly explain why spores did not germinate fully with DDA. Previous publications on *B. megaterium* have suggested that spores germinated with cationic surfactants, such as DDA, lose some of their refractiveness under phase-contrast microscopy and germinated spores may not appear as phase dark as they would if they had undergone nutrient germination (Rode and Foster, 1960, Rode and Foster, 1961). Therefore, difficulties in estimating the number of phase bright and phase dark spores during the current study may also be an issue. Germination in *C. perfringens* occurred with DDA, interestingly, it was also noted that germinated spores of *C. perfringens* were not as phase-dark in appearance as expected when compared to nutrient germinated spores (Paredes-Sabja *et al.*, 2008).

4.5 Spore germination in response to lysozyme

Lysozyme was able to germinate spores of *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B, once the spore coat had been removed. Previous studies have also shown lysozyme to have an effect on germination of spores of a wide variety of species, including *B. subtilis* and *B. megaterium* by activating part of the germination mechanism, independent of the germinant receptors or cortex lytic enzymes (Paidhungat and Setlow, 2002, Suzuki and Rode, 1969). In *C. botulinum*, lysozyme has commonly been used to remove vegetative cells from spore crops by digestion (Grecz *et al.*, 1962). A chemical treatment can be given to spores which disrupts the disulphide bonds in the spore coat resulting in complete removal of this layer and as a result spores are permeable to lysozyme and other lytic enzymes (Gould and Hitchins, 1963, Gould and Hitchins, 1965). After spore coat removal, lysozyme-induced germination can proceed as the enzyme can access the cortical mucopeptide substrate located below the spore coat (Gould and Hitchins, 1963). However, one study has shown that spores of *B. megaterium* do not require prior treatment to permeabilise the spore coat for germination with lysozyme to occur, suggesting that the lysozyme substrate is accessible to lysozyme without the need for spore coat degradation (Suzuki and Rode, 1969). In the present study, germination of spores of *C. botulinum* Group I and Group II did not occur (as determined by change in

OD₆₀₀) in spores that had an intact spore coat, which suggests that the spore coat structure is not permeable to lysozyme. In the current work, following removal of the spore coat, germination of Group I strain Af84 and Group II strain Eklund 17B occurred with a lysozyme concentration of 10µg/ml and resulted in a fall in OD₆₀₀ of ~25% and ~55% for Group I strain Af84 and Group II strain Eklund 17B, respectively. In another study, the minimum concentration of lysozyme that initiated germination in spores of *B. megaterium* was 1.5µg/ml, which resulted in an OD₆₀₀ drop by 88% in the first 30 minutes (Suzuki and Rode, 1969).

Determining the percentage of phase-dark spores after lysozyme incubation with Group I strain Af84 was challenging due to the apparent loss of spores during the germination tests. Counts using a haemocytometer revealed a reduction in the total spore titre when incubated with lysozyme, with no spores detected after 24 hours. Whereas, for Group II strain Eklund 17B, 24-hour incubation with lysozyme had little effect on the total spore titre. Osmotic rupture of Group I strain Af84 with lysozyme but not of Group II strain Eklund 17B, suggests that there may be a difference in cortex structure between *C. botulinum* Group I and Group II. The presence of significant quantities of debris following the bursting of spores of Group I strain Af84 may explain why only a ~25% change in OD₆₀₀ was observed during lysozyme germination, compared to a ~55% fall in OD₆₀₀ for Group II. In the current study, varying concentrations of lysozyme (0, 1, 3, 10, 30, 100µg/ml) were tested to determine whether the enzyme had a toxic effect on cell growth from spores. The results showed no evidence of toxicity with similar viable counts at all of the tested concentrations of lysozyme. The concentration of lysozyme used in the present study was 10µg/ml, as this was the concentration previously shown to initiate germination in *C. botulinum* Group II strains (Lund and Peck, 1994).

4.6 Determination of which part of spore germination apparatus is damaged by heat

After determining the specific germinants and conditions that induce germination of unheated spores of Group I Strain Af84 and Group II strain Eklund 17B, the recovery of heat inactivated spores using these germinants was tested in order to begin to define which parts of the germination apparatus were damaged by heat. Studies in the literature have reported on the heat resistance of spores of *C. botulinum* in a variety of food products, however many of these studies fail to consider the effect of lysozyme, which when present

may increase spore survival (Lund and Notermans, 1992, Lund and Peck, 1994). During the current study, spores of Group I strain Af84 were not recovered on PYGS medium containing lysozyme (10µg/ml), regardless of whether the spore coat was made permeable by decoating. In contrast, plating heat-inactivated spores of Group II strain Eklund 17B with an intact spore coat on PYGS medium containing lysozyme (10µg/ml) increased spore recovery by >4.4-log. Furthermore, the recovery of heat-inactivated spores was increased to >5.8-log, when the spore coat was removed. In a similar study by Peck *et al.* (1992a), the number of colonies formed on media containing lysozyme (10µg/ml) increased by a factor of 10³-10⁶, compared to the number of colonies formed on media without lysozyme for six different *C. botulinum* Group II strains after heat inactivation at 85°C for 10 minutes. For Group II strain Eklund 17B, a 5.5-log increase in survival of spores was observed when heat-inactivated spores were plated on medium containing lysozyme (10µg/ml) and spores were still viable after 5 minutes of heating (Peck *et al.*, 1992a). Survival of decoated spores with lysozyme was also noted in a previous study by Peck *et al.* (1992b), where the recovery of spores treated with thioglycolate was increased by approximately 3-log after prior heat treatment for 120 minutes at 85°C. The ability of lysozyme to recover a fraction of spores prior to chemical decoating in the present study suggests that the spore coat of Group II strain Eklund 17B is a permeable structure, with a small proportion of spores permeable to lysozyme. This small fraction could not be detected in the current study during germination assays of the spore population using the Bioscreen. The ability of lysozyme to recover spores of Group II strain Eklund 17B after heat inactivation at 85°C for 2 minutes, suggests that DNA within the spore is not damaged by this heat treatment. The inability of lysozyme to recover spores of Group I strain Af84 is not due to lysozyme preventing cell multiplication, nor to an inability of lysozyme to degrade the spore cortex, but may be due to the lytic effect that lysozyme has on spores during germination. Furthermore, a previous report by Setlow (2014), stated that spores that had been germinated by lysozyme are prone to osmotic rupture due degradation of the germ cell wall by the muramidase. Another possible explanation of the inability of lysozyme to recover spores of Group I strain Af84 is that the spores are no longer viable (for example due to DNA damage).

A study by Alderton *et al.* (1974) found that the addition of lysozyme to the recovery medium of heat-inactivated spores increased the recovery of a *C. botulinum* Group II type E strain and a *C. botulinum* Group I type A strain. Specifically, the measured heat resistance of *C. botulinum* Group II type E strain increased by 1800-fold when lysozyme was present in the recovery medium, whereas the heat resistance of *C. botulinum* Group I type A strain

only increased up to 3-fold with the addition of lysozyme (Alderton *et al.*, 1974). The results for the Group II strain is similar to that reported in the current study and by others (e.g. Peck *et al.* (1993)). The small (3-fold) increase in recovery for the Group I strain reported by Alderton *et al.* (1974) may reflect strain dependency or a loss of spores in the current work during the washing procedure. The decoating solution was changed during the current study from dithiothreitol to sodium thioglycolate, but this change had no effect on the number of colonies formed from heat inactivated spores on medium supplemented with lysozyme (data not shown).

To determine which part of the germination apparatus in Group I strain Af84 and Group II strain Eklund 17B is damaged by heating, a nutrient and non-nutrient germinant were included in the recovery protocol for heat-inactivated spores. In the current study, recovery of heat damaged spores of Group I strain Af84 and Group II strain Eklund 17B with DDA or L-alanine in the recovery medium was unsuccessful. During a study by Trunet *et al.* (2015), attempts to recover heat killed spores of *B. weihenstephanensis* and *B. licheniformis* with medium supplemented with an alanine-inosine mix and lysozyme were unsuccessful, even though spores of *B. weihenstephanensis* and *B. licheniformis* have previously been shown to germinate in response to these stimuli (Garcia *et al.*, 2010, Madslie *et al.*, 2014). Similarly, a study by Coleman and Setlow (2009) found that the addition of L-alanine and other amino acids, such as L-arginine and L-glutamine, did not affect recovery of heat inactivated spores if glucose was omitted from the recovery medium. In the present study, CaDPA did not induce germination in spores of Group I strain Af84 or Group II strain Eklund 17B and therefore, heat inactivation and recovery of spores with CaDPA in the recovery medium was not tested. A previous publication by Cazemier *et al.* (2001) reported that the recovery of heat-injured spores increased by 0.5-log with medium supplemented with CaDPA in strains of *B. subtilis*, which prompted the hypothesis that the germinant receptor proteins were damaged by heat. At present, studies evaluating the capability of DDA to recover heat-inactivated spores are limited and therefore, no comparisons can be made between previously published data and the results obtained in the current study.

The inability to recover heat-inactivated spores of Group I strain Af84 with nutrient germinants, DDA or lysozyme leaves it unresolved as to whether part of the spore germination apparatus is damaged by heating, or whether another part of the spore is damaged. The ability of lysozyme, but not DDA or nutrient germinants, to recover heat-inactivated spores of Group II strain Eklund 17B indicates that part of the germination apparatus is damaged by heating, possibly the germinant receptors, SpoVA proteins, cortex

lytic enzymes, or another presently unknown structure. The germination pathways in *C. botulinum* Group I and II were proposed by Brunt *et al.* (2016), however, it was unknown as to whether DPA precedes cortex hydrolysis or vice versa. Some advancement has been made during the current study to try and determine this unknown and it is evident that DPA release, especially in Group II strain Eklund 17B, occurs early on during germination. However, it is still unclear whether cortex hydrolysis occurs as a result of DPA release in *C. botulinum* Group I and Group II, as attempts to determine the presence of certain cortex fragments in the supernatant of germinating spores of Group I and Group II was unsuccessful (data not shown). A previous study by Francis *et al.* (2015), demonstrated that cortex hydrolysis is initiated before DPA release in spores of *C. difficile*, which is in contrast to previous findings in which DPA release causes cortex hydrolysis in *B. subtilis* and *C. perfringens* (Paredes-Sabja *et al.*, 2009a, Setlow *et al.*, 2008).

4.7 DPA content of spores

The DPA concentration of spores of Group I strain Af84 and Group II strain Eklund 17B was estimated during the current study. The DPA concentration for spores of Group II strain Eklund 17B was calculated to be 2.1pg/spore of DPA, compared to 1.9pg/spore of DPA per spore of Group I strain Af84. The DPA concentration per spore in *Clostridium* varies between species and strain, for example, spores of *C. beijerinckii* strain DSM 791 contained 0.09pg DPA per spore, whereas spores of another strain of *C. beijerinckii* contained 4.4pg DPA per spore (Jamroskovic *et al.*, 2016). In the same study, it was found that DPA content in spores of *C. acetobutylicum* ranged from 1.98pg to 2.6pg DPA per spore. DPA in the spore core contributes to overall spore heat resistance, but there are a variety of other factors that are also involved, such as low water content, presence of small acid-soluble proteins (SASPs), and spore structures, including the coat and inner membrane (Paidhungat *et al.*, 2000, Setlow *et al.*, 2006). In the current study, the two strains of *C. botulinum* used had a similar DPA content per spore, even though Group II strain Eklund 17B is less heat resistant than Group I strain Af84, suggesting that other factors are involved in providing the spore with heat resistance properties and not solely DPA content. Similarly, there has been no correlation identified between spore heat resistance and DPA content in previous publications investigating heat resistance of spores, suggesting that a large amount of DPA in the spore core does not necessarily mean that the spore will have a high heat resistance, which also indicates that other elements, in combination, protect the spore from heat

damage (Grecz and Tang, 1970, Kort *et al.*, 2005). The lack of relationship between DPA content and heat resistance may explain why Group II strain Eklund 17B in the current study has a similar amount of DPA per spore, however is less heat resistant, than Group I strain Af84. In the study by Grecz and Tang (1970), the least heat resistant strain (*C. botulinum* Type E strain VHE) had approximately the same amount of DPA as the most heat resistant strain (*C. botulinum* Type B strain 41B), however there was a correlation between heat resistance and the rate DPA was expelled from the spore core. Less than 1% (0.26% and 0.14% for Group I and II, respectively) of the spore DPA was released in the germination tests carried out.

5. Conclusion and Future Research

Results from this study have allowed revisions to be made to the proposed germination pathways in *C. botulinum* Group I and Group II, as described by Brunt *et al.* (2016) (Figure 27). Important differences in the two germination pathways have been identified, including a reliance on alternative germinant receptors and spore lytic enzymes. Additionally spores of Group I are more heat resistant than those of Group II. In the current study, spores of Group I strain Af84 and Group II strain Eklund 17B were heated at 95°C for 4 hours and 85°C for 2 minutes, respectively. Spores of Group I strain Af84 were not recovered with any of the germinants used during this study, and it is unclear whether the heat treatment damages the germination apparatus or another part of spore, and whether this damage is reversible. The ability of Group II strain Eklund 17B to be recovered with lysozyme, but not nutrient germinants or DDA, indicates that part of the germination apparatus, possibly the cortex lytic enzymes, germinant receptors, SpoVA proteins or an unknown structure is damaged by heat. The inclusion of lysozyme in the recovery medium overcomes this heat damage.

Germination in Group I and Group II strains with nutrient and non-nutrient germinants is strain and species specific and hence, more research needs to be done on *C. botulinum* using a larger number of strains of each Group in order to define the germination pathway fully. Furthermore, deciphering the timing of DPA release and cortex hydrolysis in spores of *C. botulinum* Group I and Group II using a cortex fragment assay that detects the presence of NAG residues, will advance knowledge on germination in *C. botulinum* and potentially result in prevention of germination of spores, leading to a reduction in cell outgrowth and toxin formation. Future research will involve using the Clostron method to make mutants of spores of Group I strain Af84 and Group II strain Eklund 17B and the use of non-nutrient germinants that have been identified that bypass the germinant receptors, in order to confirm the part of the germination apparatus that is damaged by heat.

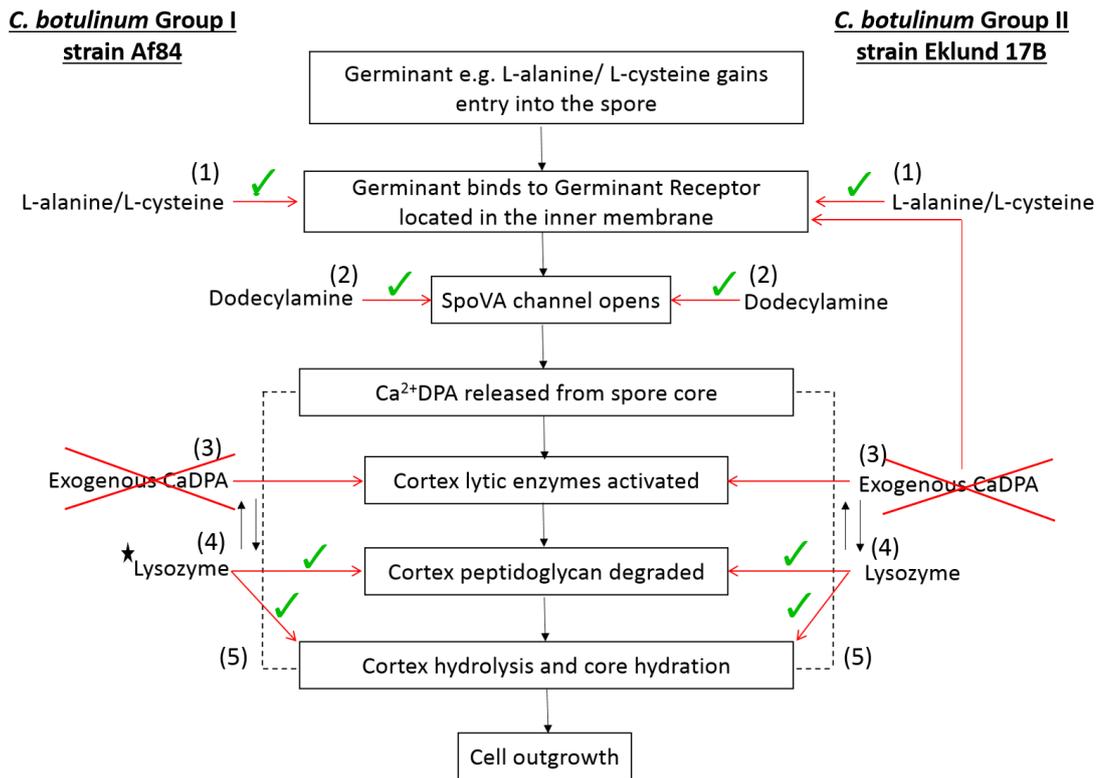


Figure 27. Revised germination pathway diagram in *C. botulinum* Group I strain Af84 and Group II strain Eklund 17B, showing whether the nutrient and non-nutrient inputs successfully germinated strains of *C. botulinum*. (1) Germination of both strains occurred with L-alanine and L-cysteine, however no recovery of heat-inactivated spores occurred with L-alanine, suggesting that the germinant receptors may be damaged by heat. (2) Dodecylamine (DDA) induces germination in both strains, possibly through SpoVA channels, however other unknown coat proteins may be required during germination because removing the coat of spores prevented germination. DDA did not recover heated spores, therefore this part of the apparatus may be damaged by heat. (3) Exogenous CaDPA did not germinate strains of *C. botulinum* used during this study, so no conclusion can be made as to whether heat damages cortex lytic enzymes in Group I and II or germinant receptors homologous to GerK in Group II. (4) Lysozyme germinates spores of both strains if the spore coat is removed. Heated spores of Group II strain Eklund 17B are recovered with lysozyme, suggesting that the DNA is not damaged but maybe an earlier part of the spore germination apparatus is damaged by heat (*Group I strain Af84 cannot be recovered with lysozyme and is prone to osmotic rupture). (5) DPA appears to be released before or at a similar time, to cortex hydrolysis.

6. References

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