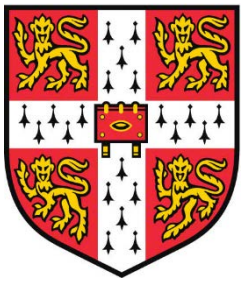
Department of Chemical Engineering and Biotechnology



University of Cambridge

Molecular analysis of GerP and spore-associated proteins of *Bacillus cereus*

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St. Edmund's College

April 2017

This dissertation is submitted for the degree of

Doctor of Philosophy

Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

This dissertation does not exceed the prescribed word limit for the relevant Degree Committee. It contains 48,495 words and 66 figures.

……

Abhinaba Ghosh

May 2nd, 2017

For my dearest Dadu and Didu...

Sri Birendra Nath Sarkar & Smt Basanti Sarkar

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Publications related to this thesis

Eijlander RT, Holsappel S, de Jong A, **Ghosh A,** Christie G, Kuipers OP. SpoVT: From Fine-Tuning Regulator in *Bacillus subtilis* to Essential Sporulation Protein in *Bacillus cereus*. ***Frontiers in Microbiology*** **(2016);7:1607**

Ghosh, Abhinaba; Manton, James D.; Rees, Eric J. and Christie, Graham. Proteins encoded by the *gerP* operon are localised to the inner spore coat in *Bacillus cereus* and are dependent on GerPA for assembly around the developing forespore (Manuscript submitted)

Abstract

Spores of various strains of *Bacillus cereus* are the causative agents of emetic and diarrheal foodborne illnesses. Typically, spores will survive thermal treatments that destroy vegetative cells, and then go on to germinate to form the vegetative cells that are associated with toxin production. The spore has to germinate in order to develop into the vegetative cells that produce toxins, hence a thorough understanding of the proteins and molecular mechanisms that underpin spore germination are of great significance from spore control perspectives. A major objective of this thesis was to use molecular genetic and fluorescence microscopy techniques to characterise the location and function of the GerP proteins in *Bacillus cereus* 14579. The GerP proteins have been identified from mutagenesis studies across the *Bacilli* as being implicated in spore germination, most likely by impacting upon the permeability of the spore coat. Data presented in this thesis reveal that the various GerP proteins all localise to the same inner-coat vicinity within the spore, as determined via the super-resolution ellipsoid localisation microscopy technique. The study also reveals that only the GerPA protein is required for the localisation of the other GerP proteins in the developing spore. A number of other coat and or germination associated proteins in *B. cereus* 14579 were examined in the course of this work. These include the GerN and GerT antiporters, which are both shown to have an involvement in inosine mediated spore germination in this strain. However, hypothetical interactions between antiporter proteins and the 'linker-like' N-terminal domain of the GerIA inosine-responsive germinant receptor protein appear unlikely since spores engineered with a truncated GerIA receptor subunit germinate normally. The protein encoded at locus BC1245 was also examined in this work, since it too had been implicated in spore germination. Data presented in this thesis indicate that this is not the case, and that the protein is a component of the spore coat. Overall, the work conducted in this project contributes to knowledge of spore assembly, spore structure and mechanisms that underpin germination, which ultimately, should permit the development of improved methodologies for spore control.

Acronyms and Abbreviations

A_{600}	Absorbance at 600 nm
a.a.	Amino acid
L-Ala	Alanine
Amp^r	Ampicillin resistance
ApE	A plasmid editor
B. anthracis	*Bacillus anthracis*
B. cereus	*Bacillus cereus*
B. subtilis	*Bacillus subtilis*
bps	base pairs
BACTH	Bacterial Adenylate Cyclase Two-Hybrid System
BSA	Bovine serum albumin
CCY	Defined media for *B. cereus* sproulation
cDNA	Complementary Deoxyribonucleic acid
CIP	Calf intestinal alkaline phosphatase
CLE	Cortex lytic enzyme
DCO	Double cross over
DNA	Deoxyribonucleic acid
DNase	deoxyribonuclease
DPA	Dipicolinic acid
E. coli	*Escherichia coli*
FM	Forespore membrane
GF	Gel filtration
GFP	Green fluorescent protein
GPLK	Glucose, proline, leucine, KBr
GR	Germinant receptor
GSP	Germination specific protease
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
IPTG	isopropyl-β-D-thiogalactopyranoside
KAM	Klenow Assembly Method

kDa	kilo Daltons
LB	Lysogeny broth
LIC	Ligation independent cloning
mRNA	messenger RNA
MES	2-(N-morpholino)-ethanesulphonic acid
MOPS	3-(N-morpholino)-propansulphonic acid
MPa	Mega pascal
MLS^r	Macrolide-lincosamide-streptogramin B resistance
MW	Molecular weight
MWCO	Molecular weight cut off
N-	Amino terminus
NAG	N-acetyl glucosamine
NAM	N-acetyl muramic acid
NEB	New England Biolabs
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PBPs	Penicillin-binding proteins
PG	Peptidoglycan
PMSF	Phenylmethanesulphonylfluoride
psi	Pounds per square inch
RBS	Ribosome binding site
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SASPs	Small acid soluble spore proteins
SCO	Single cross over
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOB	Super optimal broth
TAE	Tris-acetate-EDTA
TE	Tris-EDTA

Tet^r	Tetracycline resistance
TM	Transmembrane
Tris	Tris-(hydroxymethyl-)aminomethane
UHP	Ultrahigh pure
UV	Ultraviolet
w/v	Weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactoside

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Chapter 1. Introduction

Members of the *Bacillus* and *Clostridium* genera are able to transform into highly resistant and dormant structures called endospores under conditions of nutrient depletion[1–5]. Spores are metabolically inactive but viable and can survive for perhaps thousands of years in a dormant state without any deleterious effects[6]. Spores are exceptionally resilient to a range of harsh conditions that would be lethal to vegetative bacterial cells such as heat, desiccation, UV and γ– radiations, pressure effects, extremes of pH and a number of toxic chemicals[7–9]. Numerous studies have been conducted over the years to understand the molecular basis of spore resilience. The studies conducted indicate that defined cellular compartments are associated with different aspects of environmental resistance[8–13]. The un-germinated spore itself is typically non–hazardous, but serves to facilitate the dispersion of various diseases, including anthrax (caused by *B. anthracis*), food poisoning (caused by *B. cereus* and *C. botulinum*), tetanus (caused by *C. tetani*) and *C. difficile* associated hospital infections (CDAD)[3,14,15].

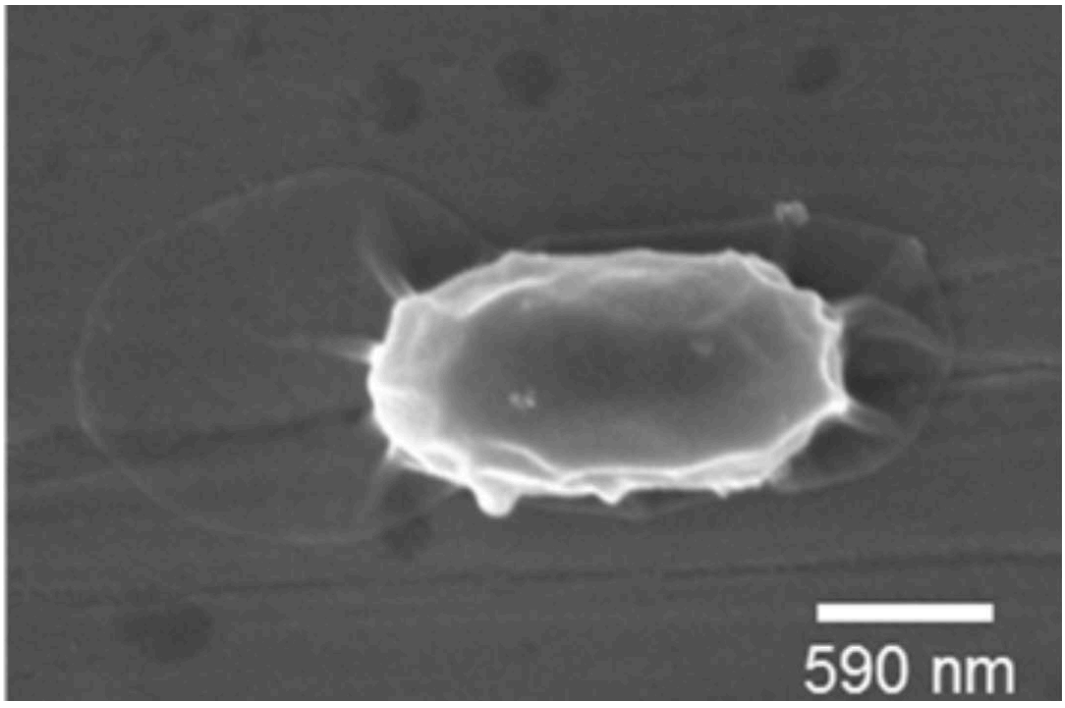


Figure 1.1 Scanning electron micrograph (SEM) of *B. cereus* ATCC 14579 dormant spores[16]

The pathogenicity and/or toxigenicity associated with these conditions require the spores to transform into vegetative cells by the process of germination, which is stimulated when conditions are favourable for growth. *B. anthracis* spores are thought to be the sole infectious cell morphotype, and their inoculation into a suitable host organism initiates the development of anthrax disease. Once the spores enter the host system, germination of the spore, which is the regulated resumption of metabolic activity within the bacterial cell, is the earliest pathogenic event that takes place[17–21]. Therefore, a thorough understanding of the mechanism of spore germination or the factors that contribute to the resistant properties of spores, might allow development of new treatments or routes to prevent various spore-borne disease(s).

1.1 The *Bacillus* genus

Bacillus species are rod-shaped, endospore-forming aerobic or facultatively anaerobic, gram-positive bacteria and a member of the phylum firmicutes[22]. The many species of the genus exhibit a wide range of physiological abilities that allow them to survive in every natural environment such as their ability to form spores. Only one endospore is formed per cell. *Bacilli* are ubiquitous in nature exploiting a wide variety of organic and inorganic substrates as nutrient sources. There are different ways adopted to classify this group based on biochemistry, lifestyles or growth on different substrates. One classification of the *Bacillus* splits them into three major classes: pathogenic, environmental, and those used for industrial purposes. The pathogenic class is represented by the toxin producers- *B. anthracis, B. cereus, and B. thuringiensis*. Collectively, these three organisms represent microbes of high economic, medical and biodefense importance. Given this significance, this group contains the highest number of closely related fully sequenced genomes, giving the unique opportunity for thorough comparative genomic analyses. Much of the disease and host specificity of members of this class can be attributed to their plasmids, which vary in size and number. Environmental Bacilli are quite diverse and include *B. subtilis, B. pumilus, B. halodurans,* and *B. coahuilensis*. The strains *B. licheniformis* and *B. megaterium* are well known representatives of industrial strains.

1.1.1 The *Bacillus cereus* group

The *Bacillus cereus* group of organisms contains the three toxin producing species of *Bacillus* genus- *Bacillus thuringiensis*, *Bacillus anthracis* and *Bacillus cereus*[23]. This group of gram-positive spore-formers forms a highly homogeneous subdivision of the genus *Bacillus.* Demonstration of their high genetic relatedness has contributed to the suggestion that *B. anthracis, B. cereus* and *B. thuringiensis* are members of a single species called *B. cereus sensu lato*[23].

B. thuringiensis is regarded as an insect pathogen alone affecting insects primarily from the orders *Lepidoptera*, *Diptera* and *Coleoptera*. It possesses *cry* genes encoding the crystal (cry) toxins located on large, transmissible plasmids[23–25].

B. anthracis, the causative agent of anthrax, encodes toxins and other virulence factors on two large virulence plasmids, pX01 (encodes the lethal tripartite toxin) and pX02 (responsible for the polyglutamate capsule), respectively[18,19,23].

B. cereus is an opportunistic pathogen, often associated with two forms of human food poisoning, characterised by either diarrhoea and abdominal distress or nausea and vomiting. *B. cereus* has been known to cause a variety of infections, including: endophthalmitis, bacteremia, septicemia, endocarditis, salpingitis, cutaenous infections, pneumonia and meningitis mostly in individuals with certain underlying conditions such as, immunocompromised patients, or patients recovering from surgery. *B. cereus* is also found as a contaminant in many food products, including dairy products. *B. cereus* ATCC 10987, a non-lethal dairy isolate phylogenetically more closely related to *B. anthracis* compared to the *B. cereus* ATCC 14579 harbors a large plasmid, pBc10987 that is similar to pXO1 in *B. anthracis.* However it lacks the virulence portion that encodes the tripartite lethal toxin and associated regulatory proteins. *B. cereus* ATCC 14579 does not harbor any large plasmid.

1.2 Sporulation

Under unfavourable conditions such as lack of nutrients in the surrounding medium, vegetative cells of *Bacillus* and *Clostridium* species irreversibly switch to a mechanism that results in the formation of spores[2,26,27]. This developmental process is termed as sporulation and follows a strictly regulated sequence of steps (Figure 1.2) which is highly conserved among spore formers[28]. Sporulation is most extensively studied in *B. subtilis*[29]. Although the scope of this work mainly focuses on germination, a brief overview of the sporulation process is presented here. More detailed analyses of sporulation are covered in several recently published reviews[30–36].

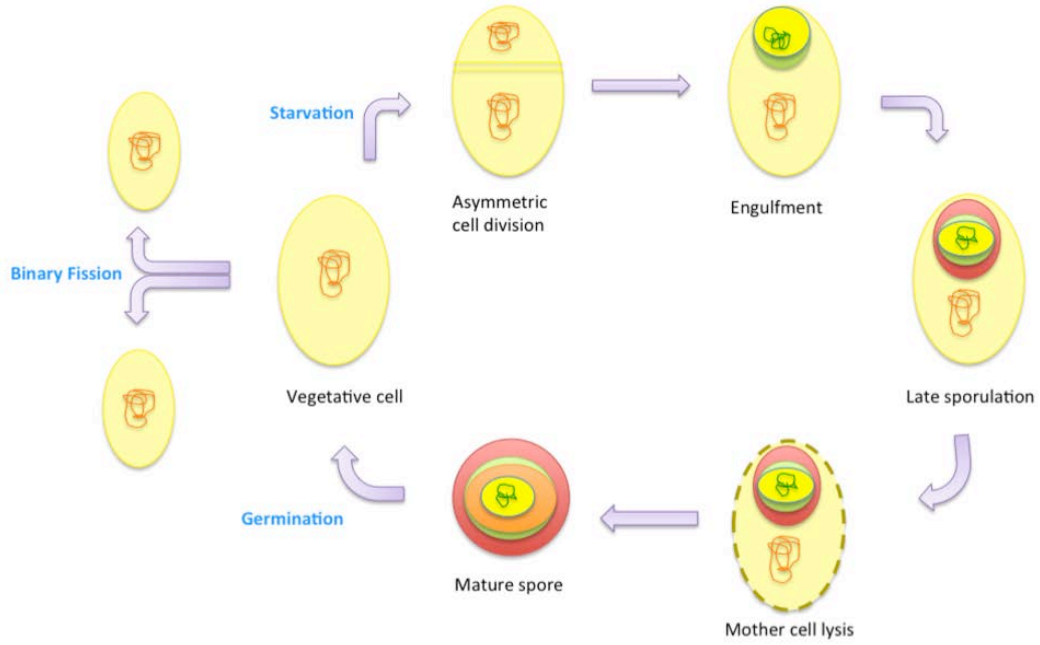


Figure 1.2 Representative illustration of the sporulation and germination cycle in *Bacillus cereus*.

During initial stages of sporulation, an asymmetric cell division creates two separate cell compartments inside the vegetative cell. In a process superficially resembling phagocytosis, the larger mother cell compartment engulfs the prespore, formed as a second compartment during the asymmetric division, isolating it from the surrounding environment with a double membrane. The primordial germ wall is assembled at the exterior of the innermost membrane and serves as precursor for the new cell wall after the spore germinates to resume the vegetative state. A thick layer of structurally unique peptidoglycan, referred to as the cortex, which is crucial in maintaining spore dormancy, is deposited between the primordial germ cell wall and the outer membrane. At or around a similar time, the multi-proteinaceous spore coat is synthesised in the mother cell and deposited onto the developing spore surface. In some species such as *B. cereus*, an additional layer called the exosporium is assembled around the spore. Thus, the resulting spore morphology consists of a set of concentrically arranged layers surrounding the cellular protoplast termed as the spore core. Finally, after approximately 8 hours, the mother cell lyses, releasing the metabolically dormant spore into the environment[28,37,38].

Although metabolically inactive, spores can constantly monitor their surroundings to determine when conditions are conducive to support spore germination and re-entry to the

metabolically active vegetative state. Germinant molecules such as amino acids, ribonucleosides or sugars, are detected by germinant receptors (GRs) located in the inner membrane of the spore. Interaction of germinants with their cognate receptors causes changes in the permeability of the inner membrane, resulting in the influx of water, while concomitant efflux of small molecules, such as metal ions and calcium dipicolinate, from the spore core. Specialised peptidoglycan lysins such as cortex lytic enzymes (CLEs) subsequently degrade the spore cortex, allowing full rehydration of the core, initiation of metabolism, and the emergence of a new cell from the fractured spore coat[39,40].

1.3 Spore structure

The bacterial spore is morphologically distinct from its vegetative cell and plays a key role in maintaining dormancy and enabling survival in extreme environments, including extra-terrestrial conditions[28]. Spores of all species belonging to the *Bacillus* and *Clostridium* genera have a similar structure, except for the presence or absence of the outermost structure called the exosporium. The anatomy of bacterial spores can be described as a series of concentrically arranged layers[39]. A *Bacillus* spore is a complex structure and consists of three main compartments–the core, the cortex and the coats[11,28,41].

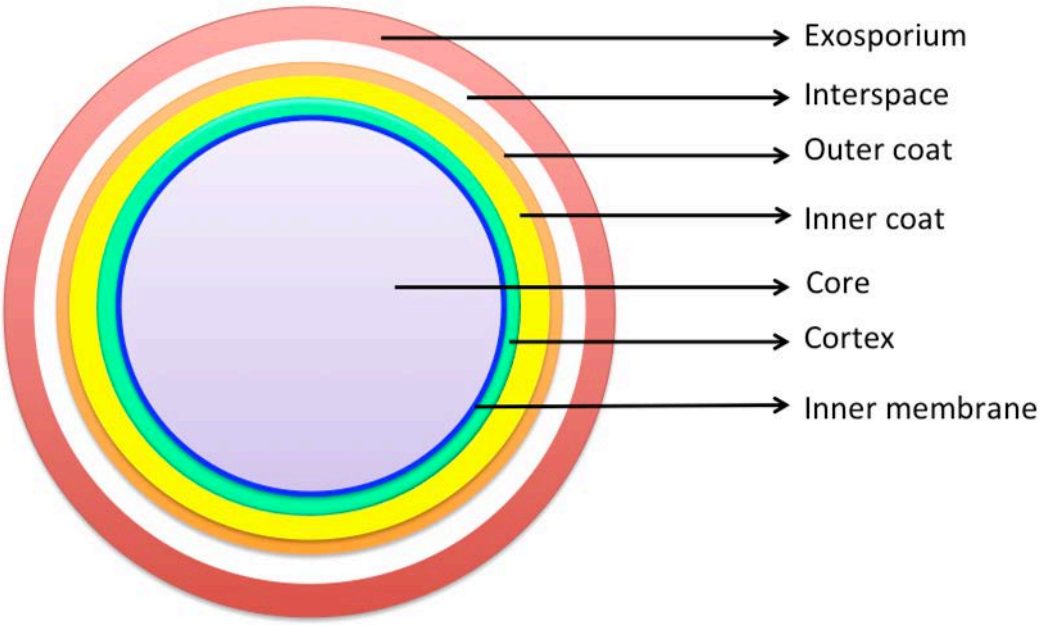


Figure 1.3 Schematic illustration of a typical *B. cereus* spore, showing its architecture of concentrically arranged shells. The DNA-hosting core (violet) is encased by the inner membrane (blue), the cortex (green), the inner (yellow) and outer coat (orange), the interspace (white) and the exosporium (red).

Figure 1.3 depicts the schematic representation of a *Bacillus* spore. The innermost region of the spore is the core. It is essentially the cellular protoplast, which comprises the bacterial genome, ribosomes and various cytoplasmic proteins[42]. The bacterial DNA is encrusted by spore-specific small acid soluble proteins (SASPs), also contained within the partially dehydrated core, where most of the water has been replaced with calcium dipicolinate[28,38]. The SASPs bind to spore DNA, thereby altering its photochemistry and resulting in increased resistance of spore DNA to desiccation, radiation and toxic chemicals[8,10,12,42,43]. Calcium dipicolinate (Ca^{2+}–DPA) in the core contributes towards the thermal resistance of the spore[12,44]. DPA is a unique spore solute and constitutes around 5 – 15% of the spore's dry weight[45–47]. The core is surrounded by the inner spore membrane that acts as the main permeability barrier towards ingress of small molecules to the spore core[42,48–50]. The inner membrane is also crucial to spore germination, as it bears many of the key components of the germination apparatus, including the germinant receptors (GRs), DPA channel proteins, cortex lytic enzymes (CLEs) and associated proteins, and

other accessory proteins that help in germination such as GerD and the GerN–type antiporter proteins[51–54]. The inner membrane is surrounded by the germ cell wall, which will act as precursor for the vegetative cell wall once the bacterium resumes vegetative growth[1,55–57]. Next to the germ cell wall is a thick layer of peptidoglycan, called the cortex, that further ensures the core's dehydrated state, and is additionally surrounded by the outer spore membrane, the precise function which is unknown[37,55,58–61].

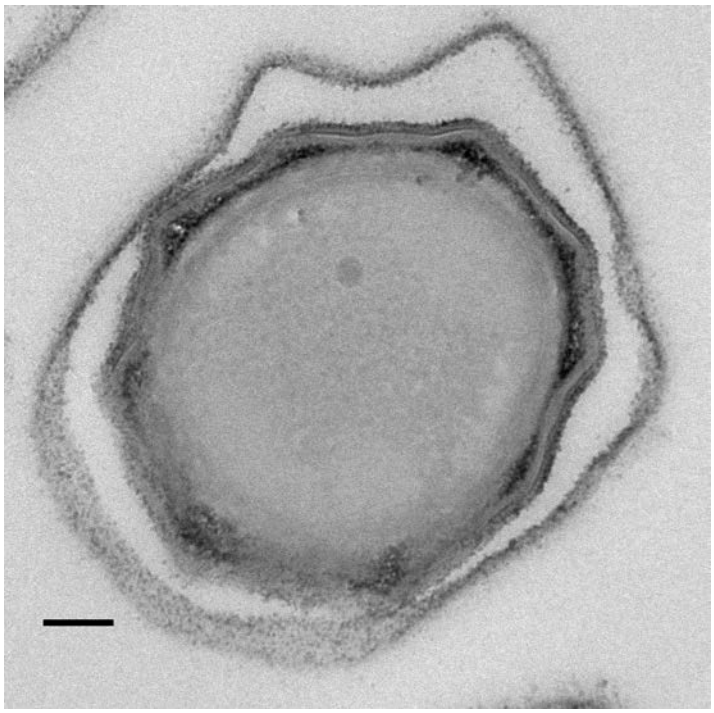


Figure 1.4 Representative thin section electron micrograph of a *B. cereus* spore. Size bar represents 100 nm.

The spore coat comprises a series of thin, concentric proteinaceous layers composed of around 80 proteins, some of which show a lamellar structure when viewed under the electron microscope[62–64]. Depending on the species, Transmission Electron Micrographs (TEMs) of the spore reveal that the spore coat may comprise a lamellar inner coat and a coarse outer coat[28,37,38,65]. Regardless of inter-species differences, the spore coat serves as a physical barrier between the spore and its environment and is selectively permeable towards different molecules by functioning as a molecular sieve and restricts access to potentially lytic enzymes and other deleterious agents that could potentially damage the core contents including harmful UV radiation[9,66–69]. The GerP proteins that have been shown to have a role in germination by mutational analysis, perhaps by mediating access

of germinants to the inner membrane located germinant receptors, are hypothesized to be located in the spore coat[6,70–72]. The outermost layer of spores of some species are characterised by the presence of a layer that is distinct from the spore coat, referred to as the exosporium[73–75]. Thin section TEMs of *B. cereus* family spores have revealed that the exosporium is a balloon-like layer that loosely surrounds the underlying spore coat and is separated from the spore coat by a defined interspace[28,76]. The precise function of the exosporium has not yet been established, although it may contribute towards spore protection, adherence, or virulence in pathogens[77–79]. Spores of *B. subtilis*, however, do not have an exosporium, but instead have a glycoprotein-containing layer termed the crust, as identified by ruthenium red stained thin-section TEMs[80,81]. The presence of glycosylated proteins in the crust led to the suggestion that the crust may actually represent a rudimentary exosporium structure in *B. subtilis*[28,80–84]. The best studied exosporium is of *B. cereus* spores, which is composed of protein (43 – 52 % dry weight), lipids (15 – 18 % dry weight), and carbohydrate (20 – 22 % dry weight) , respectively[85,86].

1.4 Spore germination

Bacterial spores can sense the presence of small germinant molecules and respond by germinating, losing the specialised structures of the dormant spore and resuming active metabolism, before outgrowing into vegetative cells[35] The process of germination can be divided into two main stages[87].

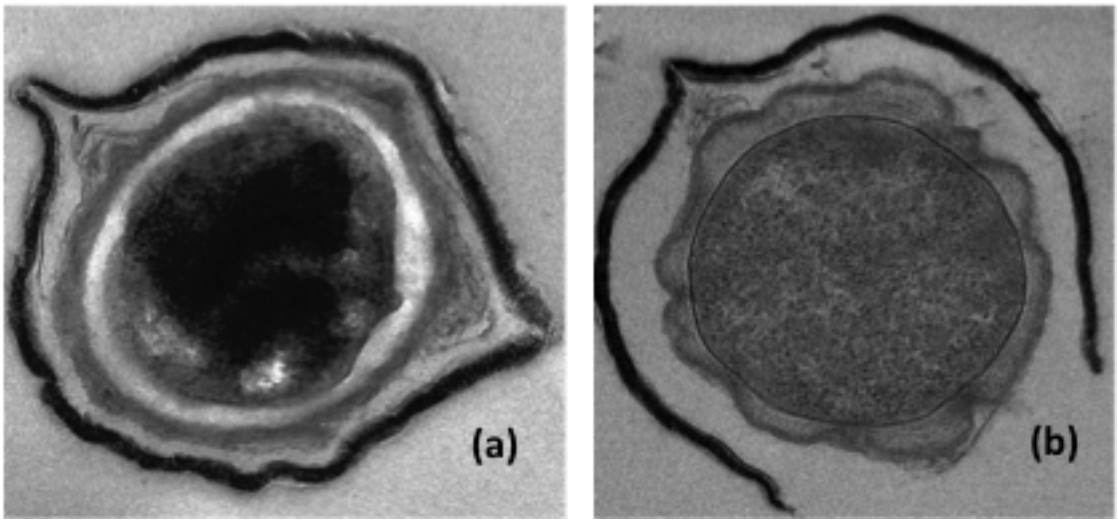


Figure 1.5 Thin section TEMs of (A) dormant, and (B) germinated *B. megaterium* spores. The spore in (B) has undergone both Stage I and II germination events, evidenced by the lack of cortex, partial hydrolysis of the spore coat, and opening of the exosporium.

1.4.1 Stage I – early spore germination events

Germination of spores can be triggered by two distinct pathways- physiological "nutrient" induced or through a collection of "non-nutrient" germination pathways[3,5,87–89]. During nutrient-induced germination, the first event that triggers the process of germination involves presumed binding of small germinant molecules (which, depending on the species, comprises a range of defined amino acids, sugars, nucleosides and or inorganic ions) with cognate receptors that are localised in the inner membrane of the spore[5,52,89–92]. Our understanding of what happens exactly immediately after receptor-ligand interaction is unknown although it is evident that spores somehow become irreversibly "committed" to germinate, as germinants can be removed after a short period of exposure without adversely affecting the progression of germination[93]. In terms of measurable biochemical events, the release of monovalent ions (H^+, Na^+ and K^+) from the spore core represents the

earliest germination event[5,35,88,89,94]. This is followed rapidly by the release of Ca^{2+}-DPA and other small molecules from the spore core, presumably via DPA channels comprising of at least the SpoVA proteins[47,95,96]. At or around the same time as Ca^{2+}-DPA release, the spore core is partially re-hydrated, and the pH of the core increases to levels that are commensurate with metabolism[94]. However, the water content at this stage is still too low to permit metabolism, although a decrease in the spore's heat resistance is observed. Spore core pools of free amino acids, including glutamic acid and arginine, are also released early during germination[88,97]. Thus the outflow of small molecules from the spore core and uptake of water comprise Stage I germination events. However, the means by which signals are transduced from activated receptors to stimulate the opening of DPA and perhaps other channels has not been elucidated[98,99]. Likewise, the role of several non-receptor germination proteins that have been identified by mutational analysis as impacting upon the efficiency of spore germination such as the GerP and GerN proteins have not been established[6,53,100–105].

1.4.2 Stage II – later germination events

The primary event in Stage II of germination involves depolymerisation of the spore cortex by specific peptidoglycan lysins referred to as cortex lytic enzymes (CLEs)[5,56,88,106–110]. Cortical depolymerisation permits further ingress of water to the spore core, which expands concomitantly, and hydrates to levels commensurate with the resumption of metabolic activity in the germinating spore[5,88,89,111,112]. There are three major CLEs that are responsible for cortical depolymerisation in all of the Bacilli, and in some of the Clostridia, namely SleB, CwlJ, and SleL (the latter of which is a cortical fragment lytic enzyme)[113–118]. The SleC and SleM enzymes in other species of *Clostridium* conduct the same process[3,119–123]. An interesting and perplexing question that lays in Stage II of germination concerns the molecular mechanisms that hold CLEs in an inactive state in the dormant spore. The CLEs are typically present as mature enzymes in the dormant spore, and the means by which they are activated during the process of germination remains unanswered[36]. However, since experimentally it has been observed that high concentrations of exogenously applied Ca^{2+}-DPA stimulate CwlJ activity, it may be that the efflux of this solute from the spore core during Stage I germination results in activation of this particular CLE[46,124,125]. Cortical depolymerisation and core rehydration permits the

activation of proteases that degrade spore core SASP, an event that is also classified as a Stage II germination event[87]. Similarly, the integrity of the spore coat is degraded during the later stages of germination. However, the mechanisms that underpin this process of coat degradation, which involves proteolytic activity, remains to be elucidated[6,36,88].

1.5 Non-nutrient mediated spore germination

Spore germination can be triggered by various chemical and physical stimuli. A brief overview of these routes that often by-pass components of the germination apparatus are described below.

1.5.1 Pressure induced spore germination

Previous studies have shown that a brief exposure of *Bacillus* spores to pressures of 100 – 150 MPa initiates efficient germination[102,126]. Experiments conducted on strains that are null for the germinant receptors, have indicated that the germination response mediated at such high pressures is triggered by activation of the germinant receptors[127].. In contrast, spores that lack germinant receptors can only be forced to germinate when exposed to extremely high pressures (500 – 600 MPa), presumably by stimulating the opening of Ca^{2+}-DPA channels located in the inner membrane[127,128]. Thus, application of high hydrostatic pressure based germination methods are of substantial interest to the food processing industry and may provide an alternative to thermal-based pasteurisation methods employed to extend product shelf life and confer safety without affecting the organoleptic properties of food[7,129–132].

1.5.2 Ca^{2+}–DPA and dodecylamine induced germination

As previously mentioned, relatively high concentrations of exogenously applied Ca^{2+}-DPA can stimulate spore germination via the activation of the CLE, CwlJ, the mechanism of which has not been established[47,65,113,124,133]. Studies have also revealed that exogenously applied cationic surfactants, exemplified by dodecylamine, can also stimulate efficient spore germination responses that by-pass the germinant receptors[113,134]. Several studies have indicated that exposure to dodecylamine results in direct opening of inner-membrane

located DPA channels and spores germinated with dodecylamine appear phase–grey under the phase-contrast microscope as a result of incomplete core hydration[113]. Thus at sufficiently high concentration, dodecylamine can stimulate germination and exert a cidal effect on spore viability[113].

1.5.3 PrkC mediated spore germination

A study conducted on muropeptide induced germination indicated that PrkC, a eukaryotic-like Ser/Thr membrane kinase, was capable of binding to peptidoglycan fragments to induce spore germination[135]. PrkC is located in the inner membrane of spores[135]. The molecular mechanism that underpins apparent PrkC- mediated germination has yet to be elucidated. It has been suggested that the presence of muropetides signals to the spore the presence of bacteria undergoing growth in an environment conducive to the vegetative life cycle[135,136].

1.6 Spore germination proteins

Bacterial spores are specialised structures that are composed of a wide array of different kinds of proteins that contribute towards maintaining dormancy[28]. Spores also contain proteins that are involved specifically in germination, including those described below.

1.6.1 Germinant receptors and accessory proteins

Specific germinants presumably binds to their cognate GRs located in the spore inner membrane and this signal is then transduced to downstream components of the germination apparatus. Unfortunately, despite considerable research effort, the biochemical function of the GRs, and the means by which their activation initiates the germination cascade, remains poorly understood[52,137,138]. Bioinformatic analyses have revealed that most members of the *Bacilli* employ several GR orthologues (usually between 2 – 7 per genome), often with overlapping germinant recognition profiles, as ascertained from molecular genetic analyses. *Bacillus cereus* ATCC 14579, for example, has seven GRs[138].

Proteins forming GRs are encoded by genes arranged in tricistronic operons, which encode individual protein subunits (A, B and C) that interact to form a single GR complex[5,139]. Recent studies have revealed a fourth component, the D subunit, which appears to be associated with only some GRs[140,141].

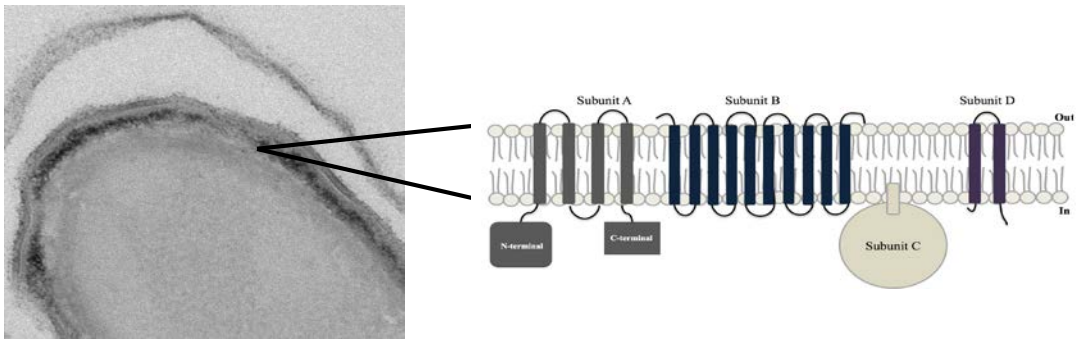


Figure 1.6 Figure showing the inner-membrane location and predicted topology of a typical GR, comprising A, B, C and D–protein subunits[105].

GRs have been characterised in various *Bacillus* and *Clostridium* species on the basis of their expression levels, function and germinant profiles[17,137,138,142]. Examples of different classes of GRs, examined *in vivo* by mutational analyses and germination profiles, are listed in Table 1.1.

Table 1.1 GR orthologues in various *Bacillus*

Organism	GR orthologues	Germinant recognition profiles	Source
B. anthracis sterne	GerH	Inosine	17,143
	GerK	Alanine and Methionine	
	GerL	Alanine, Serine and Valine	
	GerS	Histidine and tryptophan	
	GerX	Unknown	
B. subtilis 168	GerA	L-Alanine/L-Valine	144,145
	GerB	AGFK*	
	GerK	AGFK*	
B. cereus ATCC 14579	GerG	L-Glutamine	137,138
	GerI	Inosine	
	GerR	Ribonucleosides and L-alanine	
B. megaterium QM B1551	GerU	GPLK^{+}	105

*AGFK – a mixture of L-asparagine, glucose, fructose and KBr

$^{+}$GPLK– a mixture of glucose, proline, leucine, and KBr

The A and B subunits of the GRs are integral membrane proteins. Unfortunately atomic resolution structural information has not yet been published or made available for either protein. However, topology mapping experiments suggest that the A–subunit of *B. anthracis* GerH (GerHA) has four transmembrane helices, with a large hydrophilic N–terminal domain and a much smaller hydrophilic C– terminal domain[146]. These hydrophilic

domains are predicted to reside on the outer surface of the inner membrane. The B– subunit of GerH is predicted to comprise 10 – 12 transmembrane helices that are connected by small hydrophilic loops[146]. The C- protein of all the GRs is a peripheral membrane lipoprotein that is anchored to the inner membrane by a diacylglycerol moiety that in turn is attached to a conserved N– terminal cysteine residue[147]. The crystal structure of the C– subunit of the *B. subtilis* GerB receptor has been solved, revealing a multi-domain protein with a unique fold[148]. However the structure did not elucidate the precise function of the protein.

1.6.2 GerN-type antiporters

Transposon mutagenesis of the *gerI* operon, which encodes one of the GRs in *B. cereus* ATCC 10876 revealed that GerI is crucial for inosine-induced germination[137]. A study conducted on *B. cereus* ATCC 14579 has also shown that *gerI* is involved in inosine-induced germination. The GerIA protein was predicted to possess an unusually long, charged, N-terminal domain containing nine tandem copies of a 13-amino-acid glutamine- and serine-rich sequence of unknown function**.** The DNA sequence showed that the corresponding multiple tandem repeat of 39 bases are relatively conserved. The repeats were particularly glutamine rich and are reminiscent of Q-linkers[149]. Q-linkers are sequences which link two different domains of a protein and are commonly found in two-component regulatory and signal transduction systems. The glutamine-rich regions, like proline-rich regions, tend to form extended, conformationally restricted polypeptide chain structures. Thus it was hypothesised that the role of this N-terminal extension could therefore be to aid binding of the GerIA protein to some other component of the germinant apparatus or to some structural element in the spore[137].

Another accessory protein that is involved in inosine-based germination was discovered and designated as the GerN protein[53,54,103]. *B. cereus* possess a second homologue of this protein as well, designated as GerT[103]. A homologue of *gerN* was first reported in *B. megaterium* ATCC 12872 by Tn917 insertional mutagenesis and was designated as *grmA*[150]. The mutant was defective in nutrient-based germination and germinated poorly with any of the nutrient molecules that induced full germination to the wild-type strain. Bioinformatic analyses revealed that the predicted protein is homologous to NapA of *Enterococcus hirae* which is a NaH-antiporter. Thus it was hypothesised that

grmA codes for a protein that is involved in the transport of cations during the spore germination in *B. megaterium*[150].

Insertional inactivation of *gerN* in *B. cereus* 569 resulted in slower spore germination in inosine but almost normal germination in response to L-alanine. This lead to the hypothesis that GerN functions in the response to a particular germinant and that a GR may have a specifically associated antiporter that is required at the initiation of germination and which, in this case is the GerI inosine-induced receptor.

Bioinformatic analysis revealed that the GerT protein of *Bacillus cereus* shares 74% amino acid identity with its homolog GerN. Transposon mutational analysis of the single and double mutants of *gerN* and *gerT* genes revealed that GerN is required for all germination responses that involve the GerI germinant receptor. However, studies revealed that GerT does not have a significant role in germination, although it is required for the residual GerI-mediated inosine germination response of a Δ*gerN* mutant[103]. In contrast, it was observed that GerT has a significant role in outgrowth of the germinated spore as *gerT* mutant spores do not outgrow efficiently under alkaline conditions and outgrow more slowly than the wild type in the presence of high NaCl concentrations[103]. Thus it was hypothesised that the GerN protein is involved in germination involving the GerI receptor, whereas GerT contributes to the success of spore outgrowth from the germinated state during alkaline or Na^+ stress[103].

1.6.3 GerP proteins

The GerP proteins are encoded by a hexacistronic operon located in the genome of *Bacillus* species [70–72]. The six genes encoding the GerP proteins have been designated the names of *gerPA*, *gerPB*, *gerPC*, *gerPD*, *gerPE* and *gerPF*, respectively[71]. Mutational analysis of the *gerP* operon and *gerPA* to *gerPE* genes have shown a defect in the germination response of spores to both L-alanine and inosine[6,70–72]. Mutants blocked at an early stage, before loss of heat resistance or release of dipicolinate, and the efficiency of colony formation on nutrient agar from spores is reduced fivefold[70]. The last gene of the operon *gerPF* has got two other orthologues located elsewhere in the genome for all *Bacillus* speciesp[71]. Hence, the removal of two additional *gerPF*-like orthologues was necessary to achieve the germination defect observed for the other mutants[71]. The *ΔgerP* mutants were also defective in Ca^{2+}-DPA induced germination suggesting the possible

role of these proteins in this germination pathway as well. Upon physical removal of the spore coat in *B. anthracis*, the mutant lacking the full *gerP* operon no longer exhibited a germination defect, suggesting that the GerP proteins play a role in spore coat permeability[71]. Normal rates of germination, as estimated by loss of heat resistance, are also restored to a *ΔgerP* mutant by the introduction of a *cotE* mutation, which renders the spore coats permeable to lysozyme[70]. The *B. subtilis gerP* operon is expressed solely during sporulation, and is σ^K-inducible. Based on these observations, it was hypothesised that the GerP proteins are important as morphogenetic or structural components of the *Bacillus* spore, with a role in the establishment of normal spore coat structure and/or permeability, and that failure to synthesize these proteins during sporulation limits the opportunity for small hydrophilic organic molecules, like alanine or inosine or non-organic germinant molecules, such as Ca^{2+}-DPA to gain access to their normal target in the spore.

1.6.4 BC1245 protein

The possible role of the gene encoded at locus BC1245*bc1245* gene in nutrient-based germination was first reported in a doctoral thesis by Luc Hornstra (2007)[151] in his doctoral thesis where a transposon insertion as transposon mutagenesis in the gene resulted in a defective spore germination phenotype. The gene encoding BC1245 gene appears to be is a monocistronic, encoding gene putative a protein of 143 amino acids and of 115.2 kDa. The transposon mutant strain exhibited a severely delayed inosine-induced germination phenotype, plus including delayed germination in response to L-alanine, indicating but initiated normal germination, suggesting the involvement for this protein in nutrient based germination. A subsequent study Further work conducted on this gene in another lab employing insertional mutagenesis reported stated contrasting results, where the compared to the one stated in Hornstra's thesis employing transposon mutagenesis. Quantitative PCR conducted on the gene showed that it is expressed during the late sporulation phase. Insertional mutant showed no obvious defect in germination in response to either alanine or inosine, and also to exogenous Ca^{2+} DPA, suggesting the protein is not involved in germination. Extracts from the exosporium were subjected to western blotting and were found to be positive for the presence of BC1245 protein suggesting that the BC1245 protein is an exosporium protein[152].

1.6.5 BC1117 protein

The expression levels of sporulation and germination genes are fine-tuned by secondary regulator proteins that are under the control of sigma factors and enable feed forward loops in the regulatory system. An example of such a protein is SpoVT, which is expressed in the forespore compartment under the control of σ^G. SpoVT enhances the expression of some of the σ^G -dependent genes and represses others[153]. Previous work conducted in *B. subtilis* involving deletion of the *spoVT* gene resulted in spores having a defective spore coat, faster nutrient-induced germination, increased sensitivity to UV radiation and limited ability for spore outgrowth[153,154]. The SpoVT protein is highly conserved in the members of *Bacillus* species[154,155]. For DNA binding it forms a tetramer and possibly binds a yet unidentified substrate through means of the GAF domain in the C-terminal part of the protein[156]. Deletion of *spoVT* from *B. subtilis* cells results in the formation of spores that have a defective spore coat, a faster nutrient-induced germination response, an increased sensitivity to UV radiation and limited ability for spore outgrowth[154]. A recent study in *B. subtilis* has shown that levels of active SpoVT play a crucial role in determining the numbers of important germination proteins, such as nutrient germination receptors (GRs) and small acid-soluble proteins (SASPs), which affects the germination, resistance, and outgrowth properties of the spore[154]. Transcriptomic analysis of a *SpoVT* mutant strain revealed a significant number of *B. cereus*-group specific genes of unknown function to be down regulated in the mutant background. These include the gene at locus BC1117, and hence it may be that the product of this gene has a role in the process of sporulation in *B. cereus* ATCC 14579.

1.7 Objectives

Despite intense research conducted in this field spanning several decades, many fundamental aspects associated with the germination of *Bacillus* spores remain unknown. The structural integrity of the highly resistant and dormant spore is conferred, at least in part, by numerous coat proteins. However, the role of the coat or defined coat proteins in germination has not been fully elucidated. Hence, the major objective of this thesis was to confer further insight to the role of the GerP proteins, which have been postulated as being components of the coat. Further examination of the protein encoded at locus BC1245, postulated as a component of the exosporium that may influence spore germination, was a further objective. Given the genetic amenability of the ATCC 14579 strain of *Bacillus cereus*, a further non-coat associated objective was to examine the role of the GerN and GerT anti-porters in germination. Finally, the potential role of the BC1117 protein – identified as potentially being significant in spore structure from SpoVT-related work – was also investigated.

Chapter 2. Materials and methods

2.1 Bacterial growth conditions

Strains of *B. cereus* ATCC14579 employed in this work are isogenic with the *B. cereus* ATCC 14579 wild-type strain. *B. cereus* strains were routinely cultured on LB agar or broth at 30°C supplemented with antibiotics where appropriate (See Table 2.1). Competent *Escherichia coli* DH5α (NEB UK) cells, used for the preparation and propagation of plasmids and heterologous expression of proteins, were cultured in LB media at 37°C supplemented with antibiotics. *Lactococcus lactis* strains used in recombinant protein expression were grown in M17 medium (Oxoid, England) supplemented with 1% (w/v) glucose (M17G medium) and chloramphenicol (10 μg/ml) at 30°C and agitated, typically at 90 rpm. All bacterial strains were stored as 15% (v/v) glycerol stocks at -80°C.

Table 2.1 Antibiotic concentrations for *Bacillus cereus* strain

Antibiotics	Stock concentration[a] (mg/ml)	Working concentration (μg/ml)
Erythromycin[b]	10	1
Kanamycin	100	5
Lincomycin[c]	25	5
Tetracycline[c]	100	10

[a]Antibiotic stock solutions were prepared in sterile UHP water (unless otherwise stated), filter sterilised and stored at -20°C. The antibiotics were diluted appropriately in the culture media to obtain the working concentration. [b]Prepared in 100% (v/v) ethanol, [c]Prepared in 50% (v/v) ethanol: water.

2.2 Plasmids

Plasmids used in this thesis are listed in Table 2.2.

Table 2.2 Plasmids used in this thesis

Plasmids	Resistance marker/selection [a]	Source
pBAD.cLIC-GFP	LIC vector for protein expression in *E. coli*; GFP	Prof. B. Poolman
pBKJ223	*E. coli/Bacillus* shuttle plasmid; Tet^r	Dr. Toril Lindbäck
pDONORtet	*E. coli* cloning vector; Tet^r	Prof. P. Hanna
pET-SUMO	Expression vector in *E. coli;* Km^r	Dr. David M Bailey
pHT315	Low copy number plasmid maintained stably in *B. cereus* ; MLS^r	207
pHT315t	Low copy number plasmid maintained stably in *B. cereus* ; MLS^r	Modified from Arantes *et al*[207]
pMAD	SCO plasmid with ori^{ts}	Dr. Toril Lindbäck
pNFD13	*E. coli/Bacillus* shuttle plasmid; *lacZ* ori^{ts} Km^r	Prof. P. Hanna
pRE.cLIC-GFP	LIC vector for *E. coli*; Amp^r GFP	Prof. B. Poolman

[a] Amp^r, ampicillin (β-lactam) resistance; Km^r, kanamycin resistance; Tet^r, tetracycline resistance; MLS^r, macrolide-lincosamide-streptogramin B resistance; GFP, green fluorescence protein; *lacZ*, gene coding for β-galactosidase enzyme; ori^{ts}, temperature-sensitive origin of replication.

2.3 Bacterial strains used in this study

B. cereus strain 14579 was gratefully received from Dr. Toril Lindbäck (Norwegian School of Veterinary Science , Norway). Chemically competent *E. coli* NovaBlue (Novagen) or DH5α (New England Biolabs, UK) were used for transformation and propagation. *E. coli* Top10 (life technologies, Paisley, UK) were used for expression of recombinant protein. *B. cereus* strains used for localisation studies are summarised in Table 2.3.

Table 2.3 *Bacillus cereus* strains and the strains used for localisation study

Strain	Relevant phenotype or genotype
B. cereus 14579	Wild-type strain
Localisation study strains (*B. cereus* 14579 transformants)	
AG101	pHT315-Promoter-PA-GFP-PB-PC-PD-PE-PF :Ery^r
AG102	pHT315-Promoter-PA-PB-GFP-PC-PD-PE-PF :Ery^r
AG103	pHT315-Promoter-PA- PB-PC-GFP-PD-PE-PF :Ery^r
AG104	pHT315-Promoter-PA-PB-PC-PD-GFP -PE-PF :Ery^r
AG105	pHT315-Promoter-PA- PB-PC-PD-PE-GFP-PF :Ery^r
AG106	pHT315-Promoter-PA- PB-PC-PD-PE–PF-GFP :Ery^r
AG107	pHT315-Promoter-BC1245-GFP:Ery^r
AG108	pHT315-Promoter-BC1117-GFP:Ery^r

The double cross over mutant strains used in this study is summarized in Table 2.4.

Table 2.4 *Bacillus cereus* double cross over mutants

Strain	Relevant phenotype or genotype
AG109	Δ*gerPA*
AG110	Δ*gerPB*
AG111	Δ*gerPC*
AG112	Δ*gerPD*
AG113	Δ*gerPE*
AG114	Δ*gerPF*
AG115	Δ*gerP*
AG116	Δ*bc1245*
AG117	Δ*bc1117*
AG118	Δ*gerI*
AG119	Δ*gerN*
AG120	Δ*gerN:*Δ*gerT*

The double-cross over mutant strains were complemented with the respective gene along with the promoter in an episomal plasmid (pHT315) bearing erythromycin and lincomycin resistance markers. The complemented strains are summarized in Table 2.5.

Table 2.5 *Bacillus cereus* double cross-over mutants' complementation strains

Strain	Relevant phenotype or genotype
AG121	Δ*gerPA*::pHT315-Promoter-*gerPA*:Ery^r
AG122	Δ*gerPB*::pHT315-Promoter-*gerPB*:Ery^r
AG123	Δ*gerPC*::pHT315-Promoter-*gerPC*:Ery^r
AG124	Δ*gerPD*::pHT315-Promoter-*gerPD*:Ery^r
AG125	Δ*gerPE*::pHT315-Promoter-*gerPE*:Ery^r
AG126	Δ*gerPF*::pHT315-Promoter-*gerPF*:Ery^r
AG127	Δ*gerP*::pHT315-Promoter-*gerP*:Ery^r
AG128	Δ*bc1245::* pHT315-Promoter-*bc1245*:Ery^r
AG129	Δ*gerI*:: pHT315-Promoter-*gerI*:Ery^r
AG130	Δ*gerI*:: pHT315-Promoter-Truncated *gerIA*:Ery^r
AG131	Δ*gerN*-Δ*gerT*:: pHT315-Promoter- *gerN*-Promoter-*gerT*:Ery^r

The dependency based localisation study was conducted on individual *ΔgerP* strains expressing various GerP-GFP fusion proteins in an episomal plasmid. The various strains generated are shown in tables 2.6 to 2.11 respectively.

Table 2.6 Strains used for dependency study in the *ΔgerPA* mutant

Strain	Relevant phenotype or genotype
AG132	Δ*gerPA*::pHT315-Promoter-*gerPA-gfp*:Ery^r
AG133	Δ*gerPA*::pHT315-Promoter-*gerPB-gfp*:Ery^r
AG134	Δ*gerPA*::pHT315-Promoter-*gerPC-gfp*:Ery^r
AG135	Δ*gerPA*::pHT315-Promoter-*gerPD-gfp*:Ery^r
AG136	Δ*gerPA*::pHT315-Promoter-*gerPE-gfp*:Ery^r
AG137	Δ*gerPA*::pHT315-Promoter-*gerPF-gfp*:Ery^r

Table 2.7 Strains used for dependency study in the *ΔgerPB* mutant

Strain	Relevant phenotype or genotype
AG138	Δ*gerPB*::pHT315-Promoter-*gerPA-gfp*:Ery^r
AG139	Δ*gerPB*::pHT315-Promoter-*gerPB-gfp*:Ery^r
AG140	Δ*gerPB*::pHT315-Promoter-*gerPC-gfp*:Ery^r
AG141	Δ*gerPB*::pHT315-Promoter-*gerPD-gfp*:Ery^r
AG142	Δ*gerPB*::pHT315-Promoter-*gerPE-gfp*:Ery^r
AG143	Δ*gerPB*::pHT315-Promoter-*gerPF-gfp*:Ery^r

Table 2.8 Strains used for dependency study in the *ΔgerPC* mutant

Strain	Relevant phenotype or genotype
AG144	Δ*gerPC*::pHT315-Promoter-*gerPA-gfp*:Ery^r
AG145	Δ*gerPC*::pHT315-Promoter-*gerPB-gfp*:Ery^r
AG146	Δ*gerPC*::pHT315-Promoter-*gerPC-gfp*:Ery^r
AG147	Δ*gerPC*::pHT315-Promoter-*gerPD-gfp*:Ery^r
AG148	Δ*gerPC*::pHT315-Promoter-*gerPE-gfp*:Ery^r
AG149	Δ*gerPC*::pHT315-Promoter-*gerPF-gfp*:Ery^r

Table 2.9 Strains used for dependency study in the *ΔgerPD* mutant

Strain	Relevant phenotype or genotype
AG150	Δ*gerPD*::pHT315-Promoter-*gerPA-gfp*:Ery^r
AG151	Δ*gerPD*::pHT315-Promoter-*gerPB-gfp*:Ery^r
AG152	Δ*gerPD*::pHT315-Promoter-*gerPC-gfp*:Ery^r
AG153	Δ*gerPD*::pHT315-Promoter-*gerPD-gfp*:Ery^r
AG154	Δ*gerPD*::pHT315-Promoter-*gerPE-gfp*:Ery^r
AG155	Δ*gerPD*::pHT315-Promoter-*gerPF-gfp*:Ery^r

Table 2.10 Strains used for dependency study in the *ΔgerPE* mutant

Strain	Relevant phenotype or genotype
AG156	Δ*gerPE*::pHT315-Promoter-*gerPA-gfp*:Ery^r
AG157	Δ*gerPE*::pHT315-Promoter-*gerPB-gfp*:Ery^r
AG158	Δ*gerPE*::pHT315-Promoter-*gerPC-gfp*:Ery^r
AG159	Δ*gerPE*::pHT315-Promoter-*gerPD-gfp*:Ery^r
AG160	Δ*gerPE*::pHT315-Promoter-*gerPE-gfp*:Ery^r
AG161	Δ*gerPE*::pHT315-Promoter-*gerPF-gfp*:Ery^r

Table 2.11 Strains used for dependency study in the *ΔgerPF* mutant

Strain	Relevant phenotype or genotype
AG162	Δ*gerPF*::pHT315-Promoter-*gerPA-gfp*:Ery^r
AG163	Δ*gerPF*::pHT315-Promoter-*gerPB-gfp*:Ery^r
AG164	Δ*gerPF*::pHT315-Promoter-*gerPC-gfp*:Ery^r
AG165	Δ*gerPF*::pHT315-Promoter-*gerPD-gfp*:Ery^r
AG166	Δ*gerPF*::pHT315-Promoter-*gerPE-gfp*:Ery^r
AG167	Δ*gerPF*::pHT315-Promoter-*gerPF-gfp*:Ery^r

Δ*gerP* mutant was transformed with GerP-GFP fusion proteins to check if the fusion of GFP to the C-terminal have any functional effect on the functionality of the GerP proteins. Six strains were generated as shown in the table 2.12.

Table 2.12 Strains used for functional analysis of the GerP-GFP fusion proteins

Strain	Relevant phenotype or genotype
AG168	Δ*gerP* ::pHT315-Promoter-PA-GFP-PB-PC-PD-PE-PF :Ery^r
AG169	Δ*gerP* ::pHT315-Promoter-PA-PB-GFP-PC-PD-PE-PF :Ery^r
AG170	Δ*gerP* ::pHT315-Promoter-PA- PB-PC-GFP-PD-PE-PF :Ery^r
AG171	Δ*gerP* ::pHT315-Promoter-PA-PB-PC-PD-GFP -PE-PF :Ery^r
AG172	Δ*gerP* ::pHT315-Promoter-PA- PB-PC-PD-PE-GFP-PF :Ery^r
AG173	Δ*gerP* ::pHT315-Promoter-PA- PB-PC-PD-PE–PF-GFP :Ery^r

2.4 Molecular biology techniques

2.4.1 Isolation of *B. cereus* genomic DNA

QIAmp DNA mini kit (Qiagen, UK) was used for the isolation of genomic DNA from *B. cereus* broth culture, following the manufacturer's protocol supplied with the kit.

2.4.2 Polymerase Chain Reaction (PCR)

Primers used in the PCR reaction were designed using software FastPCR 6.3 (PrimerDigital Ltd., Helsinki, Finland) and Serial Cloner 2.6 (Franck Perez, SerialBasics) and then ordered through Sigma-Aldrich Custom DNA Oligo Service. The dehydrated primer pellets were re-suspended in TE-1 buffer (Appendix II) to a concentration of 100 µM and stored at –20°C as stocks. 10 µM of the stocks were used for PCR reaction.

Q5 High-Fidelity Hot Start Polymerase (supplied by New England Biolabs, UK) was used for the amplification of DNA with a view of subsequent cloning of inserts into a designated plasmid. Amplified PCR fragments were purified using QIAquick DNA purification kit (Qiagen) and stored at –20°C.

Colony PCRs conducted *E. coli* colonies while cloning were screened for the correct construction by colony PCR using 2x master mix aliquots, comprising: 0.5 µl NEB buffer N2, 0.5 µl NEB Klenow, 2.5µl 30% trehalose plus 2µl UHP water (produced by Dr. David Bailey, Christie Group).

2.4.3 Isolation of total RNA

WT *B. cereus* cells were cultured at 30°C in 200 ml CCY media[157] adjusted to an OD_{600} = 10 (~$5x10^8$cells/ml) and collected on an hourly basis following entry into the stationary phase (designated as t0 in sporulation), for the isolation of total RNA from sporulating cells. The t_0 was determined from the plateau of OD_{600} values, recorded every hour during the growth phase of the culture. The collected samples were centrifuged immediately (16,000xg for 1 min), washed with 1 ml of RNA Protect Bacteria Reagent (Qiagen Ltd.) and the cell pellets were stored at –80°C until analysis. RNA was subsequently extracted and purified from thawed cell pellets, within 4 weeks of sample collection, using RNeasy mini-kit (Qiagen Ltd.) and then stored at –80°C.

2.4.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) technique

Approximately 1 μg of RNA from each sample was converted to cDNA using a QuantiTect Reverse Transcription kit (Qiagen Ltd., Manchester, UK) and Random Hexamers (Life Technologies Ltd., Paisley, UK) as primers for cDNA synthesis. The cDNA samples for each time point served as templates for PCR reactions employing NovaTaq DNA polymerase (Merck Chemicals Ltd., Nottingham, UK) and gene specific primers designed to amplify ≤300 bp fragments of the genes of interest.

2.4.5 Agarose gel electrophoresis

For estimating the size of DNA fragments, 1% (w/v) agarose gels containing 0.5μg/ml ethidium bromide were prepared in 1x TAE buffer. Generally, 7μl of samples (5μl of DNA sample + 2μl of 5x DNA loading buffer) were loaded into the wells next to a DNA size ladder (Hyperladder I, Bioline) and electrophoresis was performed at 80 V for 1 h in 1X TAE buffer. The separated DNA fragments were then visualised using an UV transilluminator (UVItec, Cambridge, UK).

2.4.6 Digestion of plasmids using restriction endonucleases

Plasmids were typically digested in 50μl reactions, supplemented with 1x cutsmart buffer and 20U of high-fidelity restriction enzyme (commercially available, New England Biolabs, UK), following the manufacturer's instructions. All the digested plasmids were gel purified and de-phosphorylated using calf intestinal alkaline phosphatase (CIP, Novagen) before gel purification to prevent recircularisation. Products of restriction digests were purified and stored at -20°C until further use.

2.4.7 Klenow Assembly Method (KAM) reaction for joining multiple DNA fragments

KAM is an unpublished seamless cloning method developed within the Christie laboratory, for joining multiple, overlapping DNA fragments. Inserts and vectors are designed to produce overlapping regions of between 15-30bp. Appropriate DNA fragments were amplified using PCR and gel extracted. KAM master mix aliquots comprised: 0.5μl NEB buffer N2, 0.5μl NEB Klenow, 2.5μl 30% trehalose and 2μl UHP water. The master mix

was dried down in PCR tubes, with the lid removed, at 37ºC for 24 hours. Dried master mix tubes were stored in the dark at room temperature. Fragments of DNA to be assembled were made up to 5-10µl with UHP water and this was used to resuspend the dried KAM master mix, subsequently incubated for 20-40 minutes at 37ºC. Reactions were stopped by cooling on ice or storing at -20ºC, prior to transformation.

2.4.8 Transformation of chemically competent *E. coli* cells

Chemically competent *E. coli* cells were routinely stored at –80°C. In order to transform *E. coli*, the cells were thawed on ice followed by addition of the ~5µl of ligation mixture. The reaction was then incubated on ice for 30 min, heat shocked at 42°C for 30 s and cooled on ice for 5 min. Pre-warmed SOB media (250µl) was added to the cells and allowed a recovery time of 1 h at 37°C, 225 rpm. The transformants were then selected at 37°C overnight on LB agar plates supplemented with appropriate antibiotics.

2.4.9 Isolation of plasmid DNA

QIAprep Spin miniprep kits (Qiagen) were used for the isolation of plasmid DNA as stated in the manufacturer's protocol and the plasmids were stored in elution buffer at –20°C.

2.4.10 Sequencing of DNA

The Department of Biochemistry Sequencing Facility (University of Cambridge) and the Genewiz, UK performed sequencing of the cloned plasmids. Thereafter, analysis was done using CLC Sequence Viewer 6.0 software (CLCbio, Denmark).

2.4.11 Nucleic acid quantification

Nucleic acids (DNA and RNA) quantification is a crucial step in molecular biology. Concentration of nucleic acids and its purity in a sample was measured using a NanoDrop (ND-1000 Spectrophotometer).

2.4.12 Transformation of *Bacillus cereus* using electroporation

Transformation of *B. cereus* electro competent cells was performed by electroporation using a method adapted from Bone and Ellas, 1989. 5ml overnight culture of *B. cereus* was used to inoculate 100ml of Tryptone Soya Broth (with antibiotics where appropriate)

and incubated at 37°C with constant agitation in 225rpm until the OD of 0.5 was reached. Thereafter the cells were twice washed with 50ml Solution A and finally with 50ml electroporation buffer, before the cell pellet was resuspended in 1ml electroporation buffer. 50µl aliquots of the electrocompetent cells were rapidly frozen in liquid nitrogen and stored at -80°C immediately.

Electroporation was conducted using Gene Pulsar and Pulse Controller and Biorad electroporation cuvettes by mixing 500ng of plasmid with 50µl of thawed electrocompetent cells, applying electric field at 200Ω, 2kV and 25 µF. Recovery was done for 3 hours before plating on Tryptone Soya Agar supplemented with appropriate antibiotics.

2.4.13 Construction of reporter gene fusion strains

(a) Construction of GFP-GerP fusion strains and BC1245-GFP fusion strain for localisation studies

A series of plasmids were designed to create GerP-GFP fusion strains to permit GerP localisation studies. The KAM technique was used to create plasmids from the backbone of linearised pHT315 (an episomal plasmid that is maintained at low copy number in *Bacillus*), in which six variant *gerP* operons were cloned. The inserts contained the native *gerP* regulatory sequences, plus variant *gerP* ORFs in which individual *gerP* genes were fused in-frame with *gfp*. DNA coding for the various GerP proteins was PCR-amplified using purified *B. cereus* ATCC 14579 genomic DNA as template. Table 2.13 details the primers employed, and the various PCR amplicons used to create the six plasmids are detailed in Table 2.14.

Table 2.13 Primers used to create GerP-GFP fusion strains for localisation studies

Primer	Sequence (5' → 3')	Overlap
GFP-For	agtaaaggagaagaacttttcactggagttgtcccaattcttgttgaatt	--
GFP-Rev	ttatttgtatagttcatccatgccatgtgtaatcccagcagctgttacaa	--
pHT315_Prom_GerPA_For	CAGCTATGACCATGATTACGCCAAGCTTTCATTTGGCATAAAATGTAG	pHT315
GFP_GerPA_Rev	ccagtgaaaagttcttctcctttactAGTTGAGCCAATTATCGCTTGATC	GFP
GFP_GerPB_For	gcatggatgaactatacaaataaAGGAGGCAATTGCATTGAATTTTTA	GFP
pHT315_GerPF_Rev	GTTGTAAAACGACGGCCAGTGAATTCTTACGCTGTTCCAATCTGATCTTG	pHT315
GFP_GerPB_Rev	gtgaaaagttcttctcctttactAGAAGAACTAGTTGATGGTTTGATAG	GFP
GFP_GerPC_For	ggcatggatgaactatacaaataaGACATCCTTGTCATAAATATATAGCA	GFP
GFP_GerPC_Rev	ccagtgaaaagttcttctcctttactCTCCTTTCGGAAATTTCCCGGTAT	GFP
GFP_GerPD_For	ggcatggatgaactatacaaataaGTATGTATGAACCTTAACGTTGTAAA	GFP
GFP_GerPD_Rev	ctccagtgaaaagttcttctcctttactACCTGGAGTAGGAGGGACATCT	GFP
GFP_GerPE_For	ggcatggatgaactatacaaataaGGAGACTGAAAAATTATGTTGCATCA	GFP
GFP_GerPE_Rev	ccagtgaaaagttcttctcctttactTTGTGCGGAAGGTTCATCTGTAAT	GFP
GFP_GerPF_For	catggcatggatgaactatacaaataaTTCATATAGTAATTACAAAGTTT	GFP
GFP_GerPF_Rev	gtgaaaagttcttctcctttactCGCTGTTCCAATCTGATCTTGATCTG	GFP
pHT315_GFP_Rev	GTTGTAAAACGACGGCCAGTGAATTCttatttgtatagttcatccatgcc	GFP

Table 2.14 DNA fragments used for KAM

	DNA fragment	Forward primer	Reverse primer	Base pairs
PA-GFP	-Prom-PA-GFP-	pHT315_Prom_GerPA_For	GFP-Rev	1333
	-PB-PC-PD-PE-PF-	GFP_GerPB_For	pHT315_GerPF_Rev	1751
PB-GFP	-Prom-PA-PB-GFP-	pHT315_Prom_GerPA_For	GFP-Rev	1554
	-PC-PD-PE-PF-	GFP_GerPC_For	pHT315_GerPF_Rev	1477
PC-GFP	-Prom-PA-PB-PC-GFP-	pHT315_Prom_GerPA_For	GFP-Rev	2236
	-PD-PE-PF-	GFP_GerPD_For	pHT315_GePF_Rev	856
PD-GFP	-Prom-PA-PB-PC-PD-GFP-	pHT315_Prom_GerPA_For	GFP-Rev	2437
	-PE-PF-	GFP_GerPE_For	pHT315_GerPF_Rev	634
PE-GFP	-Prom-PA-PB-PC-PD-PE-GFP-	pHT315_Prom_GerPA_For	GFP-Rev	2839
	-PF-	GFP_GerPF_For	pHT315_GerPF_Rev	222
PF-GFP	-Prom-PA-PB-PC-PD-PE-PF-GFP-	pHT315_Prom_GerPA_For	pHT315_GFP_Rev	3098

Plasmids were introduced to *B. cereus* vegetative cells via electroporation and transformant colonies selected on LB medium supplemented with erythromycin.

Similarly, the plasmid for BC1245 localisation study in *B. cereus* spores was created by c-terminal fusion of GFP to the BC1245 in plasmid pHT315. Primers employed for construction of this plasmid are shown in Table 2.5.

Table 2.15 Oligonucleotides used to create BC1245-GFP fusion strains

Primer	**Sequence (5' → 3')**	**Overlap**
pHT315_Prom_SP_For	CTATGACCATGATTACGCCAAGCTTGTATCTACTTTCGTTACTTCATGTC	pHT315
GFP_SP_Rev	cagtgaaaagttcttctcctttactTTTAGCTGGACCAGGATCCCTTGAG	GFP
SP_GFP_For	CTCAAGGGATCCTGGTCCAGCTAAAagtaaaggagaagaacttttcactg	SP
pHT315_GFP_Rev	GTTGTAAAACGACGGCCAGTGAATTCttatttgtatagttcatccatgcc	pHT315

(b) Construction of SNAP-BC1245 fusion strain for localisation studies

SNAP-tag is a 20 kDa mutant of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase that reacts specifically and rapidly with benzylguanine (BG) derivatives leading to irreversible covalent labelling of the SNAP-tag with a synthetic probe.

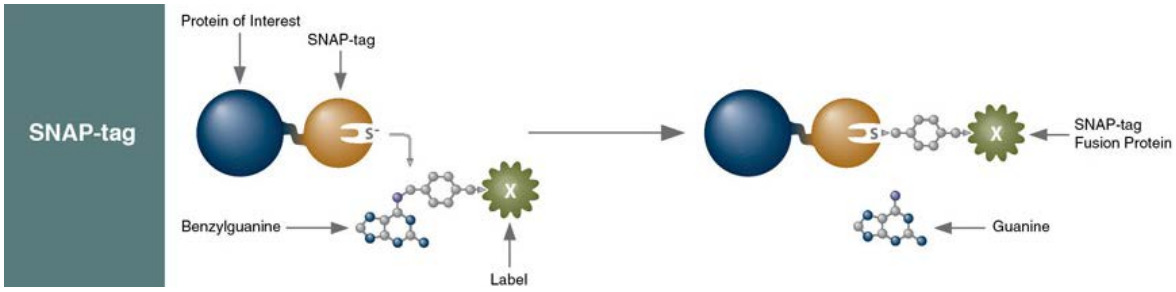


Figure 2.1 SNAP-tag Mechanism: SNAP-tag or CLIP-tag fused to the protein of interest labels itself with "X" releasing guanine or cytosine. Here X is SiR-SNAP (Source: New England Biolabs).

The plasmid for the BC1245 localisation study in *B. cereus* spores was created by C-terminal fusion of SNAP-tag to BC1245 in pHT315. Primers employed for construction of the plasmid are shown in Table 2.16.

Table 2.16 Oligonucleotides used to create BC1245-SNAP fusion strains

Primer	Sequence (5' → 3')	Overlap
pHT315_Prom_SP_For	CAGCTATGACCATGATTACGCCAAGCTTGT ATCTACTTTCGTTACTTCATGTCGTAC	pHT315
Linker_SP_Rev	GCTGCTGCTTCTCCGCCTCCTTTAGCTGGAC CAGGATCCCTTGAG	GFP
Linker_SP_For	CTCAAGGGATCCTGGTCCAGCTAAAGGAGG CGGAGAAGCAGCAGC	SP
pHT315_SNAP_Rev	CGTTGTAAAACGACGGCCAGTGAATTCTTA ATTAACCTCGAGCCCGGGGG	pHT315

2.4.14 Construction of markerless null mutant strains

A series of *gerP, bc1245, gerI, gerN, gerT* and *bc1117* null mutant strains in the *B. cereus* 14579 background were constructed using a variant of the markerless allelic exchange method developed by Janes and Stibitz[158,159]. To delete *gerPA* (BC1145), for example, PCR was used to prepare an amplicon comprising approximately 500 bp of sequence upstream and inclusive of the *gerPA* start codon. A second amplicon comprising 500 bp of downstream sequence starting from, and including the *gerPA* stop codon was also prepared by PCR. Primers were designed to include approximately 15 bp of overlapping sequence between the 3' end of the upstream amplicon and the 5' end of the downstream amplicon. Additionally, the 5' end of the upstream amplicon contained 15 bp of overlapping sequence with the 5' end of linearised pMAD vector, as did the 3' end of the downstream amplicon with the 3' end of pMAD. The fragments were assembled using a klenow-based ligation independent cloning technique, and then used to transform *E. coli* to carbenicillin resistance. Transformant *E. coli* were screened by colony PCR to identify clones with the correct construct, and plasmids subsequently purified and validated by DNA sequencing.

Similar procedures were used to prepare plasmids designed to individually delete the remaining *gerP* genes (*gerPB* [BC1144], *gerPC* [BC1143], *gerPD* [BC1142], *gerPE* [BC1141], and *gerPF* [BC1140]), and also to delete the entire operon.

pMAD derived plasmids were subsequently introduced to *B. cereus* by electroporation, with transformants being selected at 30ºC on LB plates supplemented with 1ug/ml erythromycin, 5ug/ml lincomycin and 90 µg/ml X-gal. Electroporation was conducted using a Gene Pulsar instrument (Biorad), operating at 200 Ω, 2 kV and 25 µF, and cuvettes that contained 500 ng of plasmid DNA plus 50 µl of thawed electrocompetent cells. Clones that had integrated plasmids by homologous recombination were selected by incubating blue colonies at 37ºC on fresh LB plates, re-streaking after 24 and 48 hours. Plasmid pBKJ223, which encodes the I-SceI restriction enzyme, was introduced by electroporation to single crossover cells, which were recovered on LB medium containing 10 µg/ml tetracycline. Transformant colonies were subsequently passaged every 24 hours in fresh LB medium containing tetracycline at 37ºC, with aliquots being plated and screened on LB agar containing tetracycline and X-gal for white colonies that were sensitive to erythromycin and lincomycin. Candidate colonies that had undergone a second recombination event to excise the integrated plasmid, leaving behind the truncated gene, were validated by PCR and sequencing, and then passaged on LB medium minus antibiotics to promote excision of the pBKJ223 plasmid.

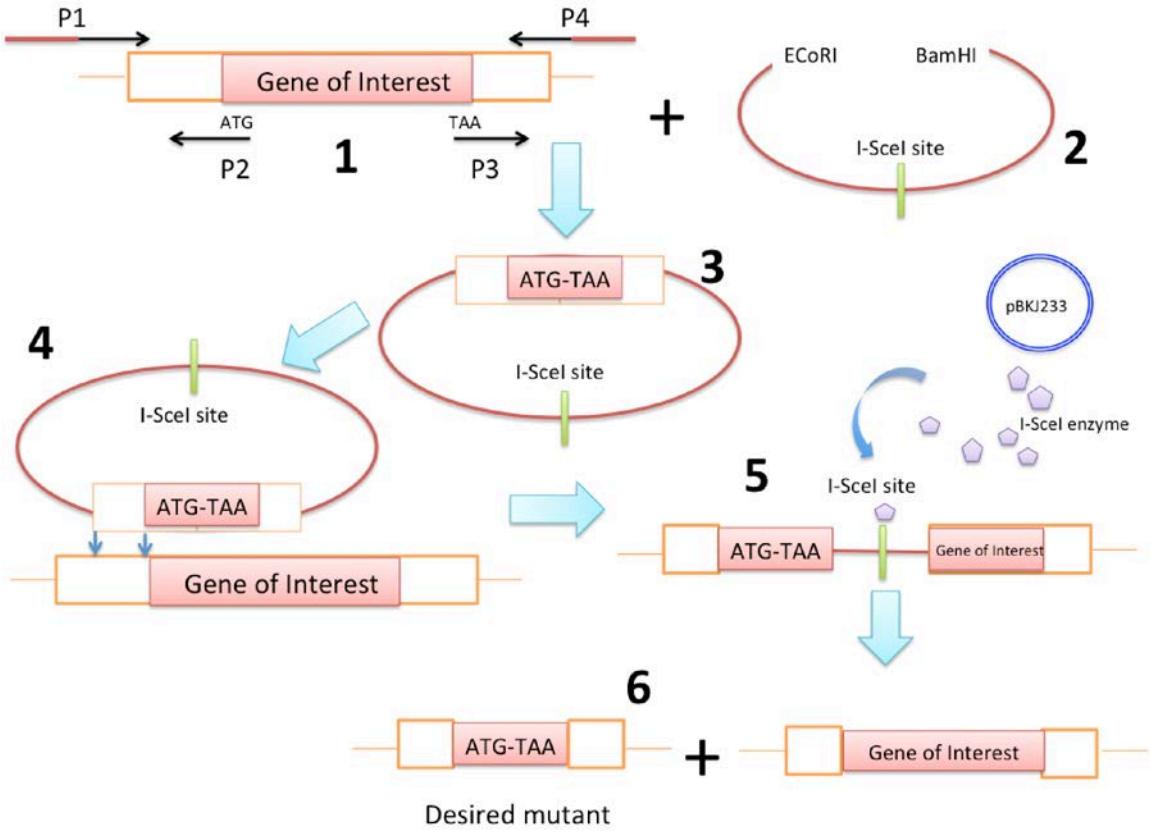


Figure 2.2 Schematic of strategy employed to create markerless null mutants in *B. cereus* 14579 strain. (1) P1and P2; P3 and P4 are the two sets of primers employed to amplify 500 bp of DNA upstream and downstream of the gene of interest with only ATG and TAA in between. P1 and P4 have around 25 bp of homology to the pMAD plasmid. (2) pMAD plasmid is double digested with *EcoRI* and *BamHI*. It has temperature sensitive origin of replication site and I-SceI cleavage site. (3) Using KAM technique, all the three fragments (upstream, downstream fragment and the digested pMAD plasmid) are assembled to give an intact pMAD with our desired inserts. (4) The plasmid is transformed into *B. cereus* ATCC 14579 strain and homologous recombination takes place when the incubation temperature is raised. (5) The second transformed plasmid pBKJ223 (encoding I-SceI enzyme) creates a double-stranded break that is a potent substrate for host systems that can repair the break by recombination. (6) This leads to a population in which approximately 50% will have undergone the desired gene replacement.

2.5 *Bacillus cereus* spore preparation and purification

Spores of *B. cereus* (wild type and mutants) were prepared by inoculating 200 ml of CCY media with 1 ml of a mid-log phase culture of the appropriate strain in a 2 L baffled flask. The flasks were then incubated at 30°C (unless otherwise indicated) for 96 h with agitation (225 rpm). Thereafter the spore suspension was mixed with 0.1% Tween 20 solution and left at 4°C overnight. On the next day, spores were harvested and purified from the culture by centrifugation (4,300 g for 10 min at 4°C), followed by repeated rounds of washing with 0.1% Tween 20 in ice-cold water, to remove the upper layer of vegetative cell debris. The wash procedure was repeated until the spore pellet was adjudged by phase-contrast microscopy to comprise >99% phase-bright spores and then stored on ice.

2.6 Spore germination assay

The spore germination assay involved measuring the loss in optical density of a 300 µl spore suspension in a 96-well plate, using a Perkin-Elmer EnVision-Xcite multilabel plate reader fitted with a 600 nm photometric filter. *B. cereus* spores spore suspensions (OD_{600} ~ 50) were at first heat activated by incubating at 70°C for 15 minutes and then cooled on ice for at least 2 min prior to conducting germination assays in the liquid medium. Typically germination assays were conducted over 90 min at 37°C, with 10 s of orbital shaking to prevent spores settling prior to reading absorbance every minute. All experiments were performed in triplicate, with at least two independent spore preparations for each strain. Germination buffer media served as negative control whereas 10mM L-alanine and 10mM inosine in 10mM Tris-HCl served as positive control for all germination assays. All the spores used in this work were free (>99%) from growing or sporulating cells, germinated spores and cell debris as determined by phase-contrast microscopy.

2.7 Light Microscopy

2.7.1 Phase contrast and fluorescent microscopy

Sporulating *B. cereus* samples and mature spores were imaged with an Olympus BX53 fluorescent microscope fitted with a QImaging Retiga 2000R CCD camera and filter sets for blue and green light excitation. Images were processed with QCapture Pro 7 Software, ImageJ and Adobe Photoshop Elements 11. The lipophilic fluorescent stain FM4-64 (life

technologies, Paisley, UK) was used to visualise cell membranes during sporulation.

2.7.2 Immunocytochemical assay for GFP localization

Mature spores (OD_{600}=10) were incubated with gentle agitation in phosphate buffered saline (PBS, pH 7.4) containing 2 % (w/v) bovine serum albumin (BSA), for 1 h (PBS-BSA). Spores were harvested by centrifugation (4,000 g for 5 min), resuspended in PBS-BSA and incubated with anti-GFP antibody (ab290, Abcam, 1:500 dilution) for 40 min, followed by 3 washes with PBS-BSA. The spores were pelleted, resuspended in PBS-BSA and incubated with Dylight 594-conjugated anti-Rabbit IgG antibody (ab96885, Abcam, 1:500 dilution) for 40 min, washed as described earlier, and analysed by fluorescence microscopy.

2.8 Scanning and transmission electron microscopy

Scanning and transmission electron microscope images of *B. cereus* spores used in this thesis were processed at the Multi-Imaging Centre, University of Cambridge by Dr. Jeremy Skepper.

2.9 Bioinformatics analysis

B. cereus 14579 genomic analyses were performed using Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg-bin/show_organism?org=bce). BLAST programs provided by the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) were used for protein and DNA homology searches. Other programs and online tools employed for this study are listed in the table 2.17.

Table 2.17 List of bio–informatics tools used in this thesis

Software/Tool	Application	Source
Biomath Calculators	Calculating molar ratio of insert to vector for cloning	Promega Ltd.
BVTech Plasmid 5.1	Drawing plasmid maps	Biovisuals Tech.. Inc, USA
Box-shade server	Shading similar residues upon protein sequence alignment.	EMBnet, Swiss Institute of Bioinformatics (SIB)
Clustal Ω	Aligning multiple protein sequences.	EMBL-EBI, UK
ExPASy Translate	Translation of DNA sequence to protein sequence	SIB
Fast PCR 6.4	Designing PCR primers	Primer Digital, Helsinki, Finland
NEB Cutter V2.0	Mapping restriction sites and ORFs on DNA fragments	NEB
Protein weight calculator	Calculating expected protein molecular weight.	EnCor Biotechnology Inc, Florida, USA
Tm Calculator	Calculating primer melting temperature and and finding appropriate annealing temperature for PCRs	NEB

2.10 Study of protein-protein interactions

Bacterial Adenylate Cyclase Two-Hybrid System (BACTH, Euromedex) was used to perform the protein-protein interaction experiment for various GerP proteins as per the manufacturer's protocol.

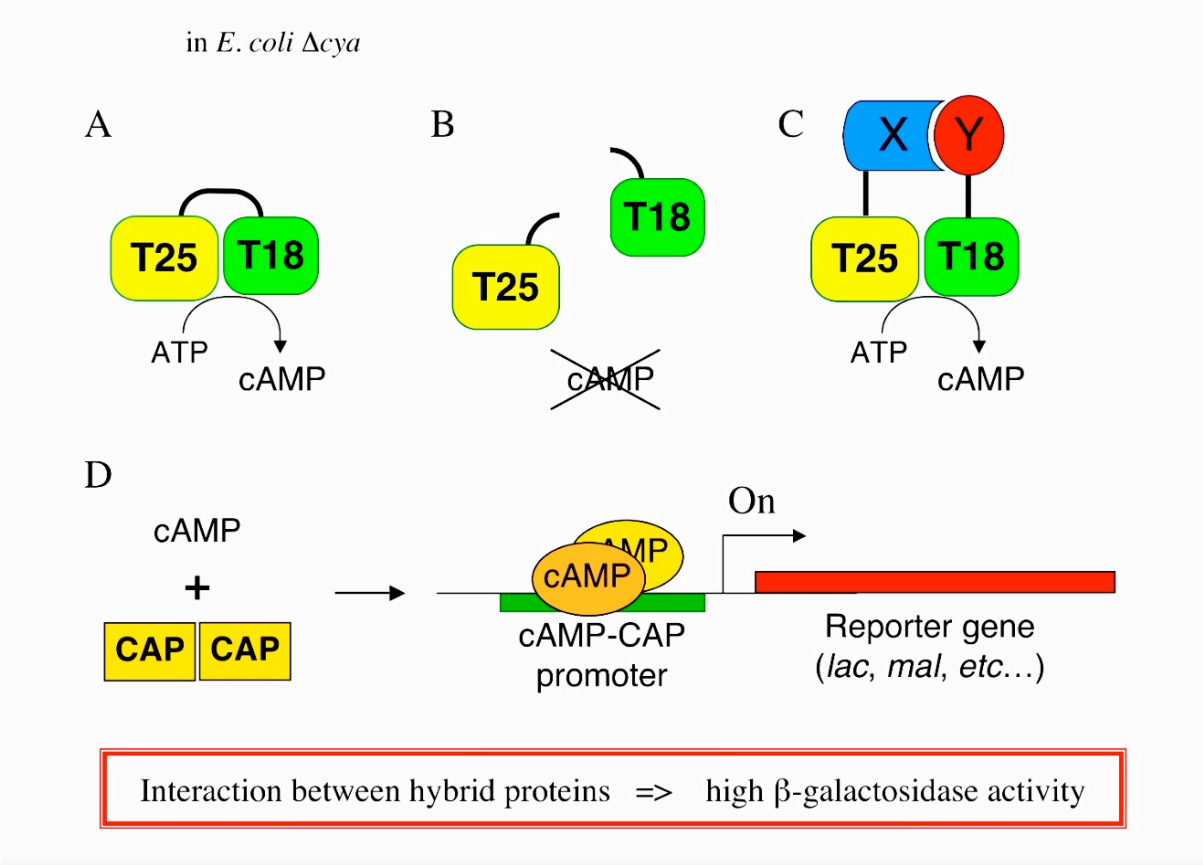


Figure 2.3 Principle of Bacterial Adenylate Cyclase Two-Hybrid System (BACTH, Euromedex). It exploits the fact that the catalytic domain of adenylate cyclase (CyaA) from *Bordetella pertussis* consists of two complementary fragments, T25 and T18 (A), that are not active when physically separated (B). When these two fragments are fused to interacting polypeptides, X and Y, heterodimerisation of these hybrid proteins results in functional complementation between T25 and T18 fragments and, therefore, cAMP synthesis (C). Cyclic AMP produced by the reconstituted chimeric enzyme binds to the catabolite activator protein, CAP. The cAMP/CAP complex is a pleiotropic regulator of gene transcription in *E. coli*. It turns on the expression of several resident genes, including genes of the *lac* and *mal* operons involved in lactose and maltose catabolism (D). Therefore, bacteria become able to utilize lactose or maltose as the unique carbon source and can be easily distinguished on indicator or selective media.

2.11 Ellipsoid Localisation Microscopy (ELM)

The ellipsoid localisation microscopy is a set of MATLAB procedure that fits models corresponding to spherical and ellipsoidal shells to fluorescence micrographs. Briefly, an image analysis pipeline is used to identify candidate spore images and segment them from the rest of the image. An iterative method is then used to fit simulated fluorescence data for shells of given parameters to the image data. After convergence, these parameters are used to simulate image data collected on a microscope with a much smaller point-spread function, giving a non-diffraction-limited simulated image. These parameters can also be directly used to quantify properties of the fluorescent shell, such as its major and minor axes lengths[160].

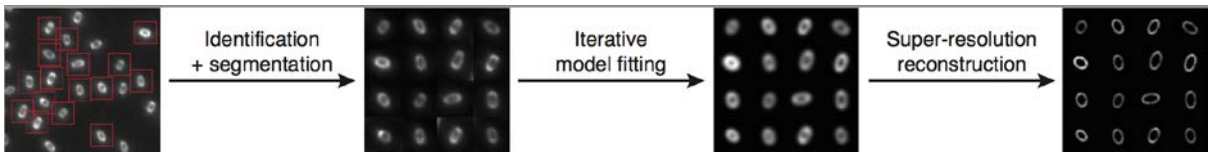


Figure 2.4 Principle of Ellipsoid Localisation Microscopy. The ELM software precisely quantifies the structure of fluorescence shells (such as bacterial spore coats) using a process of segmentation, fitting, and reconstruction. The images of individual shells are first identified and then a parametric model for a suitable shell structure (for spheres, ellipsoids, or cylinders) can be fitted to each segmented structure.

2.12 Colony enumeration of *Bacillus cereus* strains to check for viability

In order to ascertain the viability of various *B. cereus* *ΔgerP* mutants and the *gerP* null mutant, serial dilutions of spore suspensions (OD_{600}=5) were plated as triplicates on agar plates (Figure 2.5). Serial dilutions were prepared using sterile LB broth and 10 µL aliquots dispensed on solid LB medium with 90 µL LB broth dots in triplicates. The number of colony forming units (CFUs) obtained for each dilution was assessed after overnight incubation at 30°C.

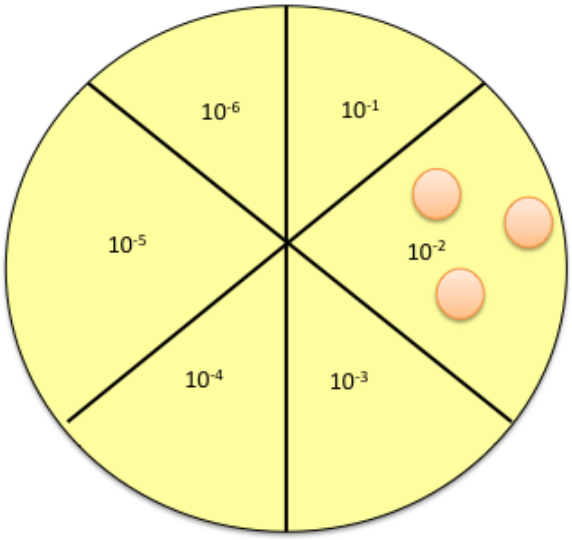


Figure 2.5 Schematic illustration of plating technique used to assess viability of various strains of *B. cereus* spores. Aliquots (10 μ L) of diluted spores were dispensed in triplicate on LB agar plates and CFUs counted after overnight incubation at 30°C.

2.13 Extraction of *B. cereus* spore exosporium by sonication

Removal of outer-most layer of *B. cereus* spores (the exosporium) was performed using a variation of the method described previously [161]. *B. cereus* spores were resuspended in 5 mL UHP water at an OD_{600}=10. Spore suspensions were sonicated in a Soniprep 150 sonicator (Sanyo) for seven 1 min bursts separated by 1-minute incubations on ice. The effect of sonication on release of exosporium material was assessed after each round of sonication by phase contrast microscopy.

2.14 Heterologous protein expression and analysis

Ligation independent cloning (LIC) was employed for the cloning and expression of various GerP proteins in this study[162].

2.14.1 Cloning of the genes for expression in *E. coli*

The six *gerP* ORFs were amplified by PCR from wild type genomic DNA using the forward (5'- ATGGGTGGTGGATTTGCT-gene specific 25 bp-3') and reverse (5'-TTGGAAGTATAAATTTTC- gene specific 25 bp -3') primer pair, which include adapter sequences to enable ligation independent cloning. Plasmids pBadcLIC-GFP were digested with *SwaI* at room temperature for 18 hours. 200 ng of digested vector and equimolar amounts of purified PCR products (gene-specific inserts) were prepared for LIC by incubation with 1X NEB buffer-2, 1X BSA, 0.5 U T4 DNA polymerase and 2.5 mM dCTP (vector) or dGTP (PCR) for 30 min at 25°C. 1 μL of the respective LIC- ready vectors were combined subsequently with 3 μL of LIC ready insert and incubated for 5 minutes at room temperature. The ligation mix was cooled on ice for 10 min and then used to transform *E. coli* TOP 10 cells to carbenicillin resistance.

2.14.2 Expression of recombinant protein in *E. coli*

Initial characterisation of the various GerP protein expression was performed based on protocols described by Geertsma & Poolman in 2010[162]. 50 mL of LB medium supplemented with 50 μg/mL carbenicillin was inoculated with 500 μL of an overnight *E. coli* culture and incubated at 37°C, 225 rpm. Once the cell density had reached an OD_{600} of 0.5-0.6, protein expression was induced by adding arabinose to a final concentration of 0.001 % (w/v) or 0.05 % (w/v), and allowed to continue for 4 hours at 30°C and overnight at 18°C. GFP fluorescence was assessed by fluorescence microscopy every hour of protein expression. After four hours or overnight of expression, samples corresponding to an OD_{600}=5 were collected by centrifugation, and the cell pellet stored at -20°C until further use. Analysis of protein expression levels was achieved by resuspending cell pellets in 400 μL disruption buffer (50 mM phosphate buffer, pH 7.5, 10% [w/v] glycerol, 1 mM $MgSO_4$, 1 mM PMSF and 0.1 mg/mL DNase), before loading into lysing matrix B tubes (MP Biomedicals, UK) and subjecting the cells to two rounds of bead beating (20 s, force 6) with 5 min cooling on ice in between eaxh disruption cycle. The supernatant was removed and aliquots subject to analysis by SDS-PAGE.

Once optimisation of the protein expression conditions were attained, larger scale protein expression experiments intended for protein purification were established by 600 mL of LB broth (supplemented with 50 μg/mL carbenicillin) with 6 ml of an overnight culture . Cultures were incubated at 18°C, 225 rpm, and protein expression induced with arabinose (final concentration 0.001% [w/v]) when the cell density had attained an OD_{600} = 0.5-0.6. The cells were permitted to grow for 12 h prior to harvesting by centrifugation at 9000xg for 15 min, 4°C. Cell pellets were washed once with sterile water (4000xg, 10 min, 4°C) and the pellet stored at -20°C until further use.

2.14.3 Purification of recombinant proteins

Recombinant spore proteins were purified by His-tag affinity chromatography, as described previously[163]. Briefly, cell pellets from 1.2 L expression cultures were resuspended in 8 mL binding buffer (20mM NaPO4, pH 7.4, 500mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 20 mM imidazole) and passed 3 times through a One-shot Cell disrupter operated at 20 kPsi. Cell lysates were centrifuged at 15000xrpm for 20 min at 4°C, before passing the supernatant through a Millex-HV syringe-driven filter unit (0.45 μm polyvinylidene difluoride [PVDF] membrane) and loading onto a 1 mL His-trap excel column fitted to an ÄKTA Pure instrument (GE Healthcare, Uppsala, Sweden). Eluted protein fractions were combined, concentrated with an Amicon ultrafiltration device (appropriate molecular weight cut-off; Millipore, USA) and stored at 4°C until further use.

2.14.4 SDS-PAGE

Protein samples were mixed with NuPAGE LDS Sample Buffer (4X) and NuPAGE sample reducing agent following the manufacturer's instructions (life technologies, Paisley, UK). Samples were incubated at 75°C for 10 min and loaded onto 4-12% Bis-Tris SDS-PAGE precast gels (life technologies, Paisley, UK) alongside appropriate protein markers. Electrophoresis was performed at 200 V for 50 min (MOPS buffer, life technologies, Paisley, UK) or 35 min (MES buffer, life technologies, Paisley, UK). The gel was subsequently stained with Colloidal Blue stain (life technologies, Paisley, UK) following the manufacturer's instructions.

Chapter 3. Analysis of *Bacillus cereus* 14579 GerP proteins

3.1 Introduction

Previous work conducted with *B. subtilis*, *B. cereus* and *B. anthracis* spores revealed that expression from the *gerP* locus is initiated during sporulation in the mother cell compartment around the time of spore coat synthesis and assembly. Also the expression of the GerP proteins is sigma K-dependent and negatively regulated by GerE, a major regulator of coat protein gene expression, suggesting that the GerP proteins could possibly be spore coat proteins. Interruption to any of the genes within the *gerP* locus results in a deleterious germination phenotype for nutrient based germination[70–72]. However, permeabilisation of *gerP* mutant spores by coat extraction procedures removes the block in the early stages of germination. These data suggest a role for the GerP proteins in spore coat permeability. Interestingly, the *gerP* mutants also exhibited a severe germination defect in Ca^{2+}-DPA dependent germination, although the physiological basis for this defect has not been identified. Since the GerP proteins have only been partially characterised since their discovery as possible spore components with a role in spore germination[70–72], the aims of this chapter were to:

(1) Establish the role of the GerP proteins in nutrient and non-nutrient based germination in *Bacillus cereus* 14579 spores.
(2) Use fluorescence microscopy to determine the precise location of the various GerP proteins in *Bacillus cereus* spores.
(3) Use fluorescence microscopy to gain insight to any dependencies that exist between GerP proteins for localisation in the spore.

3.2 Results

3.2.1 Bioinformatics analysis

Since the GerP proteins have not been studied in detail in the *B. cereus* strain employed in our laboratory (14579), the first task of this project was to conduct a bioinformatic analysis of the genome to identify the *gerP* operon, and to identify any additional *gerP*-like orphan genes (Table 3.1 and 3.2). Like *B. anthracis*, the *B. cereus* 14579 genome encodes a hexacistronic GerP locus, plus two apparently monocistronic GerPF orthologues[71].

Table 3.1 *B. cereus* 14579 putative GerP proteins

GerP proteins	NCBI Accession number	% Identity with GerP from:		
		B. subtilis	*B. megaterium*	*B. anthracis*
GerPA	BC1145	53	52	98
GerPB	BC1144	45	51	98
GerPC	BC1143	35	28	95
GerPD	BC1142	55	62	96
GerPE	BC1141	30	32	90
GerPF	BC1140	42	42	100

Table 3.2 List of orthologues of GerPF in *B. cereus* 14579

Names assigned	NCBI Accession number	Sequence identity (%)		
		GerPF1	**GerPF2**	**GerPF3**
Bc_GerPF1	BC1140	100	94	49
Bc_GerPF2	BC2276	94	100	52
Bc_GerPF3	BC4794	49	52	100

3.2.2 Construction of GerP null mutants

Defective spore germination phenotypes associated with the *gerP* operon were first observed in *Bacillus cereus* strain 569 bearing a transposon (Tn917) insertion between *gerPB* and *gerPC* (Anne ref). Our initial attempts at creating markerless deletions of the entire *gerP* operon and individual genes within the operon in this strain proved to be unsuccessful. Accordingly, the decision was made to switch the work to *B. cereus* 14579, which is more amenable to genetic manipulation. A markerless allelic exchange system was subsequently used to create a series of strains in the 14579 background where individual genes – start and stop codons aside - from *gerPA* through to *gerPF* were deleted. The same markerless approach was used to delete the entire *gerP* operon, with the exception of the start codon from *gerPA* and the stop codon of *gerPF* (Figure 3.1). In common with *B. subtilis* and *B. anthracis*, *B. cereus* 14579 has two additional *gerPF* homologues encoded on the chromosome (BC2276 and BC4794), although these were not deleted in the course of this work. Figure 3.1 shows the results of diagnostic PCR reactions used to validate the construction of the various null mutant strains.

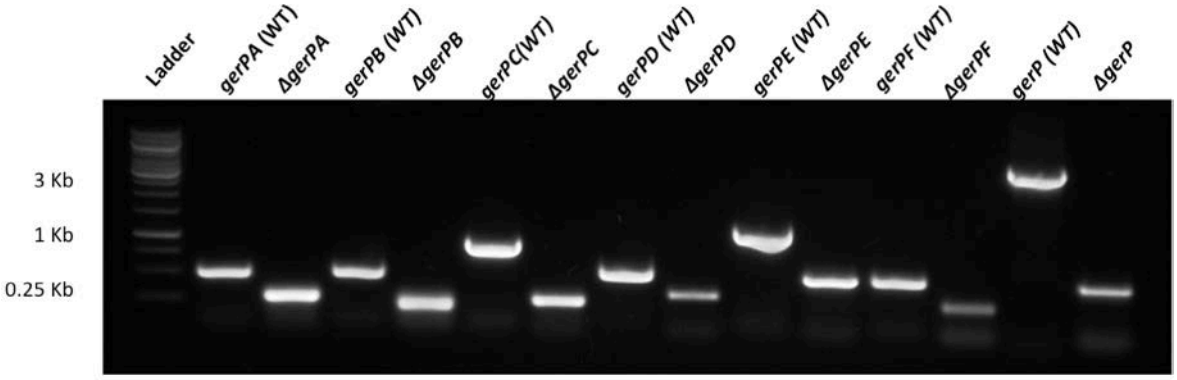


Figure 3.1 Agarose gel showing the products of diagnostic PCR reactions used to characterise the various *B. cereus* GerP null mutant strains. Primers employed in these reactions flanked the gene(s) of interest, hence in all cases PCR products associated with mutant strains are smaller than the equivalent reaction conducted with wild type DNA.

3.2.3 Investigating the role of GerP proteins in germinant receptor mediated spore germination

In order to assess whether deletion of individual and collective *gerP* genes resulted in defective germination phenotypes in the 14579 background, spores of the various null strains were subjected to a heat shock of 70ºC for 15 min, cooled on ice, and their germinative responses to L-alanine and inosine assessed by monitoring the reduction in absorbance (at 600 nm) associated with the transition of phase bright dormant spores to phase dark germinated spores. Spores of all strains, apart from the *gerPF* null strain, were defective for germination in buffer supplemented with either alanine or inosine with respect to wild type spores (Figures 3.2 and 3.3). The slight reduction in absorbance recorded after 5 min germination of mutant spores appeared to be associated with the germination of a small number of spores within the various populations, at least as adjudged by the light microscope. In contrast, the majority of wild type spores were phase grey or dark after 5 min [data not shown]. Even after 60 min, the total loss of absorbance was less than that associated with wild type spores, with microscopy revealing again the presence of many refractile dormant spores in the mutant populations. In all cases, germination defects were restored by complementation with plasmid-borne copies of the deleted gene(s) (Figure 3.4). In contrast to the above observations, the *gerPF* spore germinative responses to alanine and inosine were indistinguishable to wild type spores, indicating perhaps that the homologous genes encoded elsewhere on the chromosome can compensate for the loss of *gerPF*.

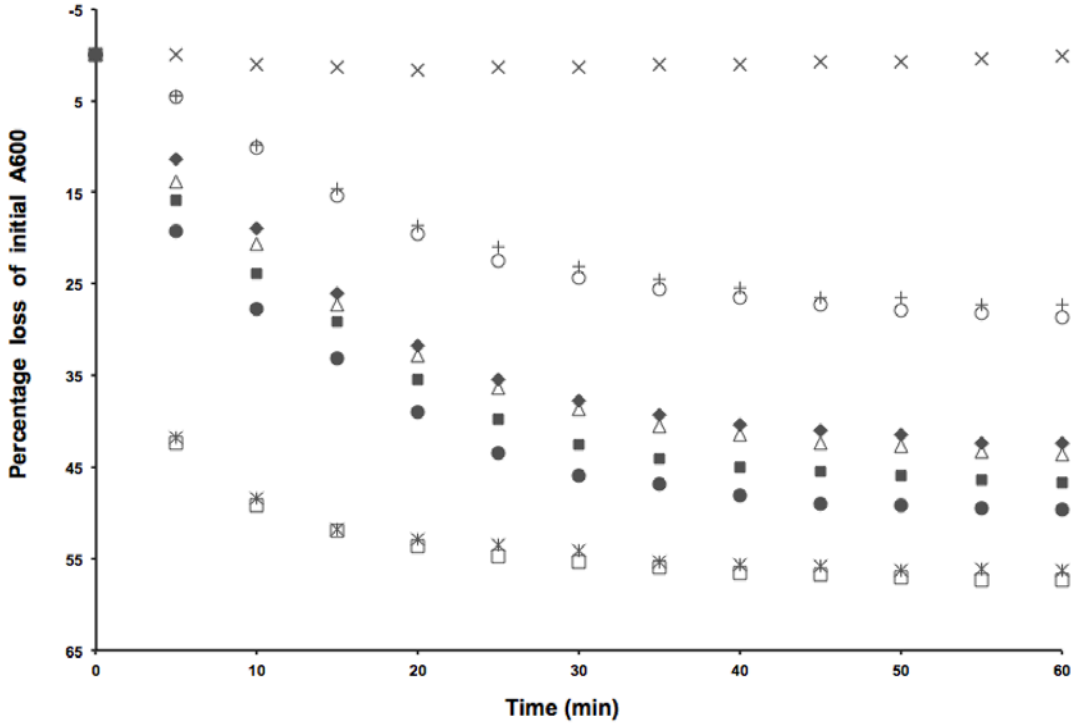


Figure 3.2 Germination of *B. cereus* GerP null mutant spores in 10 mM Tris-HCl, pH 7.4, supplemented with 100 mM L-alanine. Key: (x) buffer, (+) *gerP*, (o) *gerPA*, (Δ) *gerPB*, (■) *gerPC*, (◆) *gerPD*, (+) *gerPE*, (*) *gerPF*, (❑) wild type

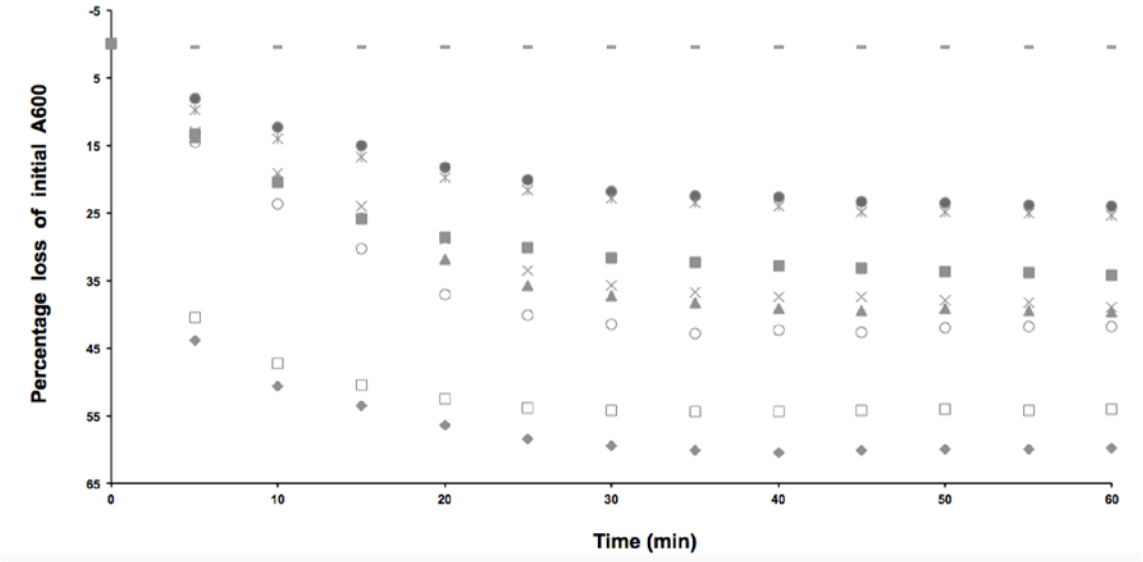


Figure 3.3 Germination of *B. cereus* GerP null mutant spores in 10 mM Tris-HCl, pH 7.4, supplemented with 10 mM inosine. Key: (-) buffer, (❑) *gerPA*, (Δ) *gerPB*, (•) *gerPC*, (o) *gerPD*, (x) *gerPE*, (❑) *gerPF*, (*) *gerP*, (◆) wild type.

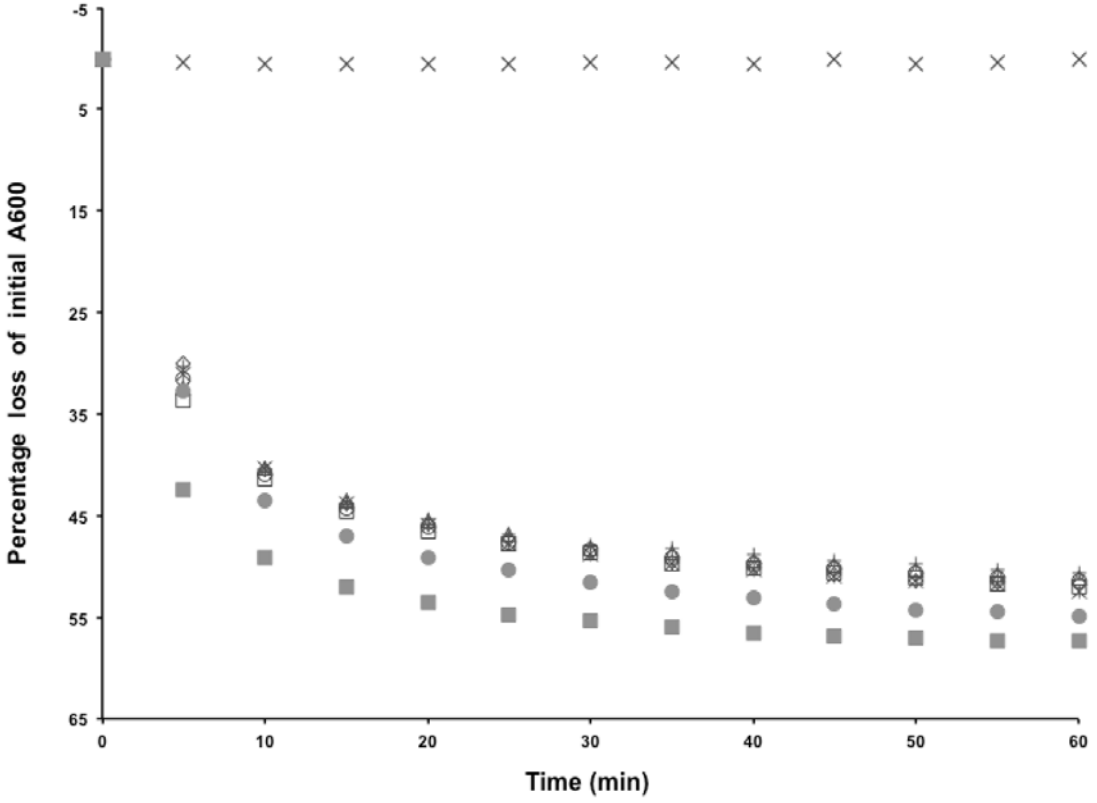


Figure 3.4 Germination of complemented *B. cereus* GerP null mutant spores in 10 mM Tris-HCl, pH 7.4, supplemented with 100mM L-alanine. Null mutant strains were complemented with a plasmid borne copy of the deleted gene(s). Key: (x) buffer, (❑) *gerPA*, (Δ) *gerPB*, (◆) *gerPC*, (o) *gerPD*, (x) *gerPE*, (❑) *gerPF*, (*) *gerP*, (■) wild type.

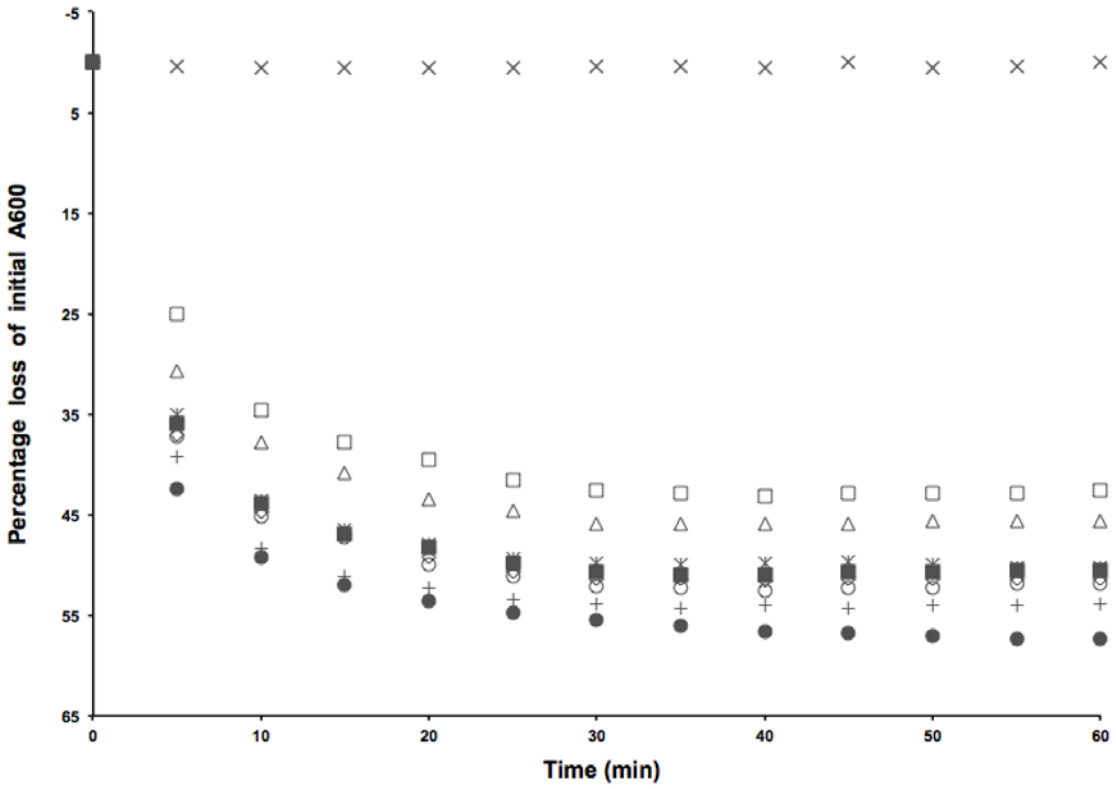


Figure 3.5 Germination of complemented *B. cereus* GerP null mutant spores in 10 mM Tris-HCl, pH 7.4, supplemented with 10 mM inosine. Null mutant strains were complemented with a plasmid borne copy of the deleted gene(s). Key: (x) buffer, (+) *gerP*, (❑) *gerPA*, (Δ) *gerPB*, (■) *gerPC*, (*) *gerPD*, (⬥) *gerPE*, (o) *gerPF*, (•) wild type.

Table 3.3 Percentage germination of GerP null mutant (and complemented) spores

(a) Germination in 100mM L-alanine

	5 minutes		60 minutes	
Strains		+*gerP*$_n$		+*gerP*$_n$
WT	70%	-	95%	-
ΔgerP	17%	50%	45%	87%
ΔgerPA	18%	57%	70%	87%
ΔgerPB	23%	53%	73%	85%
ΔgerPC	18%	51%	48%	87%
ΔgerPD	27%	53%	78%	85%
ΔgerPE	32%	50%	83%	85%
ΔgerPF	55%	68%	91%	93%

(b) Germination in 10mM inosine

	5 minutes		60 minutes	
Strains		+*gerP*$_n$		+*gerP*$_n$
WT	73%	-	98%	-
ΔgerP	10%	65%	42%	90%
ΔgerPA	10%	52%	57%	72%
ΔgerPB	10%	52%	67%	77%
ΔgerPC	13%	60%	40%	85%
ΔgerPD	23%	58%	70%	83%
ΔgerPE	10%	62%	65%	85%
ΔgerPF	62%	67%	87%	90%

+*gerP*$_n$ represents respective complements in pHT315 plasmid

3.2.4 Investigating the role of GerP proteins in non-nutrient mediated germination

Spores can be stimulated to germinate by various non-physiological routes, including by exposure to high concentrations of exogenous Ca^{2+}-DPA and to the cationic detergent dodecylamine. In order to assess the impact of deletion of the various GerP proteins in *B. cereus* 14579 on germination via these routes, spores of the various strains were prepared, incubated in Ca^{2+}-DPA or dodecylamine and germination monitored by measuring absorbance loss. Figure 3.6 shows the germinative response to Ca^{2+}-DPA, and Figure 3.7 to dodecylamine. In both cases (and for all mutant strains) a germination defect is evident as reduced absorbance loss compared to wild type spores. Light microscopy analysis of samples tat the end of each experiment revealed the spore populations to comprise a mixture of phase dark (germinated), phase grey (partially germinated) and phase bright (dormant) spores. In contrast, wild type spores were predominantly phase dark. Hence, for both non-nutrient germinative pathways examined, some spores can complete germination whereas others remain dormant. Presumably this variance is related to the degree of permeability of the spore coat in the absence of defined GerP proteins.

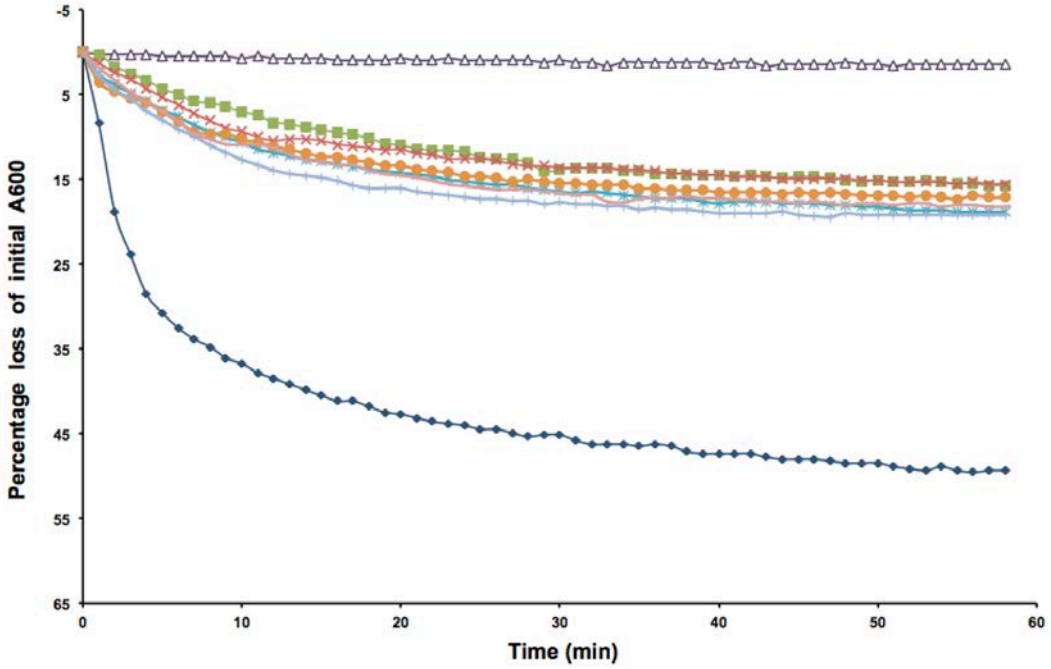


Figure 3.6 Germination of *B. cereus* GerP null mutant spores in 60 mM Ca^{2+}-DPA. Key: (Δ) buffer, (+) *gerP*, (×) *gerPA*, (□) *gerPB*, (-) *gerPC*, (o) *gerPD*, (*) *gerPE*, (◆) wild type.

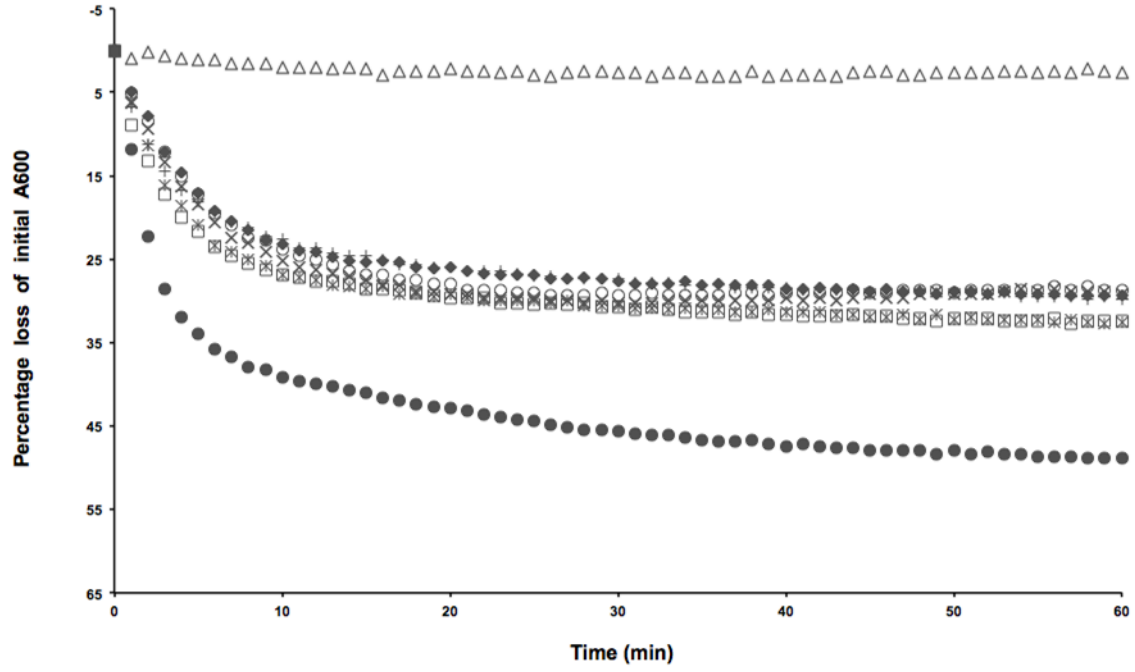


Figure 3.7 Germination of *B. cereus* GerP null mutant spores in 1mM dodecylamine. (×) buffer, (+) *gerP*, (□) *gerPA*, (Δ) *gerPB*, (■) *gerPC*, (*) *gerPD*, (◆) *gerPE*, (o) *gerPF*, (•) wild type.

3.2.5 Viability of *B. cereus* GerP null mutant spores

In order to assess the viability of the various GerP mutant spores serial dilution of spores were plated on LB plates and incubated overnight. Table 3.4 illustrates the dilutions plated and the CFUs obtained for each of the mutants.

Table 3.4 Colony forming efficiency of *gerP(A-E)* mutant strains for various dilutions

Sample	Counts from 10^{-6} dilution (cfu ml^{-1})	Counts from 10^{-5} dilution (cfu ml^{-1})
WT	35×10^{7}	5×10^{8}
ΔgerPA	7×10^{7}	2×10^{8}
ΔgerPB	6×10^{7}	3×10^{8}
ΔgerPC	5×10^{7}	2×10^{8}
ΔgerPD	6×10^{7}	2×10^{8}
ΔgerPE	7×10^{7}	1×10^{8}
ΔgerPF	2×10^{8}	2×10^{8}
ΔgerP	6×10^{7}	2×10^{8}

Evidently, the apparent viability is reduced for spores of all GerP strains on rich medium, presumably as a result of impaired permeability and access of germinants to the inner membrane located germinant receptors.

3.2.6 Fluorophore-based expression and localisation studies of GerP proteins

In order to examine the expression and localisation of the GerP proteins in *B. cereus* 14579 a series of strains designed to express C-terminal GFP fusions of the various GerP proteins were created i.e. a series of plasmids designed to create synthetic *gerP* operons in which individual *gerP* genes were fused to *gfp* were constructed (Figure 3.8). An episomal plasmid was used for the experiments, as opposed to using an integrative plasmid, to minimise the possibility of the *gfp* gene exerting downstream polar effects. Purified plasmids were used to transform *B. cereus* to erythromycin resistance, and spores prepared in CCY medium via the nutrient exhaustion method. The resultant strains were assessed by fluorescence microscopy throughout sporulation in an attempt to gain insight to the expression and localisation of the GerP-GFP fusion proteins. It was essential to maintain erythromycin throughout growth and sporulation to prevent loss of the episomal plasmid, as adjudged by the lack of fluorescence.

All six GerP-GFP proteins were monitored at regular intervals throughout sporulation. The fusion proteins localised to the developing forespore during or shortly after the development of refractile spores, starting at the mother cell proximal pole and the distal pole of the forespore and proceeding bi-directionally around the forespore (Figures 3.9 – 3.12). Encasement was complete on a still phase grey forespore. Upon maturation a bright smooth ring could be observed around the forespore and mature spores retained faint green fluorescence after release from the mother cell into the culture medium.

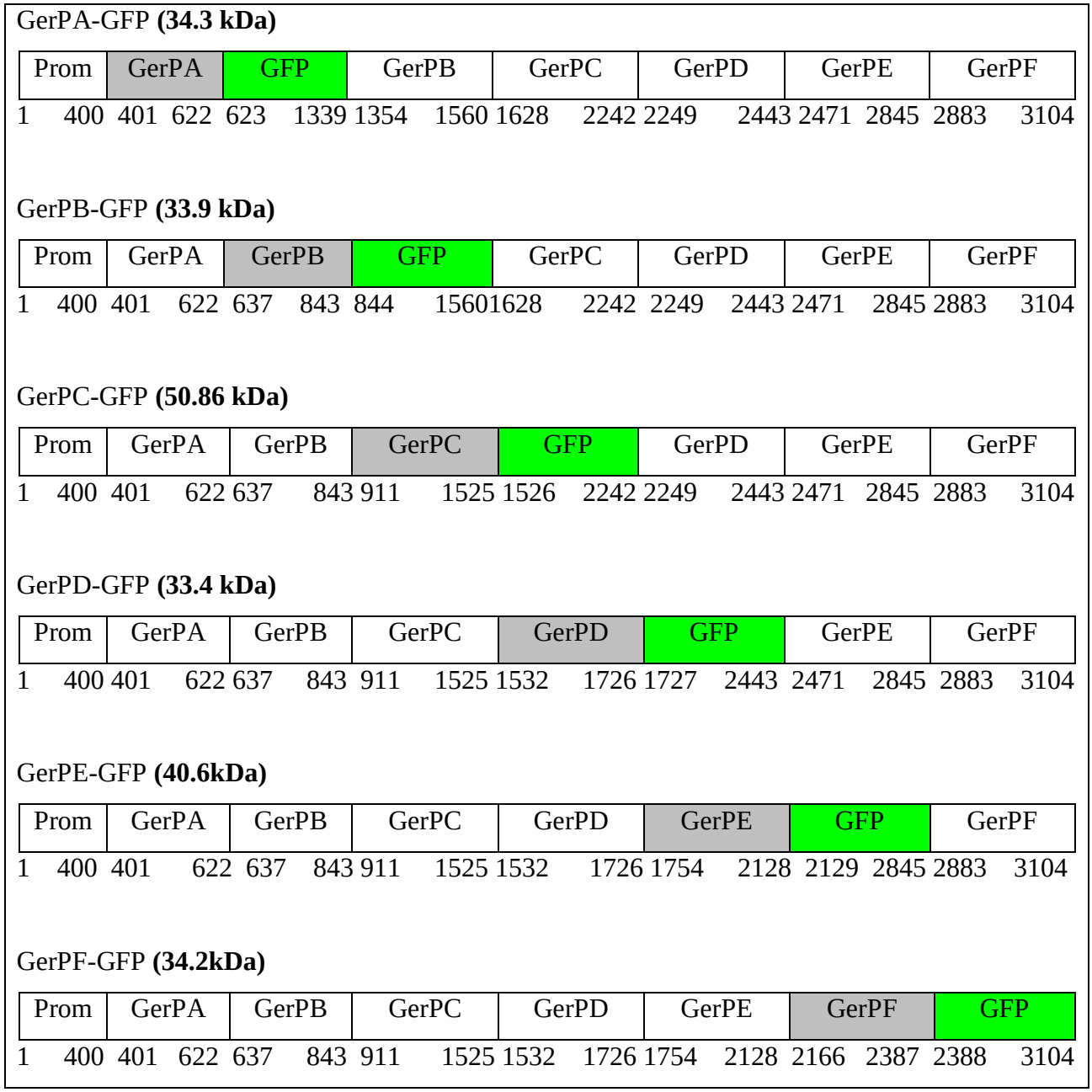


Figure 3.8 Schematic representation of variant *gerP* operons designed for the expression of GerP-GFP fusion proteins. The calculated fusion protein molecular weights are shown in parenthesis. Numbers under the schematic fusion proteins represents nucleotide numbers.

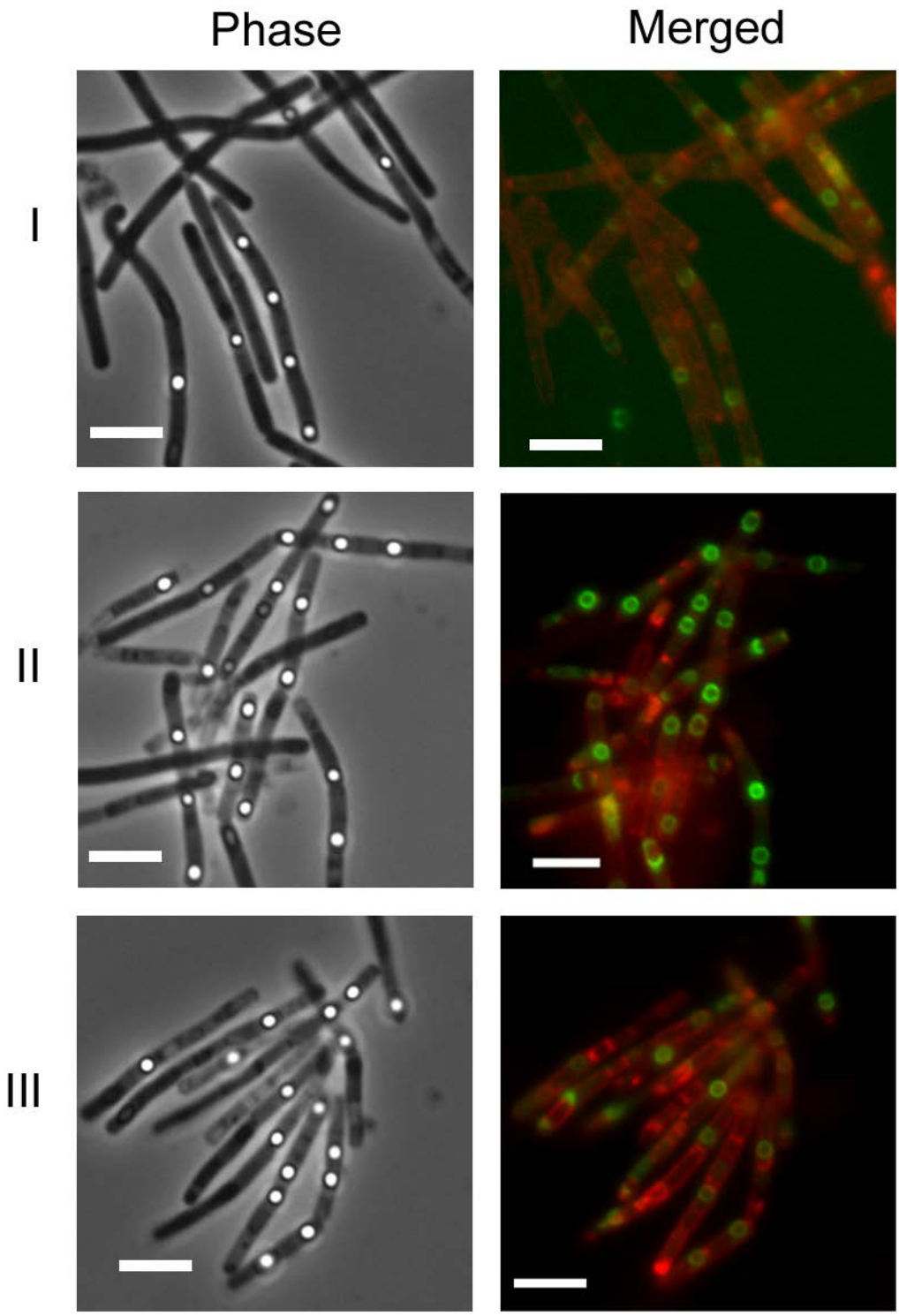


Figure 3.9 Localisation of GerP-GFP proteins in sporulating *B. cereus* 14579 cells. Sporulating cells were stained with the red-fluorescent FM 4-64 membrane stain, and viewed by phase contrast and fluorescence microscopy using appropriate filter sets. (I) *B. cereus* GerPA-GFP; (II) *B. cereus* GerPB-GFP; (III) *B. cereus* GerPC-GFP.

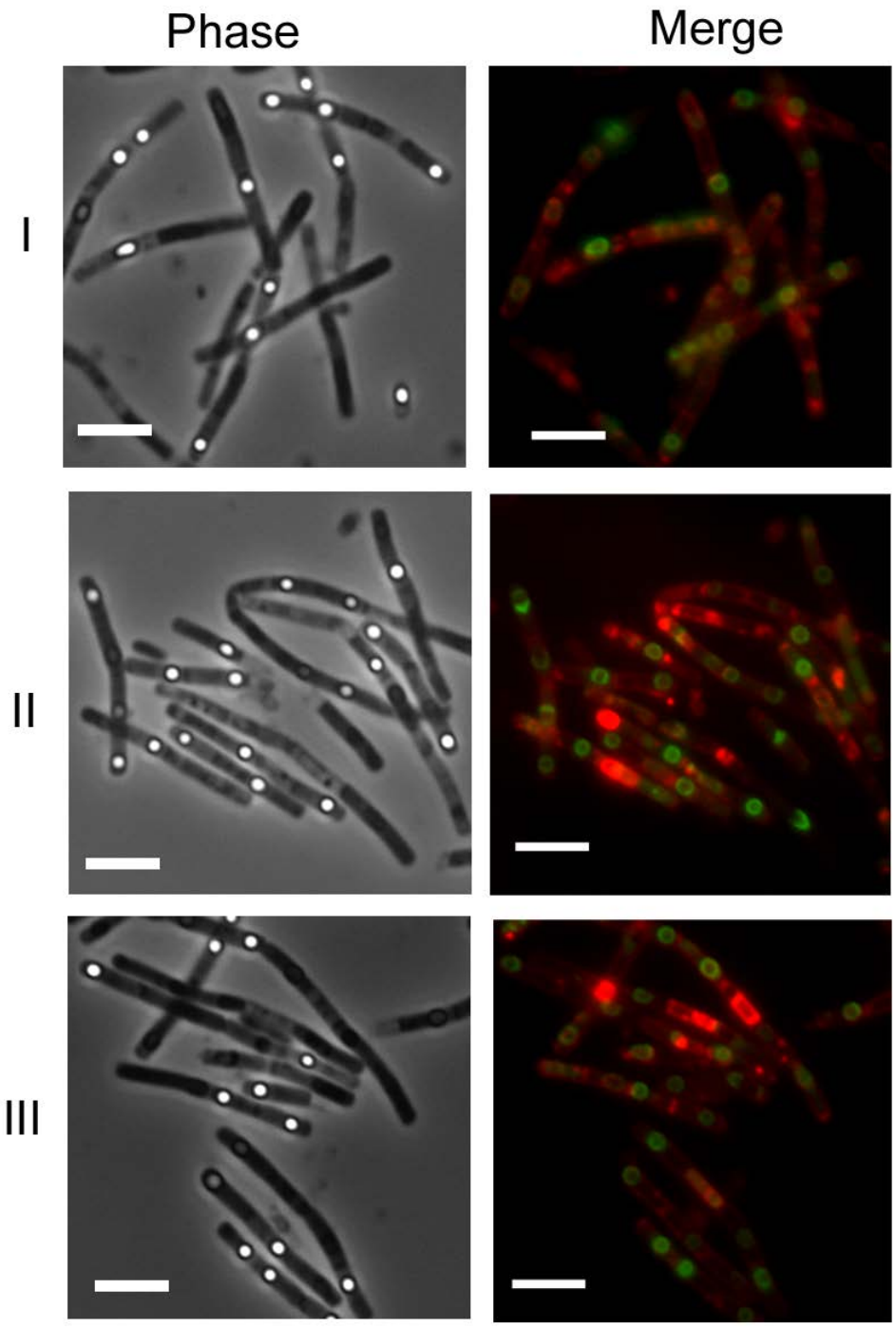


Figure 3.10 Localisation of GerP-GFP proteins in sporulating *B. cereus* 14579 cells. Sporulating cells were stained with the red-fluorescent FM 4-64 membrane stain, and viewed by phase contrast and fluorescence microscopy using appropriate filter sets. (I) *B. cereus* GerPD-GFP; (II) *B. cereus* GerPE-GFP; (III) *B. cereus* GerPF-GFP

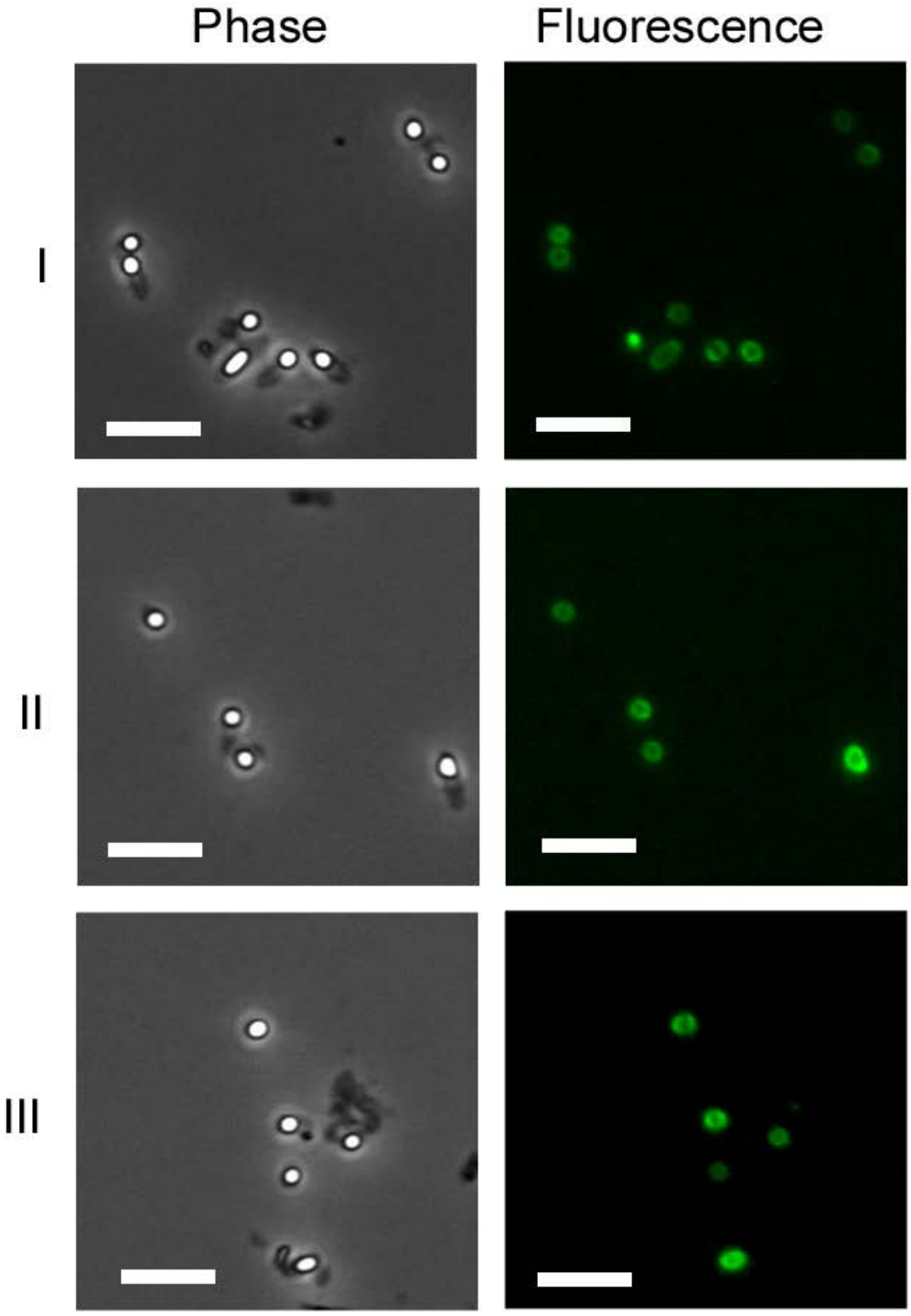


Figure 3.11 Localisation of GerP-GFP proteins in mature spores of *B. cereus* 14579. (I) *B. cereus* GerPA-GFP; (II) *B. cereus* GerPB-GFP; (III) *B. cereus* GerPC-GFP

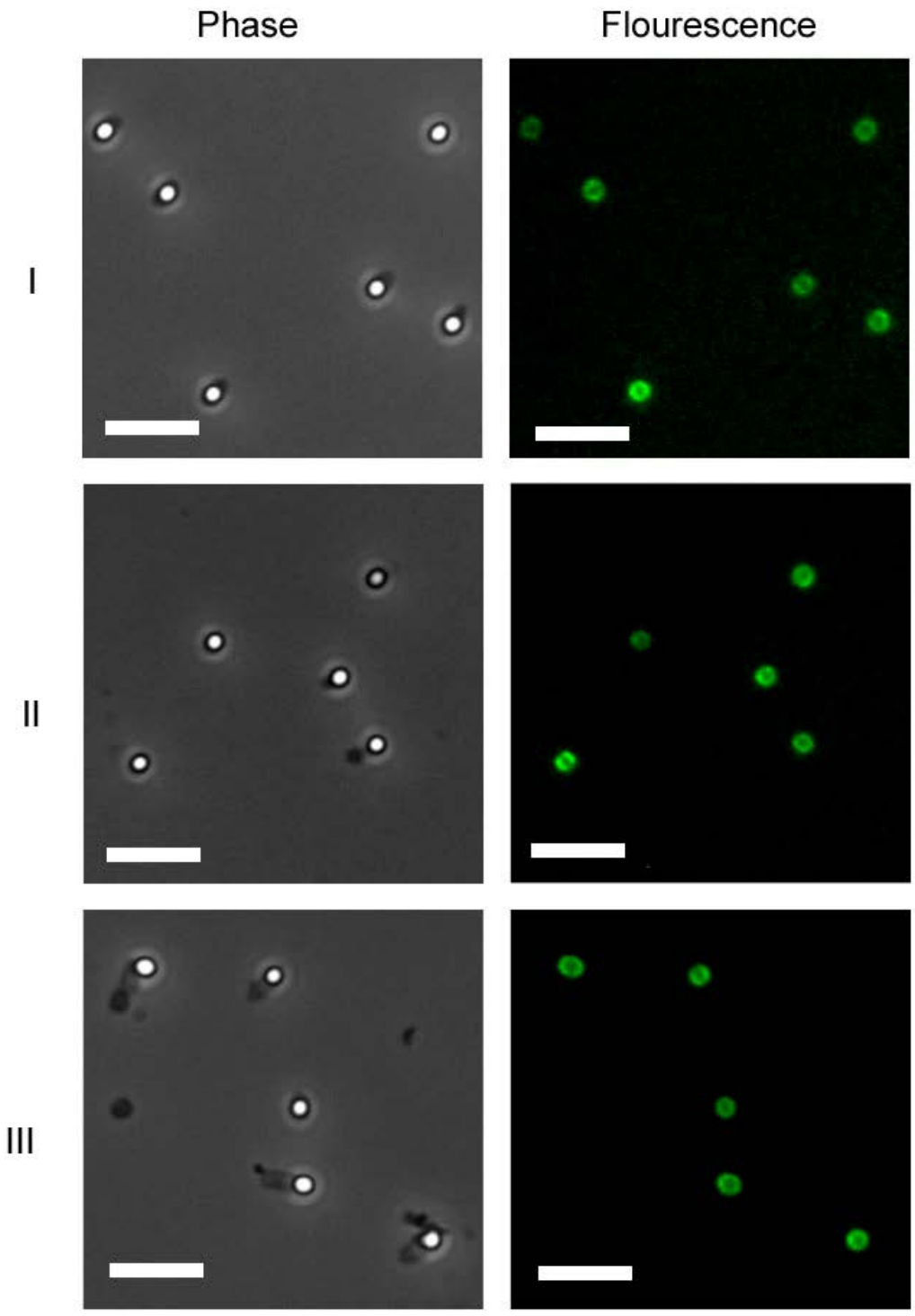


Figure 3.12 Localisation of GerP-GFP proteins in mature spores of *B. cereus* 14579. (I) *B. cereus* GerPD-GFP; (II) *B. cereus* GerPE-GFP; (III) *B. cereus* GerPF-GFP

Similar results were obtained when the various GerP-GFP plasmids were introduced to the GerP null strain (Figure 3.13).

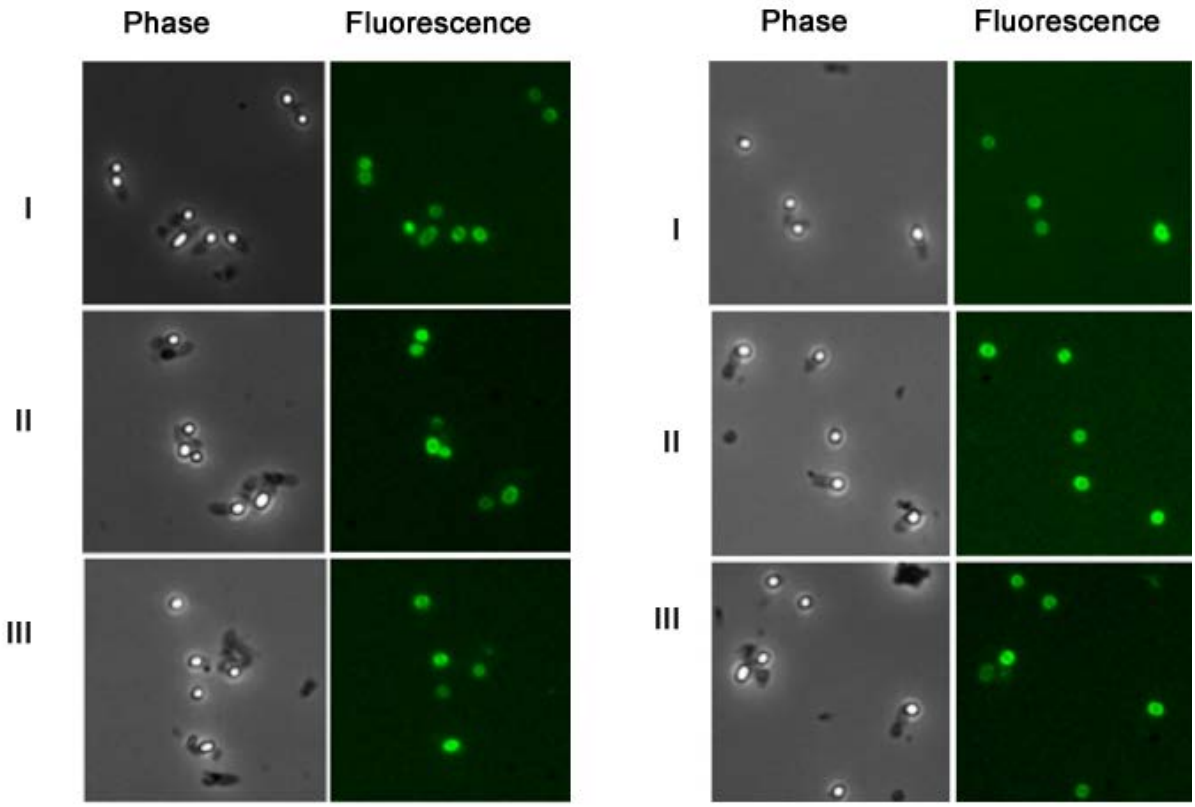


Figure 3.13 Localisation of plasmid encoded GerP-GFP proteins in mature spores of the *B. cereus* GerP null strain.

3.2.7 Immuno-detection of GerP proteins on *Bacillus cereus* spores

Anti-GFP antibodies were employed to in an attempt to ascertain whether the various GerP-GFP fusion proteins were localised to the surface of the spore (or at least accessible to antisera). Spores expressing GerP-GFP fusion proteins were incubated with anti-GFP antibodies, followed by appropriate washing and blocking steps prior to incubation with Dylight-594 conjugated anti-rabbit secondary antisera. *B. megaterium* CotX-GFP spores were used as a positive control (CotX is surface located)[160], with wild-type *B. cereus* spores serving as a negative control. Samples were washed and then examined by fluorescence microscopy. In contrast to the control *B. megaterium* CotX-GFP spores, none of the GerP-GFP spores were observed to show red fluorescence with the few red

fluorescent foci observed probably resulting from non-specific labelling indicating that the GerP proteins are not localised to the surface of the spore (Figure 3.14).

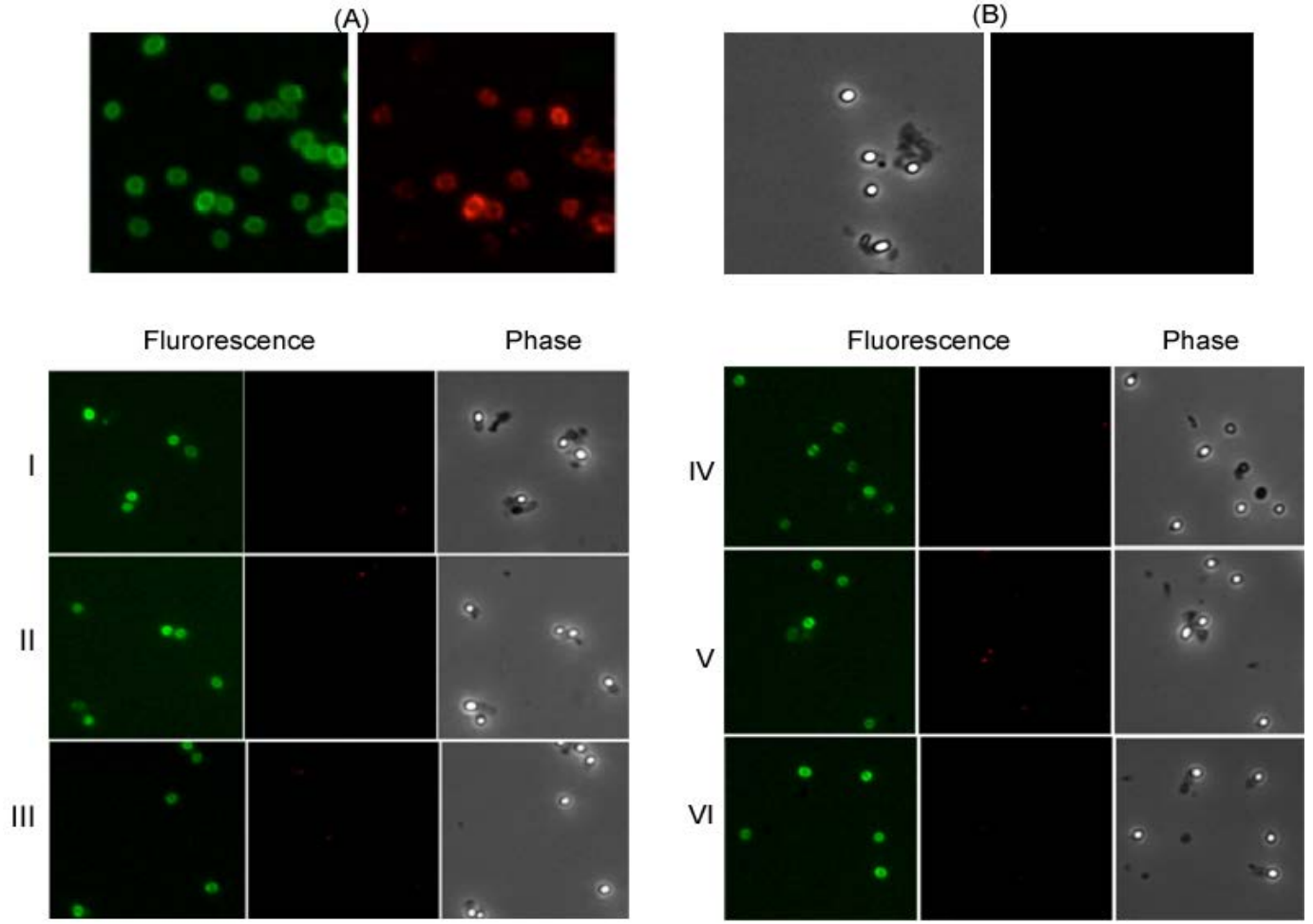


Figure 3.14 Immuno-labelling of *B. cereus* GerP-GFP spores with Dylight-594 conjugated anti-GFP antisera. (A) *B. megaterium* CotX-GFP spores [positive control]; (B) *B. cereus* WT spores [negative control]; (I) *B. cereus* GerPA-GFP spores; (II) *B. cereus* GerPB-GFP; (III) *B. cereus* GerPC-GFP; (IV) *B. cereus* GerPD-GFP; (V) *B. cereus* GerPE-GFP; (VI) *B. cereus* GerPF-GFP.

3.2.8 Ellipsoid Localisation Microscopy

In order to investigate the precise location of GerP proteins, Ellipsoid Localisation Microscopy was employed[160]. *B. cereus* spores designed to express GFP fusion proteins to the various GerP proteins, and to a range of other coat and exosporium proteins, were used for these analyses. Unfortunately, information pertaining to the location and distribution of proteins that comprise the spore coat and exosporium in *B. cereus* is sparse in comparison to that available for *B. subtilis* spores. However, previous studies have revealed that the BclA protein forms the fibre-like nap on the exterior surface of the exosporium, whereas CotD has been identified as being located in the inner coat[164]. Accordingly, strains bearing *gfp*-fusions to the 3' ends of these genes were constructed as part of our ELM analyses to serve as benchmarks for the exosporium and inner coat. Strains designed to express C-terminal GFP fusions to SleL and CwlJ were also constructed, since at least in *B. subtilis,* these cortex lytic enzymes have been localised to the inner coat[159]. Spores of additional strains, including *bxpB-gfp* (BxpB is an exosporium protein)[166], were prepared but failed to show any fluorescence, whereas *cotE-gfp* spores provided unreliable results. CotE is important in the assembly of spore outer coat proteins, and it may be that the GFP moiety disrupted its key morphogenetic role.

ELM analyses conducted to demonstrate the precise location of GerP-GFP fusion proteins in the matured spores revealed that the GerPA 'shell' has an equivalent radius of 460 nm, GerPB 437 nm, GerPC 430 nm, GerPD 410 nm, GerPE 452 nm and GerPF at 430 nm, respectively (Figure 3.15).

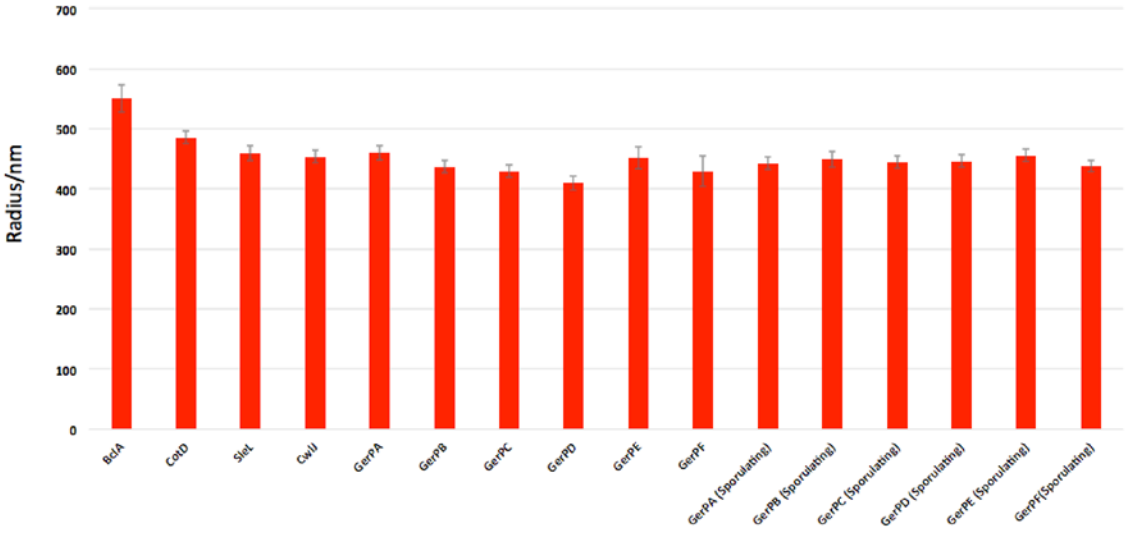


Figure 3.15 Ellipsoid Localisation Microscopy measurements of the location of various GerP-GFP proteins, plus other putative coat and exosporium proteins, in mature *B. cereus* spores.

ELM measurements of the positions of the various GerP-GFP fusion proteins was also conducted in sporulating cells. Images of all six variant strains expressing GerP-GFP fusion proteins were captured at a similar stage in sporulation in order to maintain consistency in the data collection method. All six GerP-GFP proteins were observed to have a similar equivalent shell radius in the developing spore as in the mature spore (Figure 3.15). As expected, the BclA-GFP protein was measured as having the largest equivalent radius, which is consistent with it's position on the exterior of the spore. Similarly, ELM-derived measurements indicate that the CotD-GFP protein is located to the exterior of the SleL-GFP protein. In general, mean radii associated with all six GerP proteins indicate that these proteins occupy a similar location within the inner spore coat, and that this location is interior to the position occupied by SleL (albeit residual error associated with GerPB and GerPF measurements mean that we can place these proteins with less certainty).

3.2.9 Inter-dependency between GerP proteins for spore localisation

Having established that the various GerP proteins appear to localise to broadly the same vicinity within the spore, the next objective was to identify whether the localisation of any individual GerP protein is dependent upon presumed interactions with other protein(s)

encoded within the operon. In the first instance this was achieved by introducing variant pHT315 plasmids containing the presumed *gerP* promoter sequence plus an ORF encoding the GerP protein of interest with a C-terminal GFP fusion to strains bearing markerless chromosomal deletions in single *gerP* genes. For example, six plasmids encoding GerPA-GFP through to GerPF-GFP were introduced individually to the *gerPA* null strain, and then to the *gerPB* background, and so on, creating a total of 36 new strains. Each of these strains was then sporulated by nutrient exhaustion and analysed at intervals by fluorescence microscopy until mature spores were released. The results of these analyses reveal that in all backgrounds, with the exception of *gerPA*, the various GerP-GFP fusion proteins are expressed and localise around the developing forespore in a manner reminiscent of that observed for GFP fusion proteins in the wild type background (Figures 3.16 – 3.23). Hence, in the absence of GerPB, for example, all six GFP fusion proteins were observed to localise during sporulation and to persist in the mature spore. The exception to this series of observations occurs in the *gerPA* background, where in the absence of GerPA, none of the GerPB – GerPF GFP fusion proteins were observed to localise around the developing forespore (although diffuse fluorescence was observed in the mother cell in each case). Similar observations were made when the entire *gerP* operon, modified with individual ORFs containing in-frame *gfp* fusions, was expressed in *trans* from a series of plasmids introduced to the *gerP* null background, reducing the potential for differences in expression levels between chromosomal and plasmid borne genes being responsible for the apparent dependency on GerPA for localisation of all other GerP proteins (data not shown).

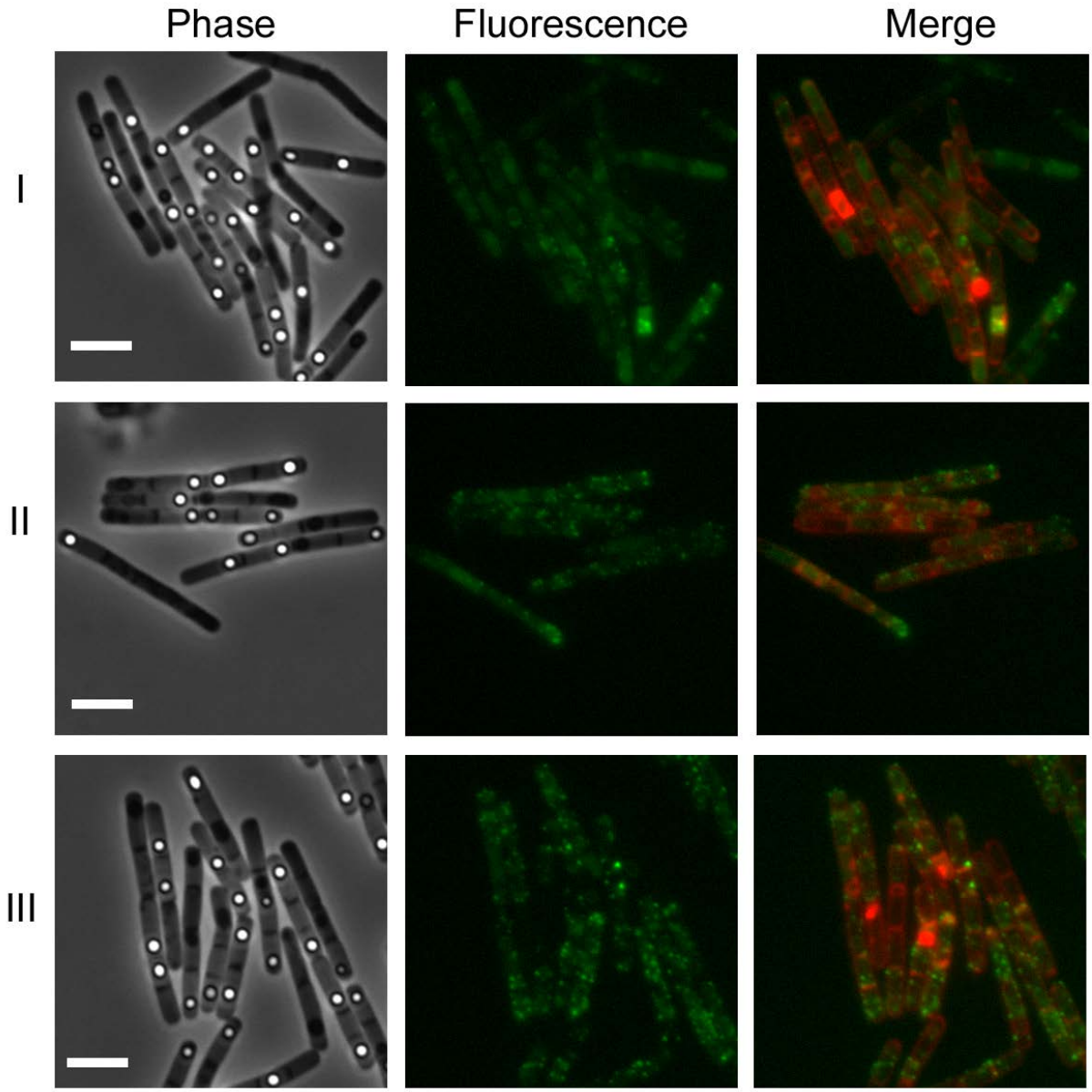


Figure 3.146 Localisation of GerP proteins in the *B. cereus* GerPA null strain. (I) GerPB-GFP (II) GerPC-GFP, (III) GerPD-GFP.

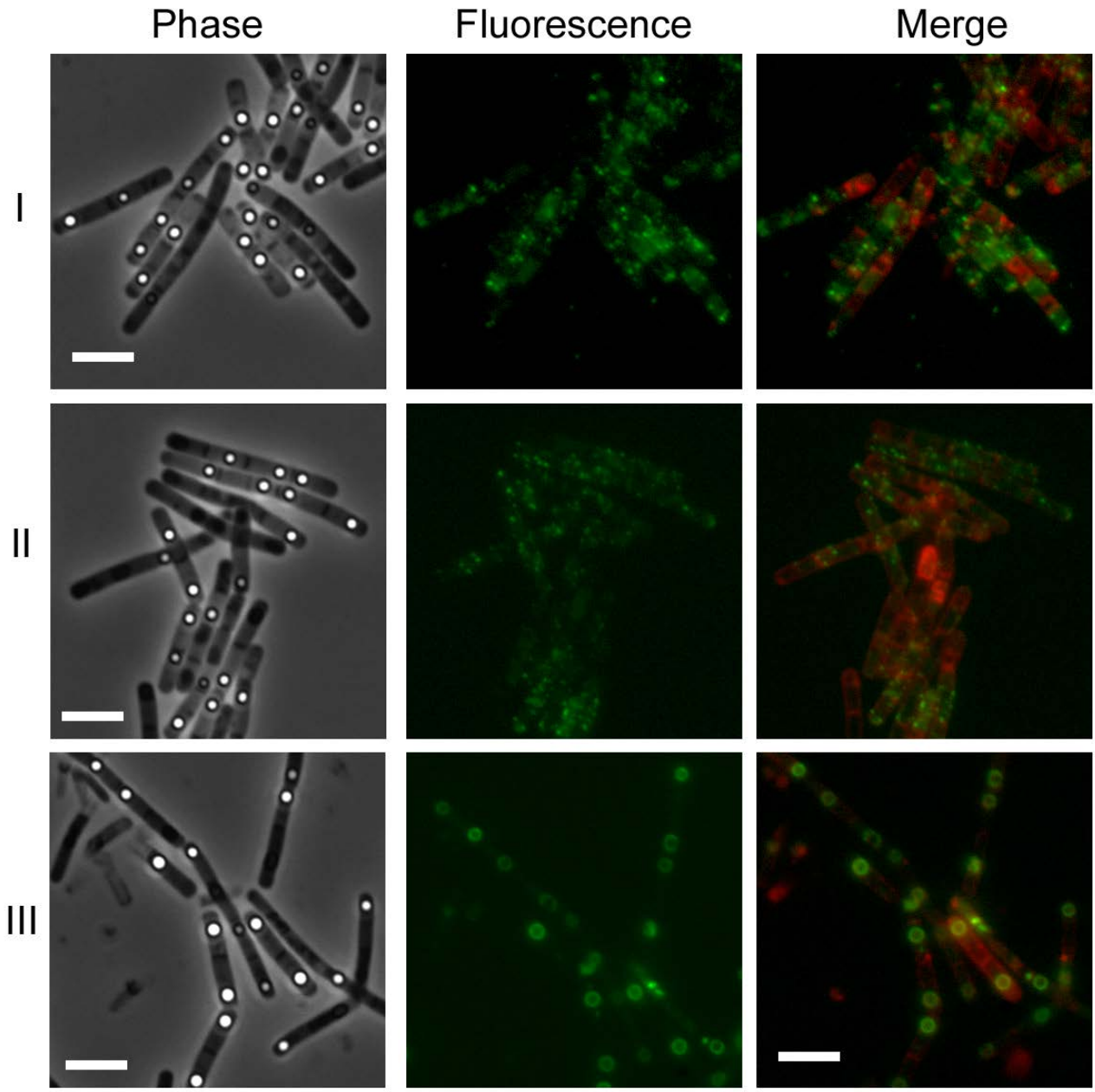


Figure 3.157 Localisation of GerP proteins in the *B. cereus* GerPA null strain. (I) GerPE-GFP (II) GerPF-GFP, (III) GerPA-GFP.

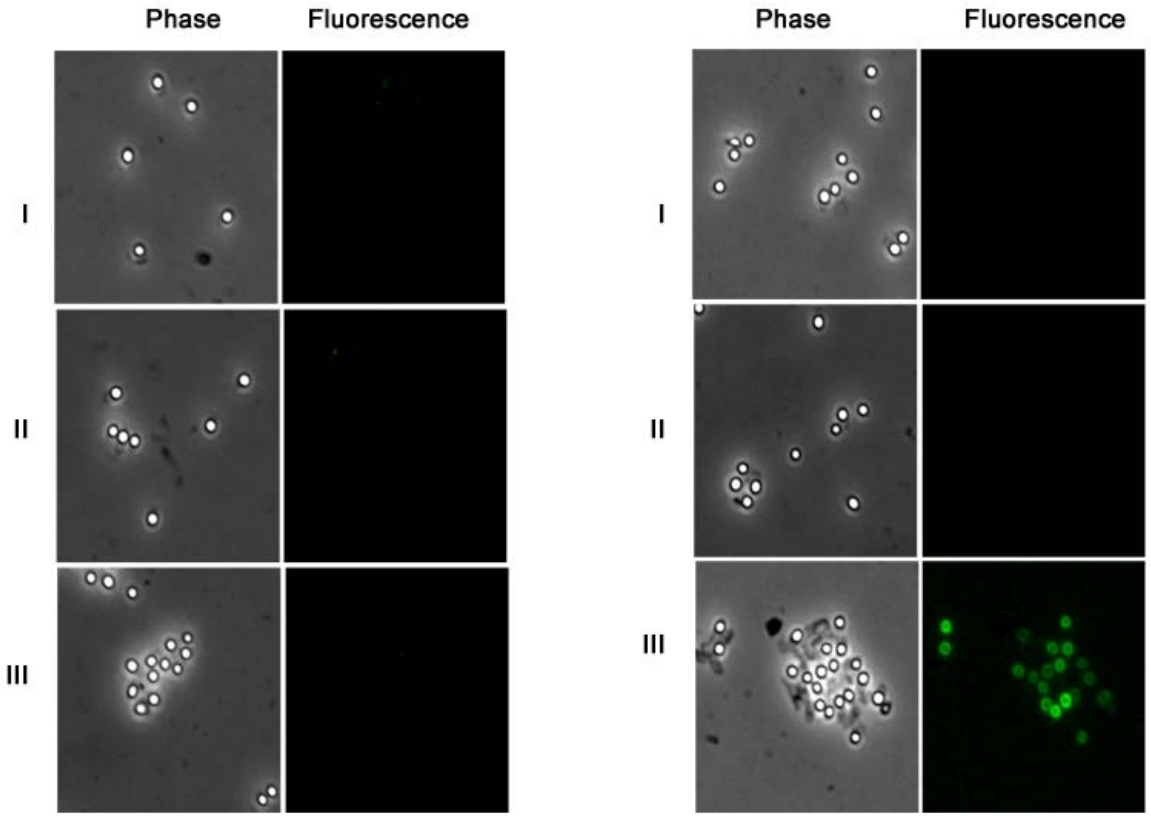


Figure 3.18 Fluorescence microscopy analysis of *B. cereus* GerP null spores complemented with {Left(I) GerPB-GFP, (II) GerPC-GFP, (III) GerPD-GFP}, {Right(I) GerPE-GFP, (II) GerPF-GFP, (III) GerPA-GFP}.

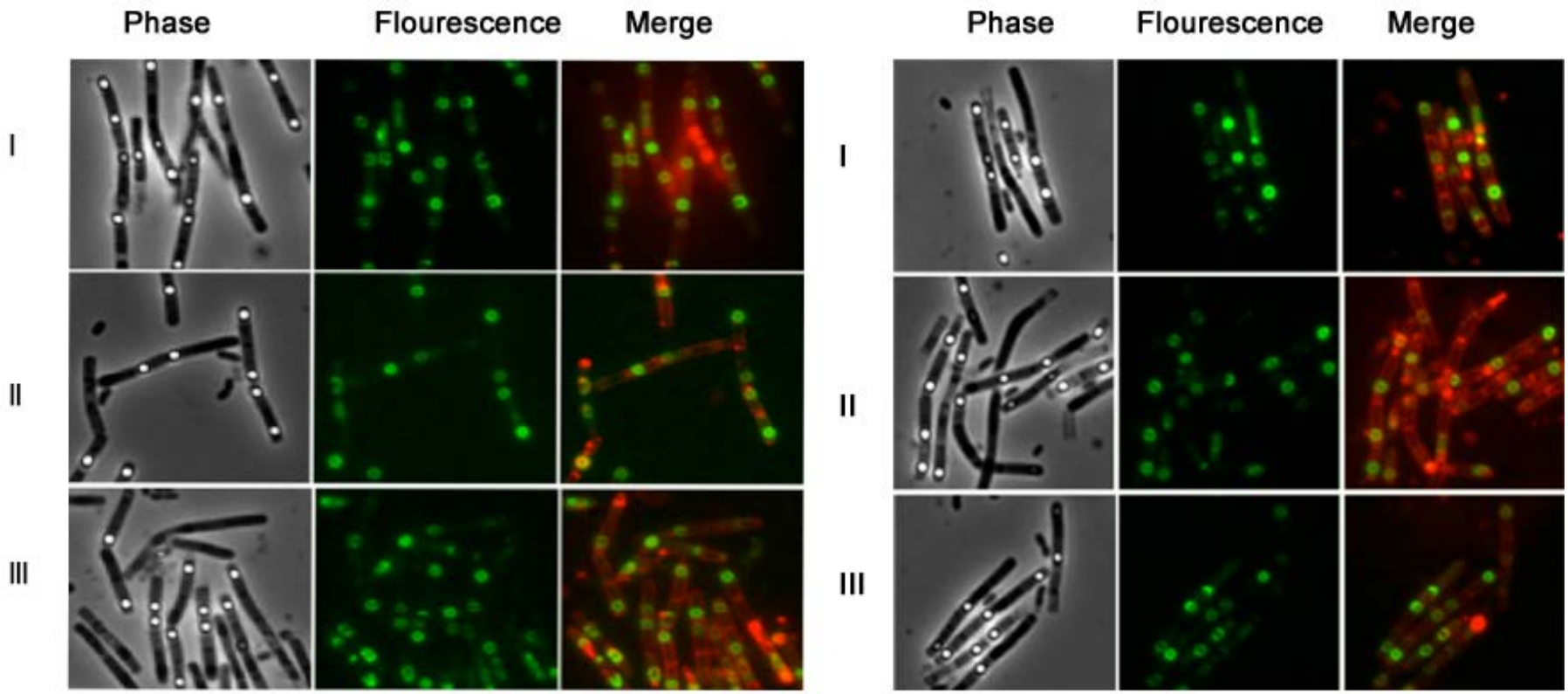


Figure 3.169 Localisation of GerP proteins in the *B. cereus* GerPB null strain.{Left (I) GerPA-GFP (II) GerPC-GFP, (III) GerPD-GFP}, {Right(I) GerPE-GFP, (II) GerPF-GFP, (III) GerPB-GFP}

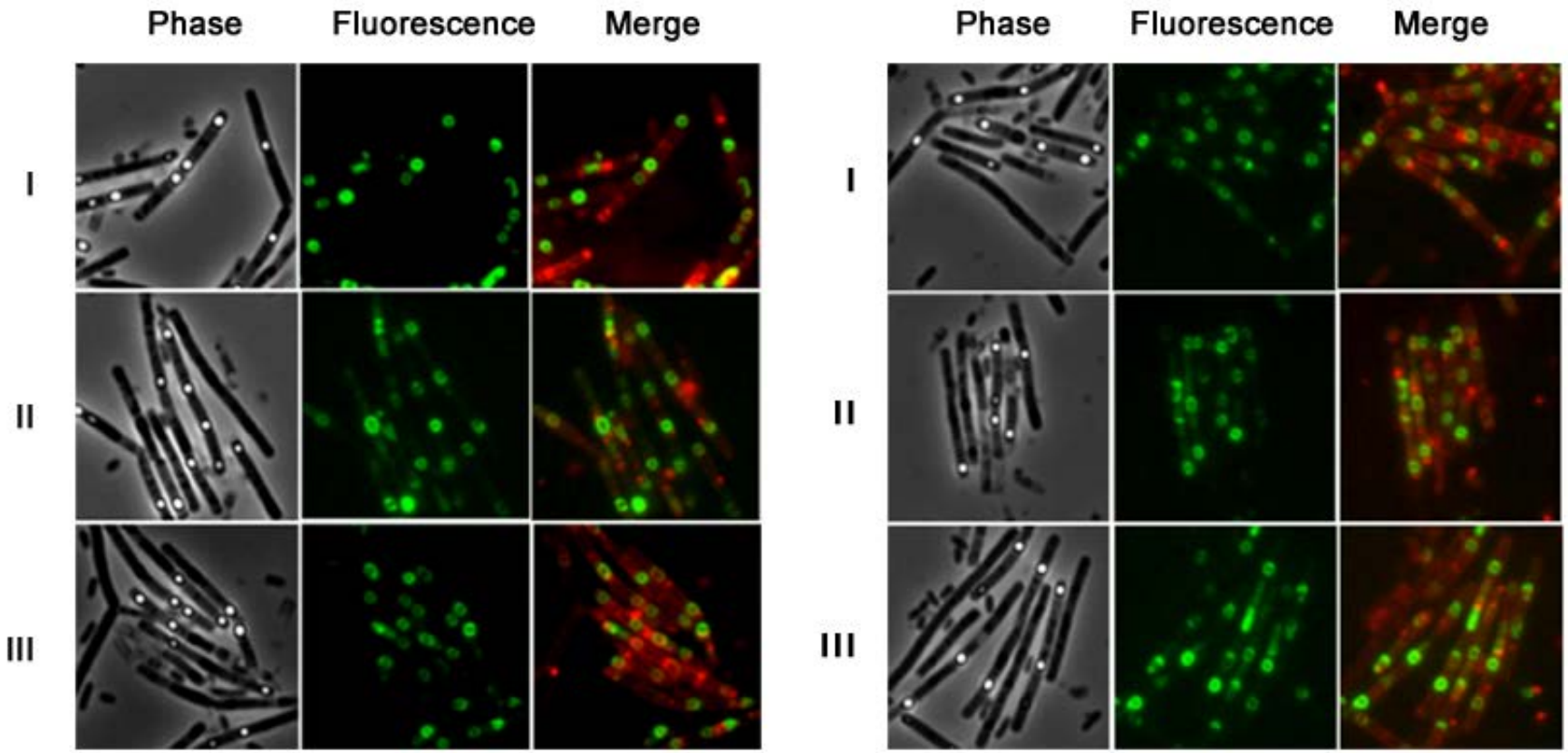


Figure 3.20 Localisation of GerP proteins in the *B. cereus* GerPC null strain. {Left(I) GerPA-GFP (II) GerPB-GFP, (III) GerPD-GFP}, {Right (I) GerPE-GFP, (II) GerPF-GFP, (III) GerPC-GFP}

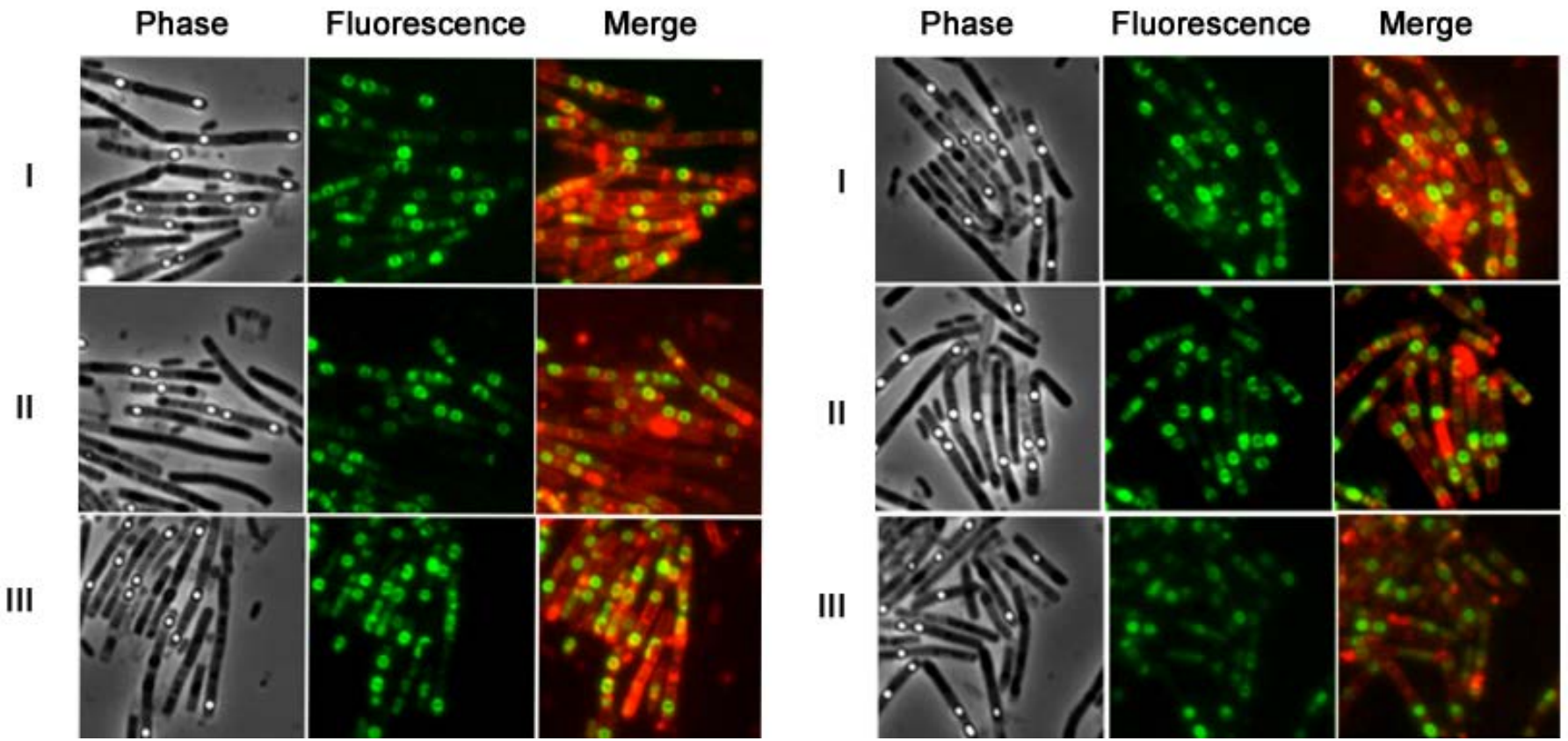


Figure 3.171 Localisation of GerP proteins in the *B. cereus* GerPD null strain. (I) GerPA-GFP (II) GerPB-GFP, (III) GerPC-GFP, (IV) GerPE-GFP, (V) GerPF-GFP, (VI) GerPD-GFP

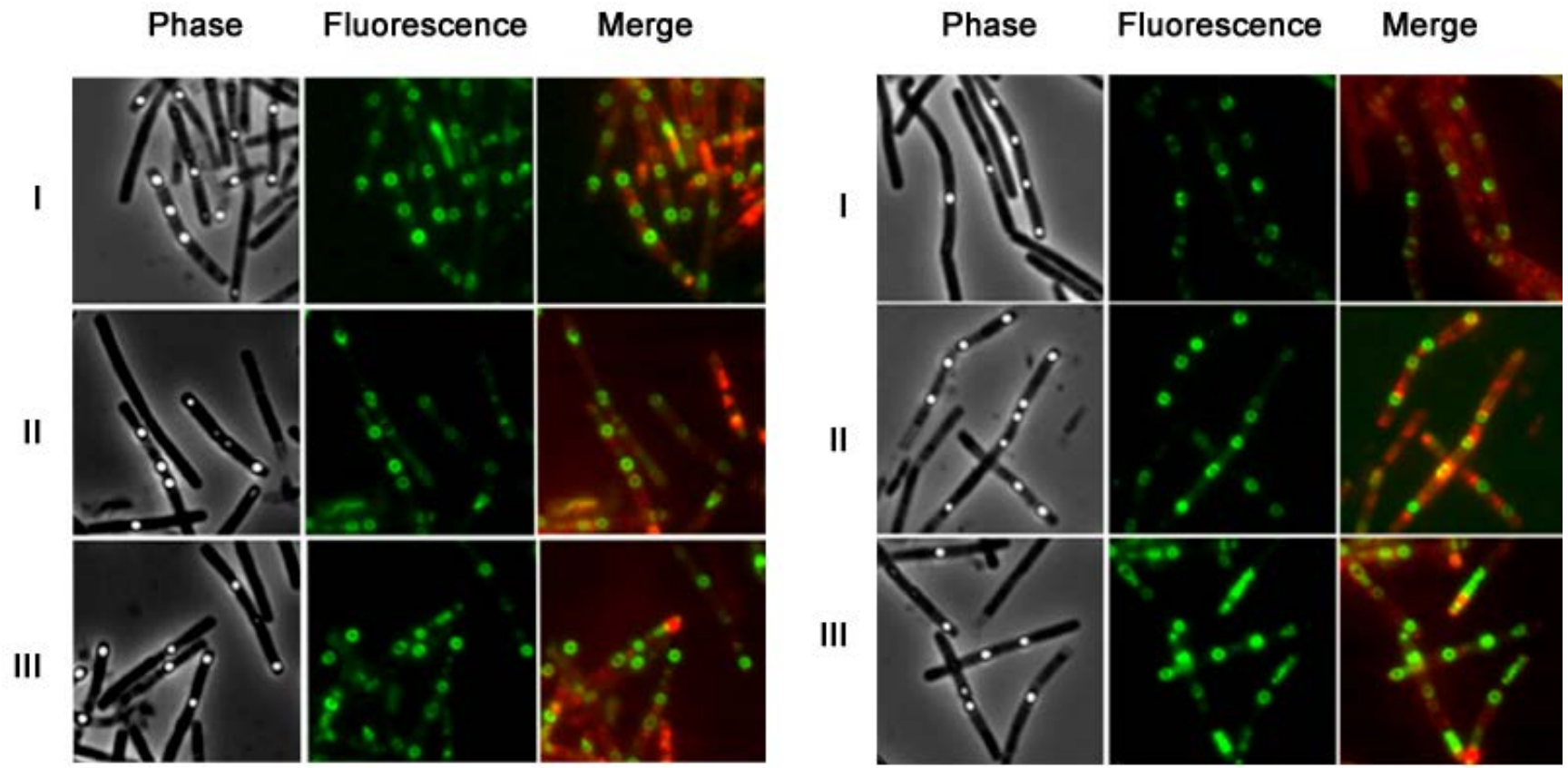


Figure 3.22 Localisation of GerP proteins in the *B. cereus* GerPE null strain. (I) GerPA-GFP (II) GerPB-GFP, (III) GerPC-GFP, (IV) GerPD-GFP, (V) GerPF-GFP, (VI) GerPE-GFP

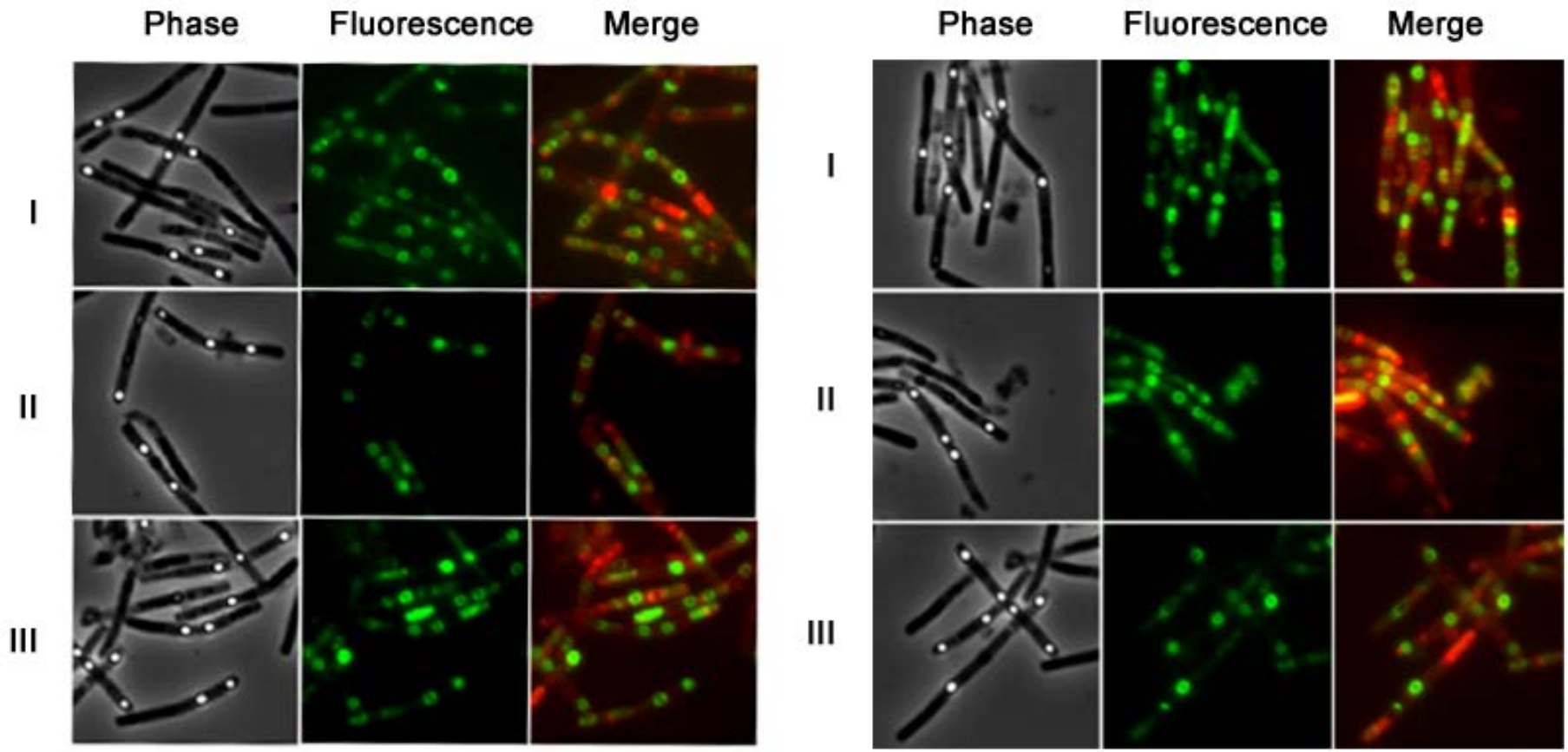


Figure 3.183 Localisation of GerP proteins in the *B. cereus* GerPF null strain. (I) GerPA-GFP (II) GerPB-GFP, (III) GerPC-GFP, (IV) GerPD-GFP, (V) GerPE-GFP, (VI) GerPF-GFP

3.2.10 Functional analysis of GerP-GFP fusion proteins

In order to determine whether the presence of the GFP moiety interfered with the function of the various GerP protein partners, the germination behaviour of the various strains of spores was examined. In all cases, the germination profiles of spores designed to express were comparable to wild type spores (Figure 3.24 and 3.25), indicating that GFP doesn't interfere with the various GerP protein functions (and presumably location within the spore).

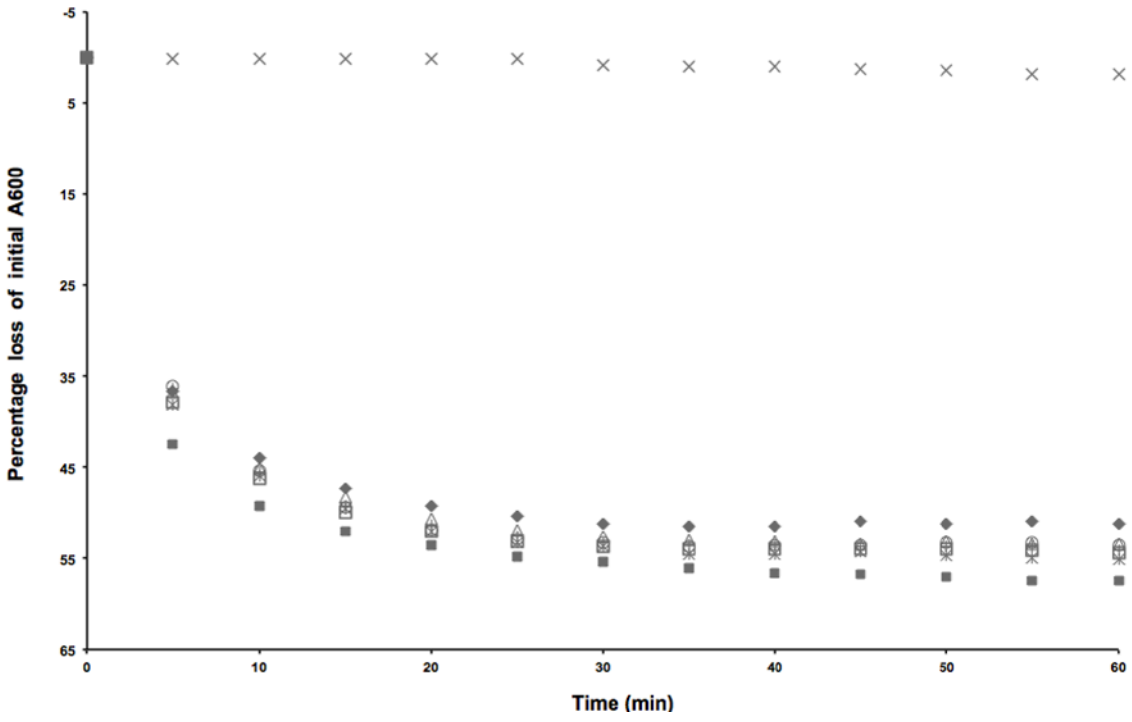


Figure 3.19 Germination of complemented *B. cereus* GerP null mutant spores in 10 mM Tris-HCl, pH 7.4, supplemented with 10 mM inosine. Null mutant strains were complemented with plasmid borne copies of individual GerP-GFP structural genes. Key: (x) buffer, (◆) *gerP*, (*) *gerPA-gfp*, (Δ) *gerPB-gfp*, (□) *gerPC-gfp*, (◆) *gerPD-gfp*, (+) *gerPE-gfp*, (o) *gerPF-gfp*, (■) wild type

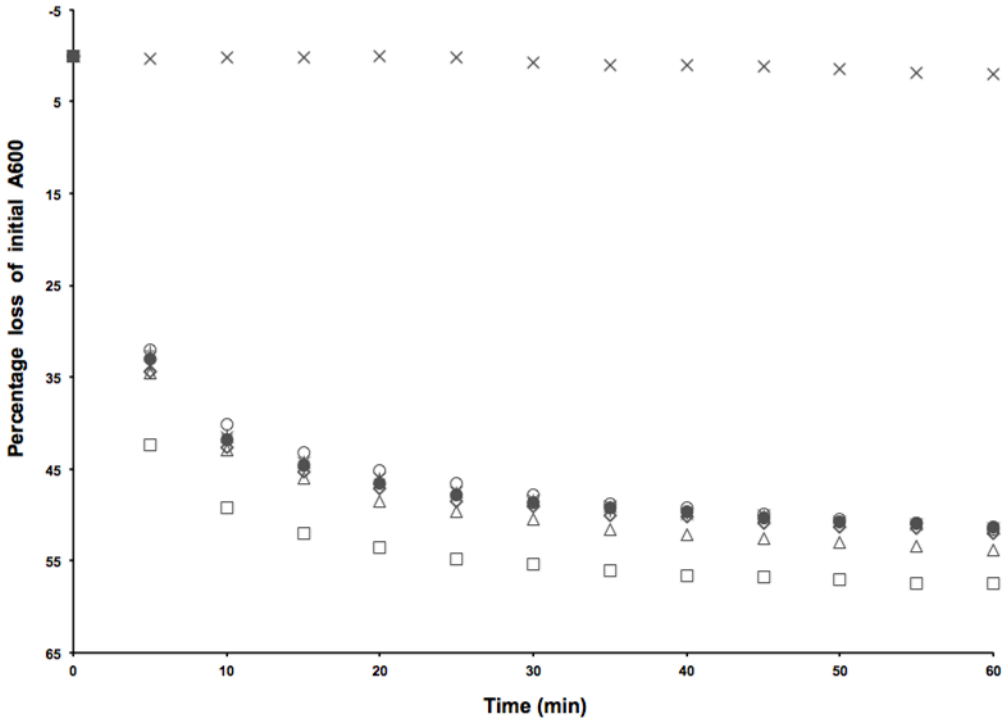


Figure 3.20 Germination of complemented *B. cereus* GerP null mutant spores in 10 mM Tris-HCl, pH 7.4, supplemented with 100 mM L-alanine. Null mutant strains were complemented with plasmid borne copies of individual GerP-GFP structural genes. Key: (x) buffer, (*) *gerPA-gfp,* (o) *gerPB-gfp,* (■) *gerPC-gfp,* (◆) *gerPD-gfp,* (+) *gerPE-gfp,* (Δ) *gerPF-gfp,* (❑) wild type

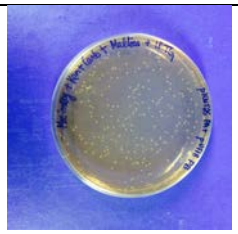
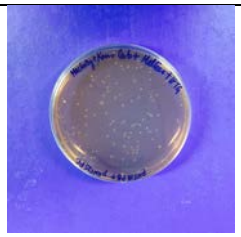
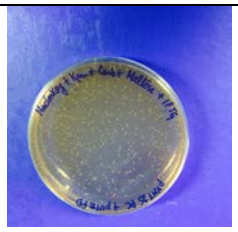
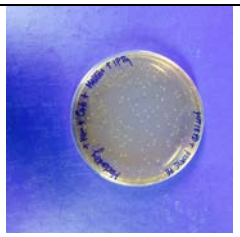
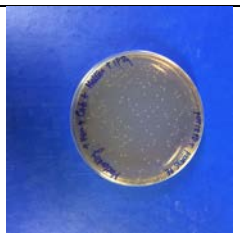
3.2.11 GerP protein-protein interactions

Mutational analyses conducted in this work, and previously, indicate that all six GerP proteins are required for their function within the spore (presumably to assist with the permeability of the inner coat) indicating that they may physically interact with each other. Fluorescence analyses conducted in this project confer further evidence for this idea, since they all appear to be located within the same vicinity of the spore, and the GerPA protein at least is required for localisation of the other GerP proteins within the developing spore. In order to investigate physical interactions between various pairs of GerP proteins, the Bacterial adenylate cyclase two-hybrid system was utilised. The various combinations of plasmids bearing T25 and T19 fragments with individual *gerP* genes as inserts are detailed in Table 3.5. In this assay, positive interactions taking lace within the *E. coli* strains

expressing the proteins of interest results in the formation of deep pink colonies. Unfortunately, with the exception of the supplied positive control, no interactions between the various GerP proteins could be detected by this method (Figure 3.26).

Table 3.5 Combination of various GerP proteins tested for interaction by BACTH

Serial Number	Plasmid combinations		Proteins tested
1	pKNT25-*gerPA*	pUT18-*gerPB*	GerPA and GerPB
2	pUT18-*gerPB*	pKNT25-*gerPC*	GerPB and GerPC
3	pKNT25-*gerPC*	pUT18-*gerPD*	GerPC and GerPD
4	pUT18-*gerPD*	pKNT25-*gerPE*	GerPD and GerPE
5	pKNT25-*gerPE*	pUT18-*gerPF*	GerPE and GerPF
6	pKNT25-*gerPA*	pUT18-*gerPD*	GerPA and GerPD
7	pUT18-*gerPB*	pKNT25-*gerPE*	GerPB and GerPE
8	pKNT25-*gerPA*	pUT18-*gerPF*	GerPA and GerPF
9	pKNT25-*gerPC*	pUT18-*gerPF*	GerPC and GerPF
10	pKNT25-zip	pUT18C-zip	Positive control

Serial Number	MacConkey Agar plate	Proteins being tested	Result
1		GerPA and GerPB	Negative
2		GerPB and GerPC	Negative
3		GerPC and GerPD	Negative
4		GerPD and GerPE	Negative
5		GerPE and GerPF	Negative

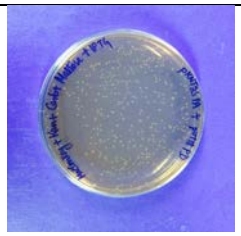
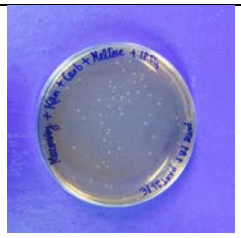
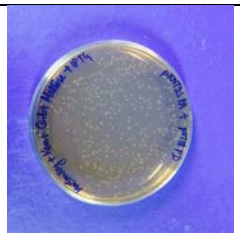
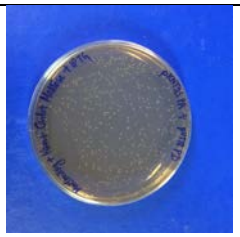
Serial Number	MacConkey Agar plate	Proteins being tested	Result
6		GerPA and GerPD	Negative
7		GerPB and GerPE	Negative
8		GerPA and GerPF	Negative
9		GerPD and GerPE	Negative
10		Positive Control	--

Figure 3.21 Outputs from the Bacterial Adenylate Cyclase Two-Hybrid System (BACTH) used to probe for GerP protein interactions.

3.2.12 Electron microscopy analysis of GerP null spores

Work conducted in this thesis has provided evidence for a coat location for the various GerP proteins in *B. cereus* spores. The defective germination phenotypes of the various GerP null strains indicate that the GerP proteins may have a morphogenetic role within the coat assembly and structure. Thin sections of spores were examined by TEM to investigate whether morphological defects were evident in mutant spores (Figure 3.27), however, no obvious differences between mutant and wild type spores were observed.

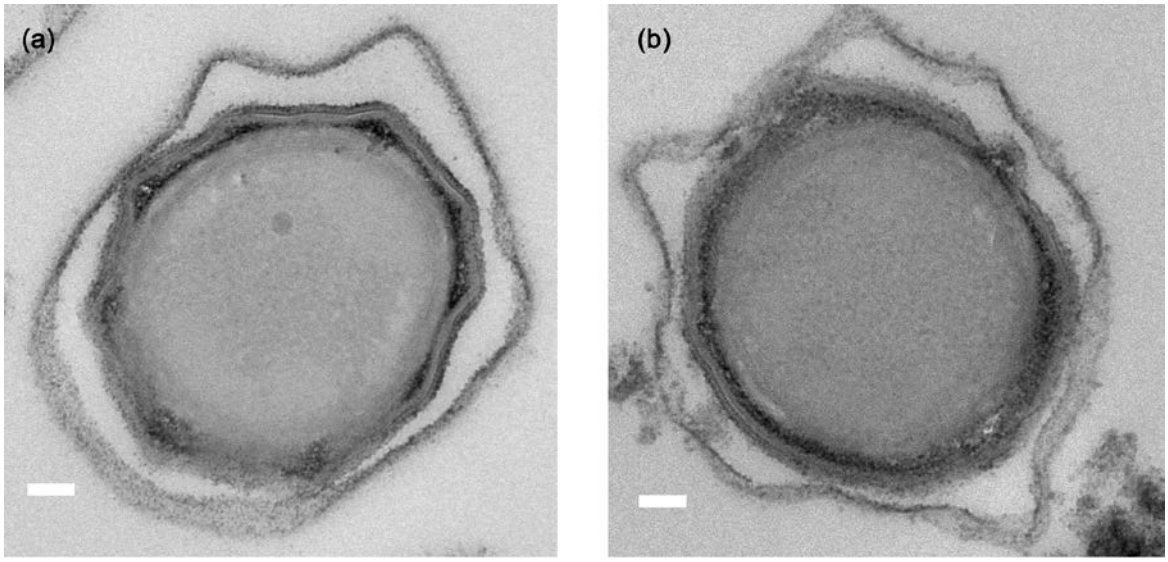


Figure 3.22 Thin section TEM micrographs of a (a) *B. cereus* 14579 wild type spore, and (b) *gerP*-null mutant spore. The marker represents 100 nm.

3.2.13 Heterologous expression of *B. cereus* GerP proteins

Preliminary analyses were conducted on the expression of recombinant GerP proteins with a view to conducting longer-term structural work. All six proteins were cloned into a pBAD-based vector for expression in *E. coli* as GFP fusion proteins. The highest levels of expression of soluble protein, ascertained by fluorescence levels, and by SDS-PAGE, were achieved by inducing expression at 18°C and incubating for >12 hours. Soluble protein was detected for GerPC, GerPD and GerPF by this method, but not for GerPA, GerPB and GerPE. The poor solubility of the proteins may explain why the BACTH system failed to detect protein interactions (Section 3.2.11). In most cases, fluorescent foci of aggregated protein were observed in the cytoplasm of *E. coli*, which was also dispersed with fluorescence (Figure 3.28).

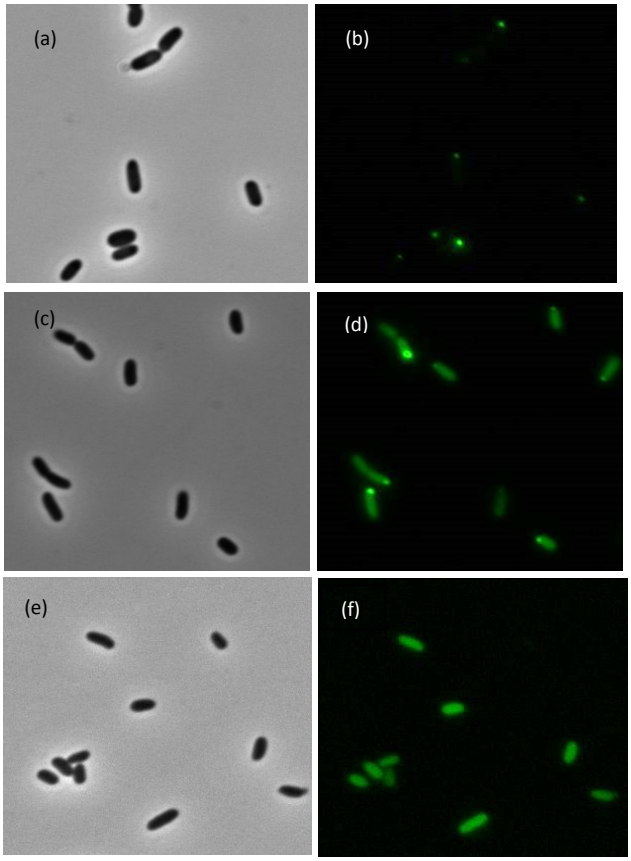


Figure 3.23 Expression of recombinant *B. cereus* GerP-GFP proteins in *E. coli*. Key: (a, b) GerPC-GFP, (c, d) GerPD-GFP, (e, f) GerPF-GFP. Expression was induced at 18^{o}C with 1 mM arabinose, and cells examined by phase and fluorescence microscopy after 12 hours of expression.

Attempts to purify recombinant GerP proteins were focussed on the GerPD-GFP fusion protein, since it showed the greatest level of expression in *E. coli*. In this case, a construct bearing a C-terminal His10 tag was cloned and used for expression to facilitate purification. An SDS-PAGE gel showing initial purification work is shown in Figure 3.28.

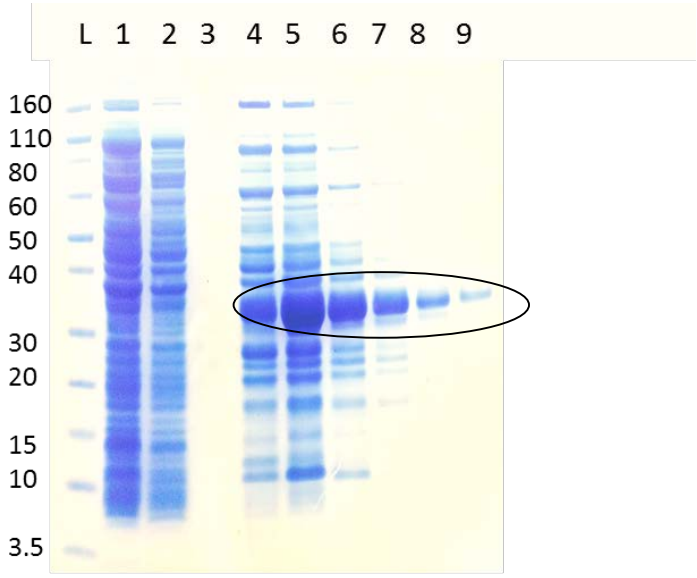


Figure 3.24 Purification of recombinant *B. cereus* GerPD. The figure shows an SDS-PAGE gel loaded with samples collected at different stages of the purification process, which used a GE Healthcare HisTrap excel column fitted to an Acta Pure system. Key: L, protein size marker (Novex Sharp, Invitrogen); Lane 1- column loading flow-through fraction; 2-3, wash fractions; 4-9 eluted protein fractions. Putative GerPD-His10 is circled.

Evidently, the use of a single affinity step is insufficient under the purification conditions applied in Figure 3.29. Several attempts conducted to optimise the affinity purification scheme failed to purify the protein to sufficient standards for crystallography trials and was thus discontinued.

3.2.14 Conclusions

The main objectives of this chapter were to use fluorescence microscopy to determine the precise location of the various GerP proteins in *Bacillus cereus* spores, and to characterise any dependencies that exist between the various GerP proteins for localisation in the spore. Significant progress towards meeting these objectives was achieved, in that results of the work indicate that the GerP proteins are located to the inner spore coat, where they presumably influence the permeability of the coat to molecules seeking access to the inner

integuments of the spore (including germinants). Additionally, the work has revealed that the GerPA protein is essential for the localisation of the remaining GerP proteins within the developing spore, raising the possibility that it forms a basal layer that recruits the other GerP proteins.

Chapter 4. Characterisation of the BC1245 protein in *B. cereus* 14579 spores

4.1 The BC1245 locus

A potential role for the protein encoded at locus BC1245 was revealed first from a preliminary transposon based study that indicated that spores with an insertion at this locus showed a germination defect in response to inosine and alanine[151]. A subsequent study employing plasmid-based insertional mutagenesis cast doubt on the initial results, as the resultant spores showed no obvious germination defect. Furthermore, immunoblotting experiments from the same study indicated that the BC1245 protein was a component of the spore exosporium[152]. With the availability of enhanced molecular genetic techniques, which enable markerless deletions free from any polar effects, and potentially super resolution microscopy analysis, the objective of this chapter was to revisit BC1245 with a view to more firmly characterising its role in *B. cereus* 14579 spores.

4.2 Bioinformatic analysis of BC1245

The open reading frame at the chromosomal BC1245 locus is predicted to be monocistronic, spanning 429 nucleotides and encodes a putative protein of 143 residues with a molecular mass of 15.2 kDa. A BLASTP search indicates that the protein is restricted to members of the *B. cereus* family. Table 4.1 details the percentage amino acid identity shared across the various species with Figure 4.1 illustrating the protein alignment.

Table 4.1 Sequence identity of *B. cereus* BC1245 with orthologues present in related species

Species	**Accession number**	**Sequence identity (%)**				
		BM	**BW**	**BT**	**BC**	**BA**
***B. mycoides* (BM)**	DJ92_3879	100	57	52	53	**53**
***B.* weihenstephanensis (BW)**	BcerKBAB4_115	57	100	77	78	**76**
***B. thuringiensis* (BT)**	BTB_c12960	52	77	100	81	**82**
***B. cereus* (BC)**	BC1245	53	78	81	100	**98**
***B. anthracis* (BA)**	**BAS1169**	**53**	**76**	**82**	**98**	**100**

Figure 4.1 Clustal omega alignment of BC1245 orthologues present in members of the *B. cereus* group.

4.3 BC1245 expression

RT-PCR was conducted on cDNA samples derived from various points of the vegetative growth curve and sporulation in order to identify if and when BC1245 is expressed. As shown in Figure 4.2, expression from the BC1245 locus occurs only – and throughout – sporulation, indicating that the protein product is a component of the spore. Faint bands observed immediately preceding entry to sporulation are probably the result of asynchronous sporulation.

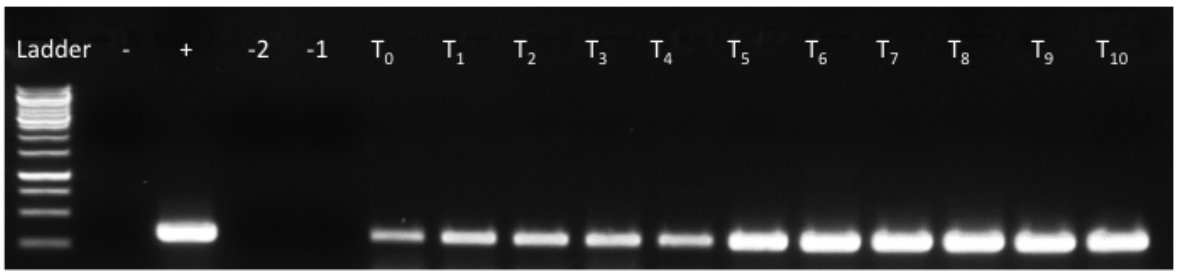


Figure 4.2 RT- PCR analysis of *B. cereus* BC1245 expression. Ladder - DNA marker (Generuler 1Kb, Thermofisher); (−) negative control with no template; (+) positive control with wild type genomic DNA; t_2 - t_1 represent samples collected prior to sporulation (identified as entry to stationary phase); t_0 – t_{10} are time points since the onset of sporulation.

4.4 Fluorescence microscopy based analyses of BC1245

Relatively crude immuno-blot analyses conducted previously indicated that the BC1245 protein was a component of the spore exosporium. In order to facilitate a more precise examination of the location of the protein a strain designed to express a C-terminal GFP fusion protein was constructed in the *B. cereus* 14579 background, using the episomal low copy number pHT315 plasmid to place the gene under control of its native regulatory sequences. The BC1245-GFP strain was sporulated by nutrient exhaustion and examined by fluorescence microscopy throughout growth and sporulation in an attempt to gain insight to the expression and localisation of the protein. Diffuse fluorescent signal from the GFP moiety coincided with the development of phase bright spores, and was observed to localise to form a non-uniform shell around the spore as sporulation continued (Figure 4.3). Fluorescence persisted in the mature spores, although again, in a non-uniform manner, and often with a distinct brighter foci observable at one of the spore poles.

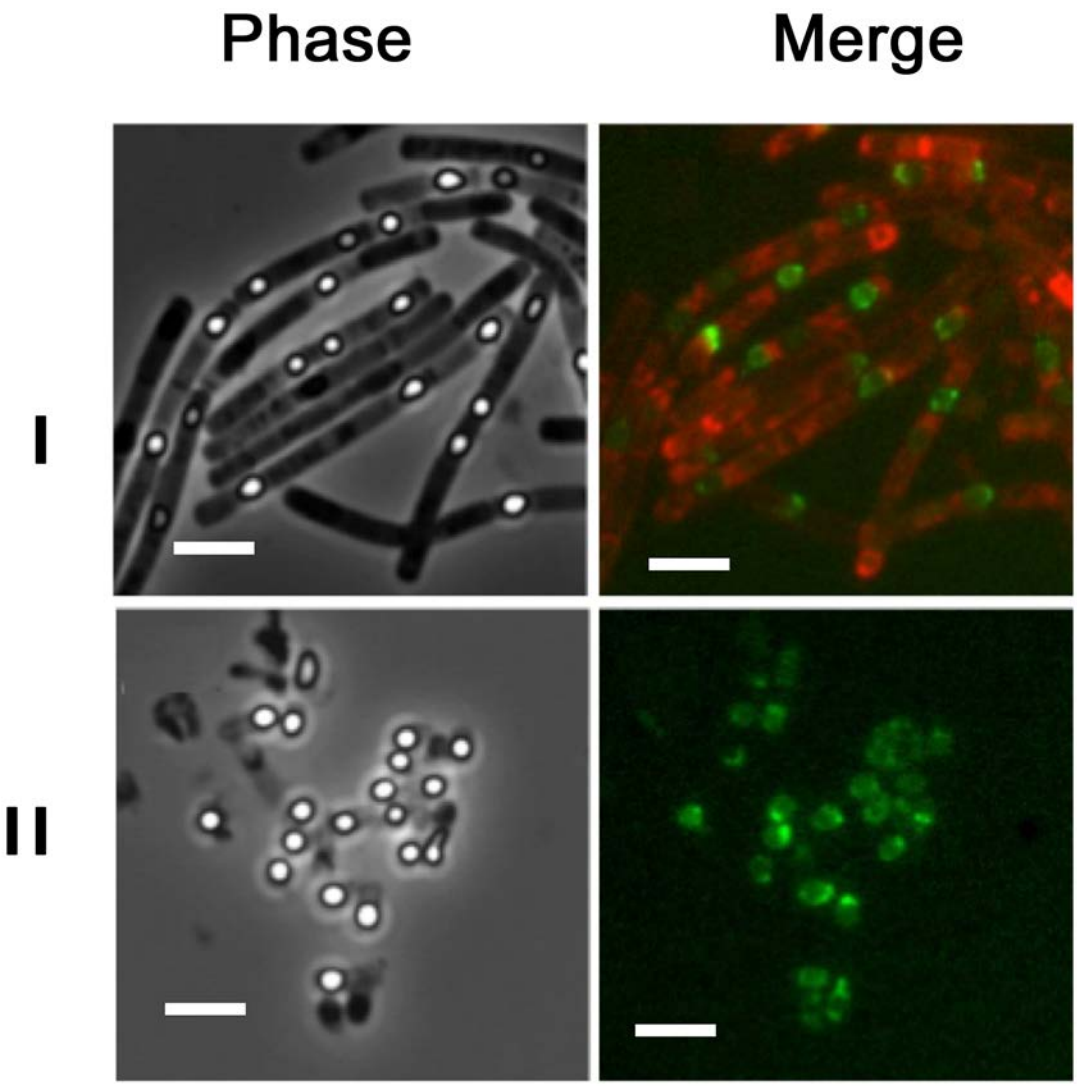


Figure 4.3 Light and fluorescence microscopy analysis of *B. cereus* 14579 (I) sporulating cells and (II) spores constructed to express BC1245-GFP. Samples were counterstained with lipophilic FM 4-64 to show the location of the cell membranes.

4.5 BC1245-SNAP tag analyses

The non-uniform fluorescence evident in *B. cereus* BC1245-GFP spores raised the possibility that the presence of the GFP moiety was somehow disrupting the localisation of the protein in the spore. In order to examine this possibility, a strain designed to express a SNAP-tag to BC1245 was constructed (with a flexible linker between the two proteins), the potential value here being that fluorophore labelling can be achieved after the formation of the mature spores (albeit the presence of the SNAP-tag moiety could be equally disruptive as GFP). *B. cereus* spores expressing the BC1245-SNAP fusion protein and subsequently labelled with Oregon Green were subject to fluorescence microscopy analysis, as shown in Figure 4.4. As with the GFP-based analyses, the BC1245-SNAP protein appears to be distributed around the spore with distinct foci at one of the spore poles.

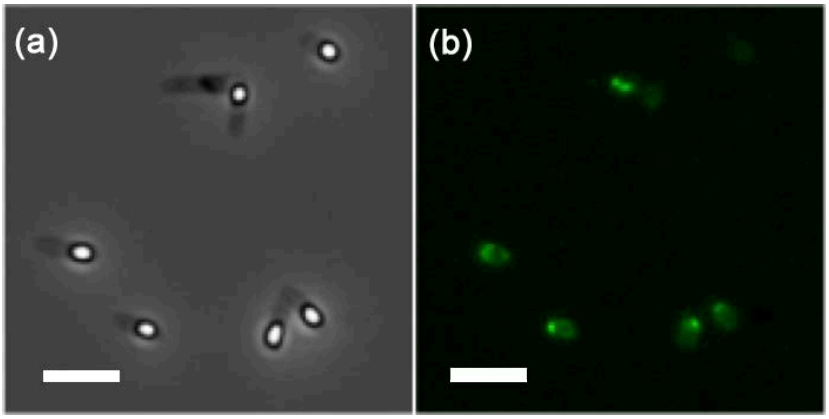


Figure 4.4 Light and fluorescence microscopy analysis of *B. cereus* BC1245-SNAP spores labelled with Oregon Green.

4.6 Immuno-detection of BC1245-GFP

Anti-GFP antibodies were employed to in an attempt to ascertain whether the BC1245-GFP fusion protein is localised to the surface of the spore (or at least accessible to antisera). *B. subtilis* ProY-GFP spores were used as a positive control (ProY is surface located), with wild-type *B. cereus* spores serving as a negative control (not shown). As shown in Figure 4.5, *B. cereus* BC1245-GFP spores are associated with a red fluorescent

signal when treated with anti-GFP antisera, indicating that the fusion protein is surface exposed, or at least accessible to antisera.

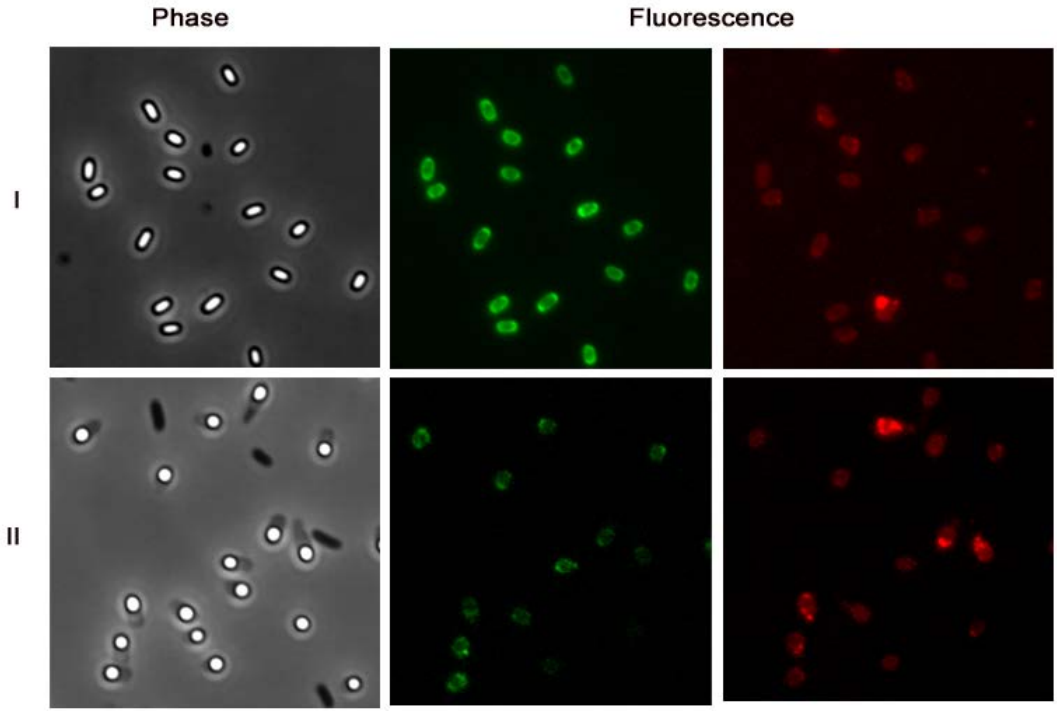


Figure 4.5 Immuno-labelling of B. cereus BC1245-GFP spores with Dylight-594 conjugated anti-GFP antisera. (I) B. subtilis ProY-GFP (II) B. cereus BC1245-GFP

4.7 Analysis of sonicated *B. cereus* BC1245-GFP spores

Unfortunately the non-uniform distribution of the BC1245-GFP protein prevented reliable analysis by ellipsoid localisation microscopy. Hence, in order to investigate further the location of the BC1245 protein in the spore, spores displaying the GFP fusion protein were subjected to several rounds of sonication with a view to shedding of the exosporium while retaining the spore coat. Spore suspensions were sonicated for seven one-minute cycles separated by 1-minute incubations on ice. The effect of sonication on release of exosporium material, and potentially BC1245-GFP, was assessed after each round of sonication by phase contrast and fluorescence microscopy.

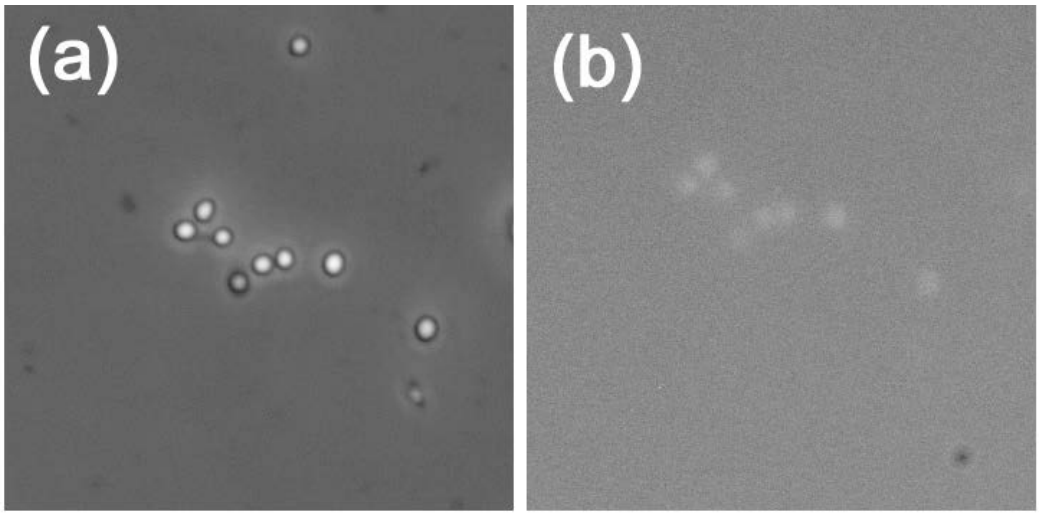


Figure 4.6 Phase contrast (a) and fluorescence microscopy (b) images of *B. cereus* BC1245-GFP spores subject to seven one-minute rounds of sonication interspersed with incubation on ice.

Analysis under phase contrast optics revealed sonication to be an effective method for removal of the exosporium, which was visibly removed from the bulk of the spores after >5 cycles. Unexpectedly, a fluorescent signal was still visibly associated with the partially disrupted spores and not with shed exosporium material. These analyses indicate that the BC1245-GFP protein is a component of the spore coat as opposed to the exosporium. From these analyses we can also infer – again unexpectedly - that the anti-GFP antisera are capable of traversing the exosporium in *B. cereus* 14579 spores to access proteins on the underlying spore coat.

4.8 *B. cereus* BC1245 null mutant spores

The pMAD based markerless gene deletion method was used to construct a BC1245 null mutant strain in the 14579 background to enable re-assessment of the germination phenotype of the resultant spores and to enable structural analysis of the mutant spores by TEM. A strain in which the entire open reading frame was deleted with the exception of the putative start and stop codons was successfully constructed as outlined in Chapter 2 and validated by PCR (Figure 4.7) and sequencing.

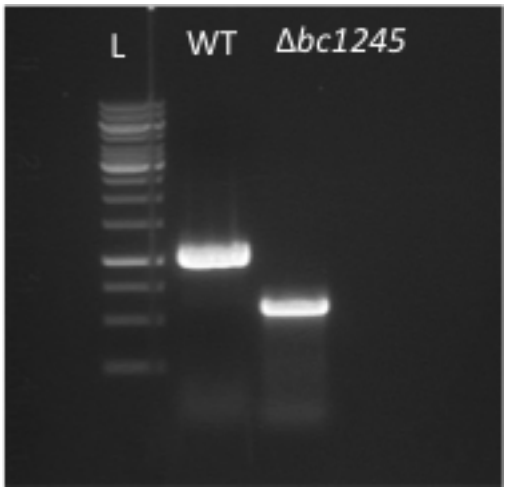


Figure 4.7 PCR conducted with primers that flank the BC1245 locus, confirming deletion of the bulk of the ORF in the null mutant strain.

4.9 Analysis of *B. cereus* BC1245 spores by electron microscopy

Sectioned wild type and BC1245 null spores were analysed by TEM to ascertain whether deletion of the protein resulted in an observable defect in the mutant spores. However, as shown in Figure 4.8, the BC1245 null spores appeared normal, indicating that the protein does not have a morphogenetic role in the assembly of the spore coat or exosporium.

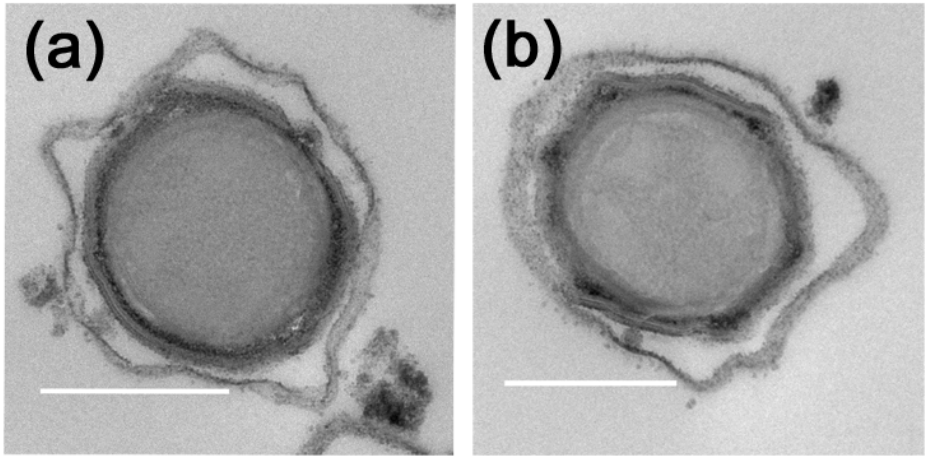


Figure 4.8 Thin section TEM images of (a) wild-type *B. cereus* 14579 spore; (b) *Δbc1245* spore. Size bar represents 500 nm

4.10 Assessing the germination properties of *B. cereus* BC1245 null spores

As mentioned in the introduction to this chapter, there are contrasting reports in the literature concerning the potential role of the BC1245 protein in *B. cereus* 14579 spore germination. With the construction of a clean null mutant strain the opportunity was taken here to re-assess the germination properties of these spores in this work. Figure 4.9 shows the germination response of wild type and the mutant spores in response to nutrient germinants (10 mM inosine and 100 mM L-alanine), whereas Figures 4.10 and 4.11 show germinative responses via the non-physiological Ca^{2+}-DPA and dodecylamine pathways, respectively.

In all cases the germinative responses of the mutant spores is comparable to germination of the wild type strain. Similarly, colony counts on rich medium are similar to those achieved with wild type spores (data not shown). However, it may be that the mutant spores are moderately defective when stimulated to germinate via the germinant receptor pathways, since the absorbance loss never reaches the same level as that achieved by wild type spores. However, neither microscopy analyses nor attempts made to characterise the kinetic parameters associated with these germinative responses delivered convincing evidence of a genuine defect (data not shown). The use of a single spore analysis method, such as the laser-tweezer raman spectroscopy method developed by Setlow and colleagues, as opposed to the population analysis methods employed in this work, is probably required to establish whether the germination phenotype of BC1245 null spores is truly defective.

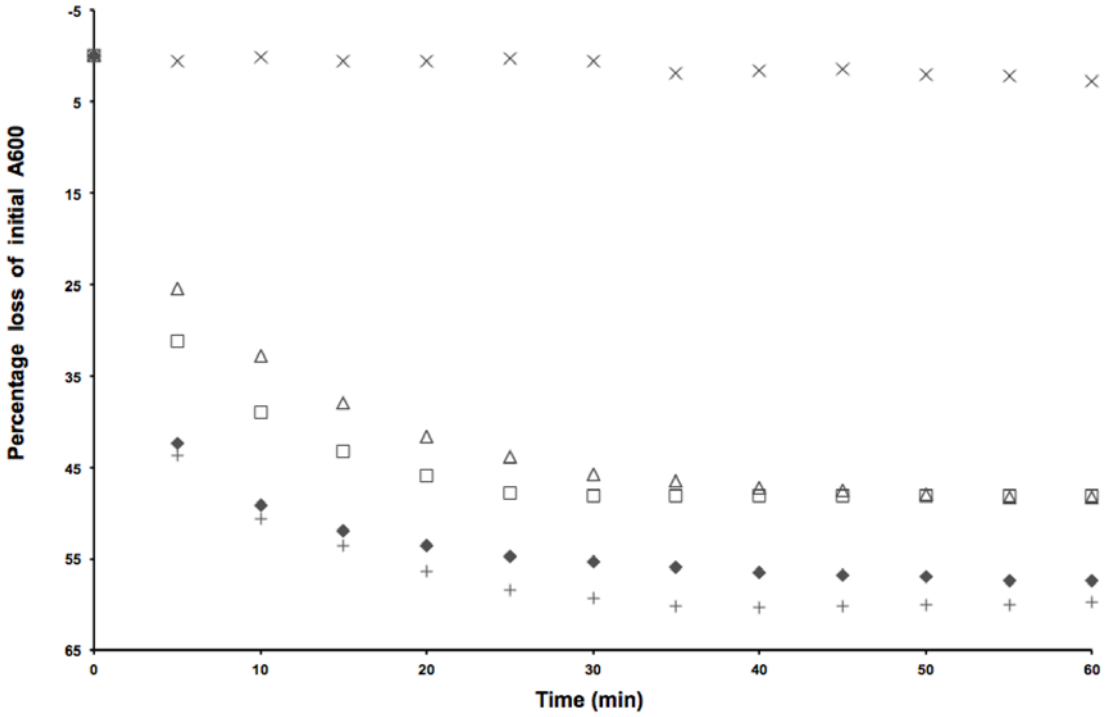


Figure 4.9 Germination of *B. cereus* BC1245 null and wild type spores in 100mM L-alanine and 10mM inosine. Spores were incubated in 10 mM Tris-HCl, pH 7.4, at 37ºC supplemented with the respective germinants. Key: (x) buffer; (Δ) BC1245 spores in 100mM L-alanine; (❑) BC1245 spores in 10mM inosine; (◆) wild type spores in 100mM L-alanine; (+) wild type spores in 10 mM inosine.

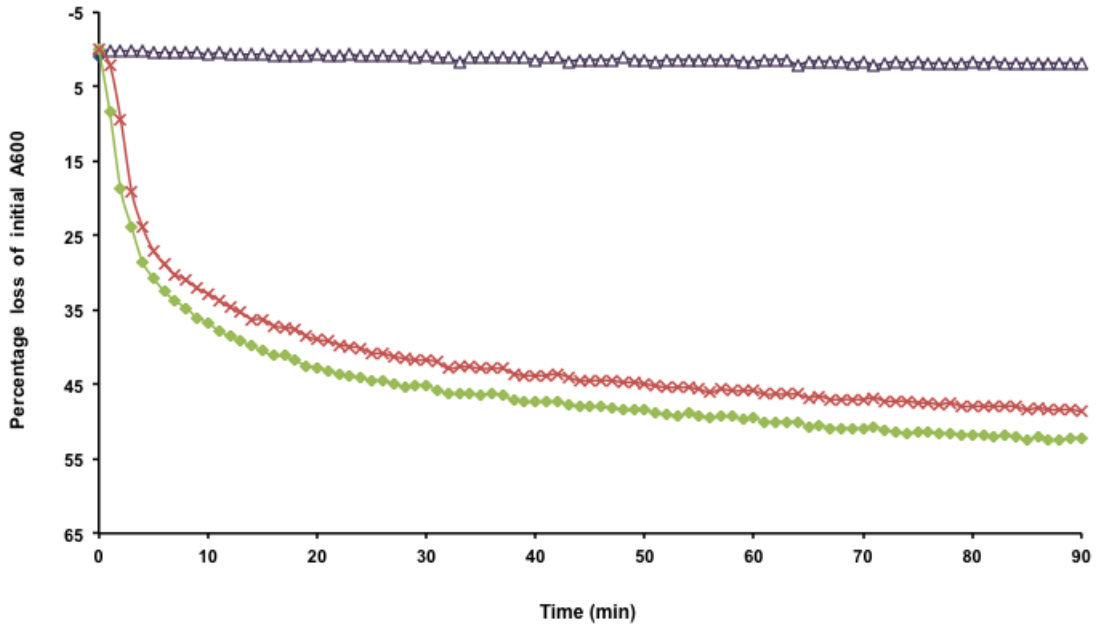


Figure 4.10 Germination of *B. cereus* BC1245 and wild type spores in response to Ca^{2+}-DPA. Assays were conducted at 37ºC for 1 hour in 60 mM Ca^{2+}-DPA. Key: (Δ) buffer; (◆) wild type spores; (x) BC1245 spores.

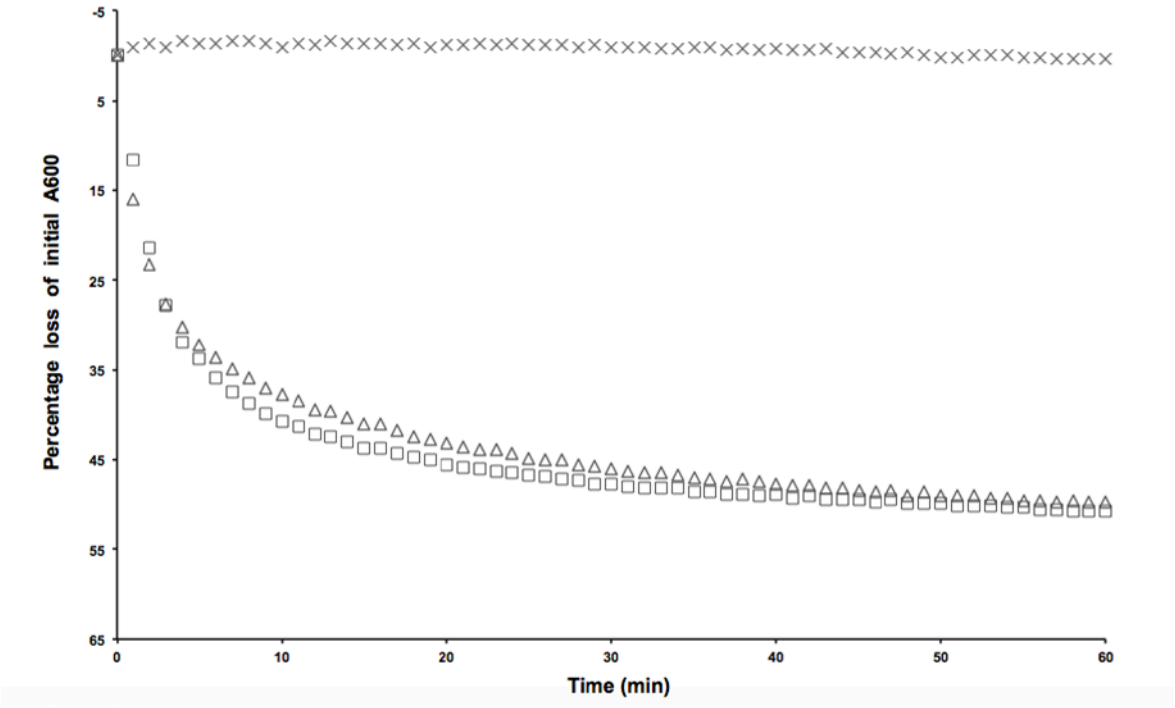


Figure 4.11 Germination of *B. cereus* BC1245 and wild type spores in response to dodecylamine. Assays were conducted at 40ºC for 1 hour in 1 mM dodecylamine. Key: (x) buffer; (❑) wild type spores (Δ) BC1245 null spores.

4.11 Conclusions

The objective of this chapter was to characterise the role, if any, of the BC1245 protein in *B. cereus* 14579 spores. A markerless null mutant and GFP (and SNAP) fusion strains were constructed for the first time to facilitate the study of this protein in *B. cereus* spores. Results can be summarised thus:

- RT-PCR based transcriptional analyses indicate that the protein encoded at BC1245 is expressed only during sporulation.
- Fluorescence microscopy analyses confirm that the protein is a component of the spore. Whereas immunolabeling experiments indicate that the protein is surface (or antibody) exposed, and therefore part of the exosporium, spores that have been partially disrupted by sonication retain their fluorescence in the absence of an exosporium, indicating that BC1245 is a component of the coat. A dual coat and exosporium location appears unlikely since shed exosporium material lacked any fluorescent signal.
- No obvious defect in spore structure was evident in thin section TEMs of BC1245 null spores indicating that it doesn't have a morphogenetic role in the assembly of the *B. cereus* 14579 coat or exosporium.
- BC1245 null spores germinate normally on rich medium and in response to the non-physiological germinants Ca^{2+}-DPA and dodecylamine. The germinative response to single trigger physiological germinants – L-alanine and inosine, respectively, may be associated with a modest defect, although single spore analysis techniques will be required to measure this precisely.

Chapter 5. Investigating the role of antiporter proteins in *Bacillus cereus* 14579 spore germination

5.1 Antiporter proteins and spore germination

Given that major changes in the permeability of the spore inner membrane to a range of small molecules, including ions, represents some of the earliest events in spore germination, a role for ion channel proteins is perhaps to be expected. Evidence for the involvement of such a spore-specific protein in germination was identified first in *B. megaterium*, where a 12872 strain bearing a transposon insertion in the grmA locus was shown to be defective in response to all single trigger germinant compounds[150]. Bioinformatic analyses revealed that the GrmA protein was a homologue of the NapA sodium-proton antiporter from *Enterococcus hirae*[150]. Subsequent mutagenesis led studies conducted in *B. cereus* 569 indicated that homologues of the GrmA protein – GerN and GerT – both have roles in the germination of spores of this species. The GerN protein seems to be particularly important since *B. cereus* spores that are null for this protein germinate relatively normally in response to alanine but show a defective germination response to inosine. Hence, it seems that the activity of the GerN protein is somehow linked specifically to the function of the germinant receptors that initiate the spore germinative response to inosine. In *B. cereus* 569 spores the GerI germinant receptor is responsible for initiating germination in response to inosine. This receptor is notable since the A-subunit of the receptor has an extended N-terminal domain comprising several tandem repeats of a glutamine and serine rich sequence hypothesised as having a potential role in interacting with other components of the germination apparatus[137]. In contrast, the orthologous GerT antiporter's activity was identified as being more subtle, appearing to have a role associated with vegetative outgrowth under alkaline or enhanced saline conditions.

Intriguingly, a later study insertional-mutagenesis conducted in the QM B1551 strain of *B. megaterium* spores failed to show any germination defect in spores null for GrmA, indicating that the role of antiporter proteins in spore germination may be species or even strain specific (equally, polar effects associated with the transposon insertion in the initial *B. megaterium* 12872 study cannot be discounted). However, subsequent unpublished work has identified significant germination defects in QM B1551 derived strains of *B. megaterium* engineered to have only single classes of germinant receptors,

conferring further evidence that in some cases the activity of an antiporter protein is associated with defined germinant receptors[167]. Given the degree of uncertainty that still exists over the role of antiporters in spore germination (*B. subtilis*, for example, appears not to use any) the objectives of work conducted in this chapter were to:

- Create markerless GerN and GerT null strains to examine the role, if any, of these proteins in spore germination in *B. cereus* 14579.
- Establish whether the extended 'linker-like' region of the A-subunit of the GerI receptor is important to the function of the inosine mediated germination response in *B. cereus* 14579 spores.

5.2 Bioinformatic analyses

A BLASTP search using *B. cereus* 10876 GerN and GerT as seeds revealed the presence of the respective orthologues in the 14579 strain. GerN is encoded at BC1612 and GerT at BC0838. The *B. cereus* 14579 GerN protein shares 99% sequence identity at the amino acid level with the orthologous protein present in the 10876 strain, whereas the GerT protein shares 74% identity. GerN comprises 387 amino acid residues and has a predicted molecular mass of 41 kDa, whereas GerT comprises 395 amino acids and has a predicted molecular mass of 42.5 kDa. An alignment of GerN and GerT from *B. cereus* 14579 is shown in Figure 5.1. For reference, the sequence identity of GerN with *B. megaterium* GrmA is 26%.

The GerIA germinant receptor protein was identified in a similar manner, using the orthologous protein from the 10876 strain as seed. The 14579 protein shares 85% sequence identity with the orthologous protein in the 10876 strain, and is predicted to include the extended N-terminal domain hypothesised as having a role in protein interactions [137](Figure 5.2).

Figure 5.1 Sequence alignment of GerN and GerT from *B. cereus* 14579 as conducted by Clustal Omega.

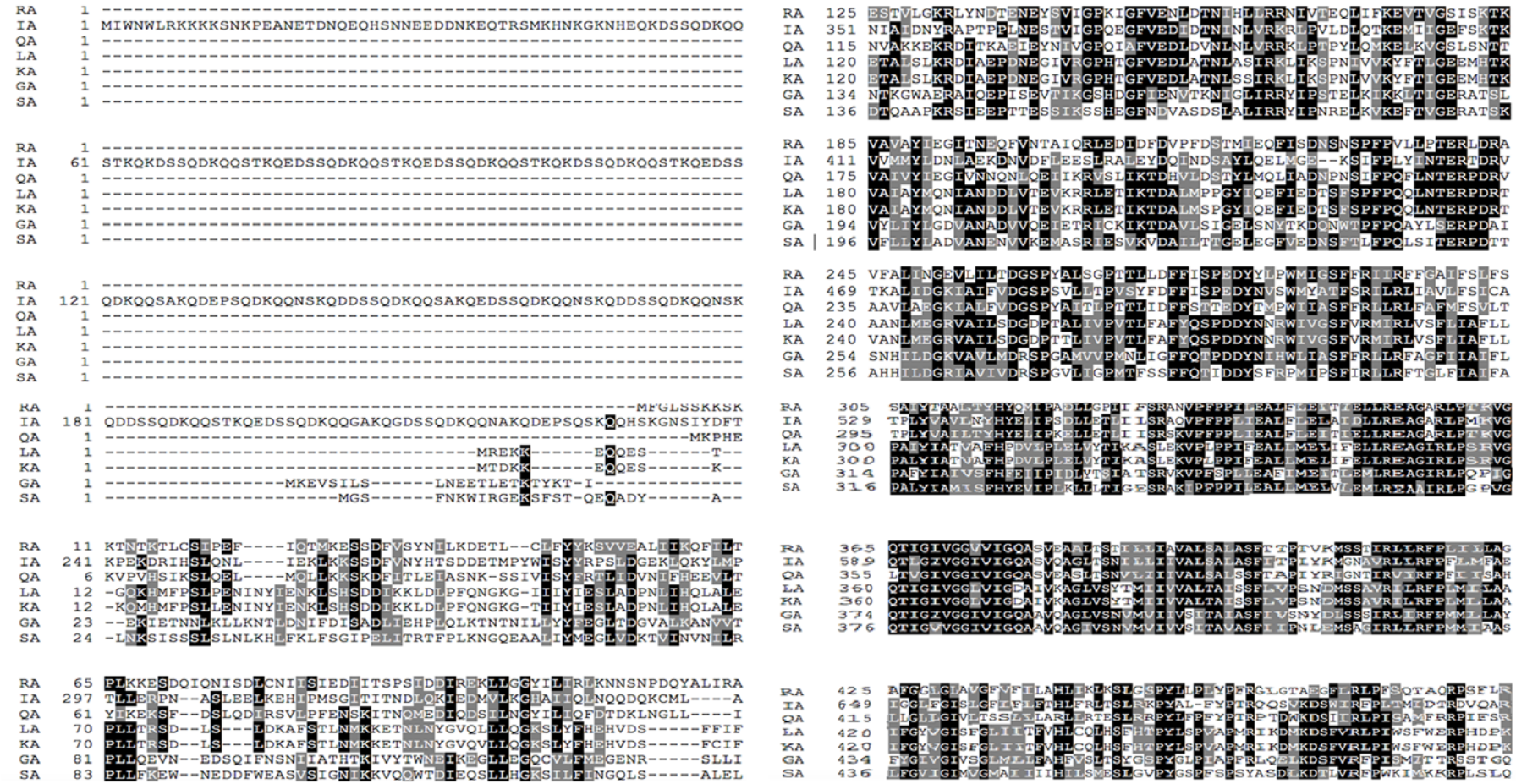


Figure 5.2 Clustal Omega sequence alignment of the seven germinant receptor A-subunit proteins present in *B. cereus* 14579.

5.3 Expression of GerN and GerT

In order to ascertain whether GerN and GerT are expressed in *B. cereus* 14579, and in which phase of growth, RT-PCR was conducted on cDNA samples collected at regular intervals throughout growth and sporulation. These analyses revealed that both genes are expressed during the latter part of sporulation, being initiated approximately five hours after entry to sporulation. Neither protein was expressed during vegetative growth, hence both are likely to be components of the spore. GerN-associated PCR products were more abundant than GerT-associated PCR products, indicating that expression of GerN may be at a higher level than GerT; equally, differences in band intensity may reflect a bias in the PCR reactions.

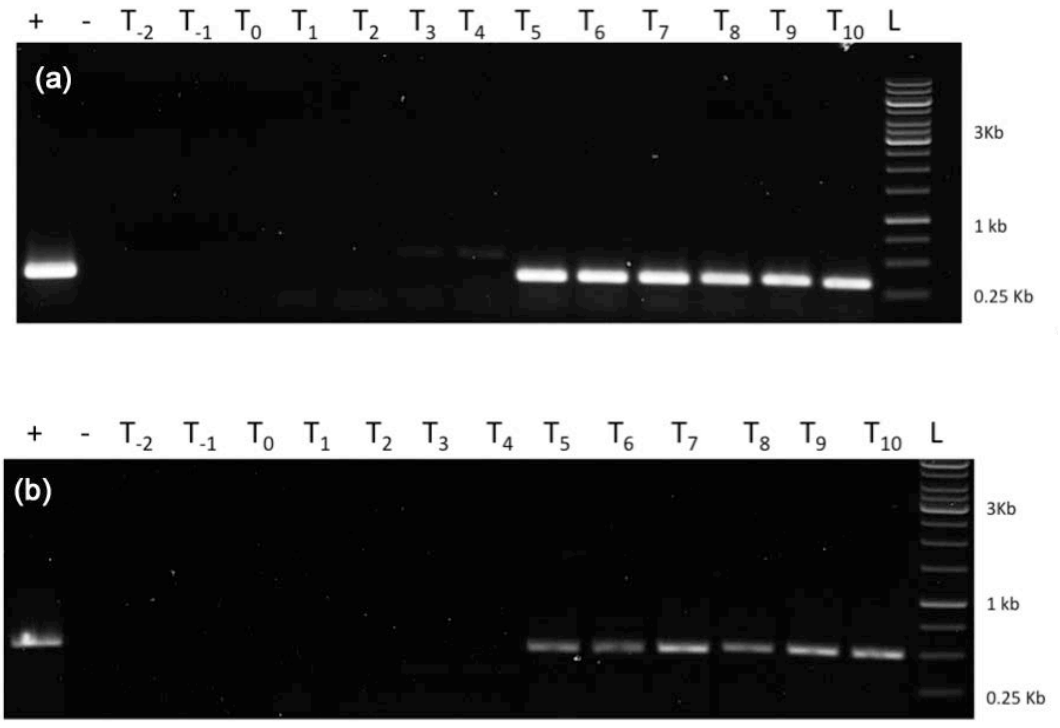


Figure 5.3 Transcriptional analysis of (a) *gerN*, and (b) *gerT* from *B. cereus* 14579 as determined by RT-PCR. Key: L, ladder; (+) genomic DNA positive control; (−) negative control (no DNA); t_2 - t_1 are time points before sporulation; t_0 – t_{10} are time points since the onset of sporulation.

5.4 Construction of GerN, GerT and GerI null mutant strains

The pMAD-facilitated method outlined in Chapter 2 and elsewhere in this thesis was used to generate markerless deletions of the entire open reading frames, except the start and stop codons, of *gerN* and *gerT* in *B. cereus* 14579 (Figure 5.4). A *gerN gerT* double mutant strain was conducted similarly. The same technique was used to delete the entire GerI operon, except the start codon of the *gerIA* gene and the stop codon of the *gerIC* gene (Figure 5.5).

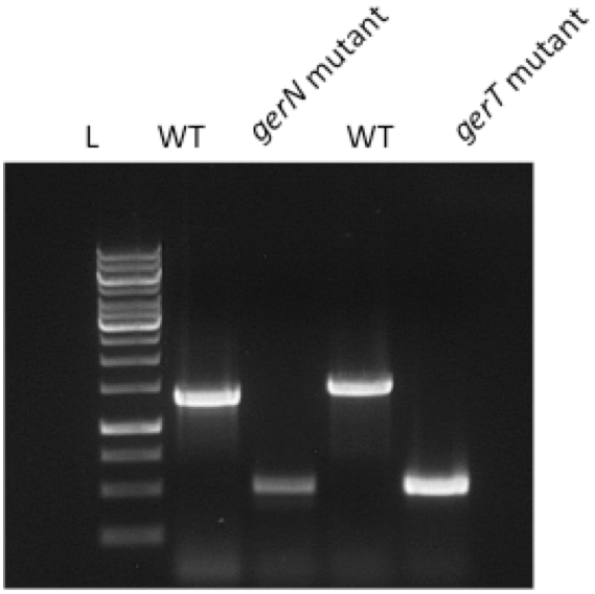


Figure 5.4 Agarose gel showing the PCR products derived from reactions conducted with primers that flank the *gerN* (BC1612) and *gerT* (BC0838) genes, confirming deletion of the bulk of the ORFs in the respective strains. L denotes size marker.

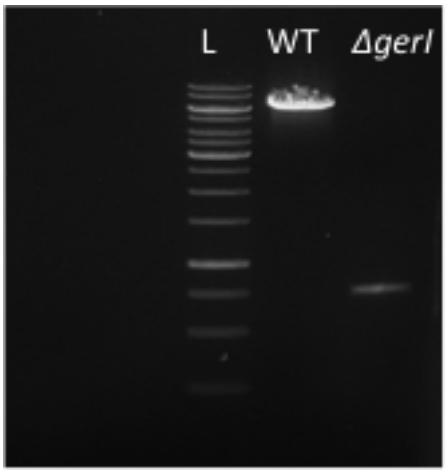


Figure 5.5 Agarose gel showing the products of a diagnostic PCR, conducted with primers that flank the operon, and confirming deletion of the genes that comprise the operon in the mutant strain. L denotes size marker.

5.5 Investigating the role of GerN and GerT in *B. cereus* 14579 spore germination

In order to assess the role of GerN and GerT proteins in the germination of *B. cereus* 14579 spores, spores of all three null mutant strains – gerN, gerT, and gerN gerT - were first prepared by nutrient exhaustion in CCY medium. Additional strains in which the deleted genes were restored by pHT315-derived plasmids were constructed to conduct complementation experiments and sporulated. In all cases, growth and sporulation appeared similar to that observed with the wild type strain. Spores of the various strains were subjected to a heat shock (80^{o}C for 20 min), cooled on ice, and then incubated in 10 mM Tris-HCl, pH7.4, supplemented with either 10 mM inosine, or a mixture of 10 mM inosine and 10 mM L-alanine. The resultant germination profiles, determined by monitoring absorbance at 600 nm, are shown in Figure 5.6 and 5.7.

The germination profiles reveal that GerN null spores have a germination defect, with an absorbance loss of approximately half of that achieved by wild type spores after 30 minutes (by which time the wild type spores have germinated completely). An absorbance loss of approximately 45% was achieved after 2 hours, but then plateaued. Microscopy analysis revealed that the spore population to comprise a mixture of phase dark and phase bright spores at this time point, indicating that some spores had germinated completely whereas others remained dormant. Spores that were null for GerT showed a similar pattern

of absorbance loss to the GerN mutant strain, indicating that this protein has a role in inosine mediated germination, and which is in contrast to the results reported for the 10865 strain[103]. The double mutant strain showed a serious germination defect, losing ~10 % absorbance after 2 hours of incubation, reinforcing the idea that both GerN and GerT are involved in inosine mediated germination in *B. cereus* 14579. The germination defect in the double mutant strain was complemented by plasmid borne copies of the genes. Similarly, the presence of alanine in the medium restored germination to levels comparable with wild type spores, suggesting that the alanine mediated response is not affected by deletion of *gerN* or *gerT*. Germination of *gerN* and *gerT* null spores and the *gerN: gerT* null mutant showed germination phenotype similar to the wild-types train in L-alanine suggesting alanine mediated response is not affected by deletion (Figure 5.8).

The influence of GerN and GerT on non-nutrient germination was also examined, in this case the response to the cationic detergent dodecylamine. Representative germination profiles are shown in Figure 5.9. In all cases, the loss of absorbance is slightly reduced in the mutant strains compared to wild type spores, although the defect – if any, is evidently subtle.

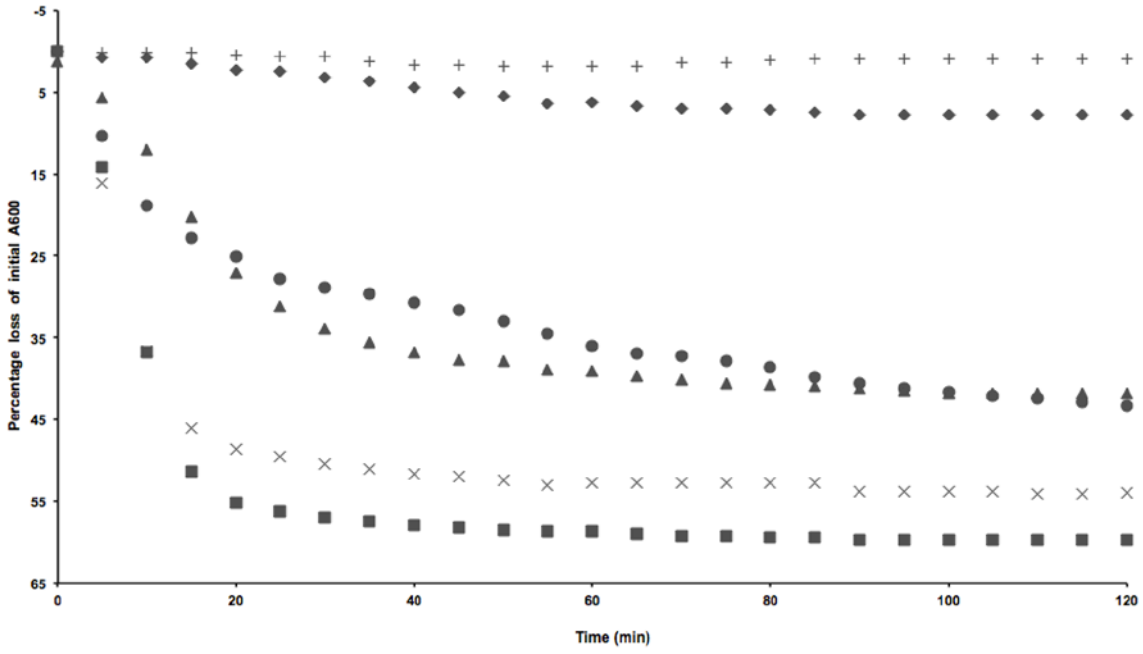


Figure 5.6 Germination of *B. cereus* 14579 strains null for *gerN* and/or *gerT* in 10 mM Tris-HCl, pH 7.4, supplemented with 10 mM inosine. Key: (+) buffer, (•) *gerN*, (Δ) *gerT*, (⬥) *gerN gerT* double mutant, (×) complemented *gerN gerT*, (■) wild type.

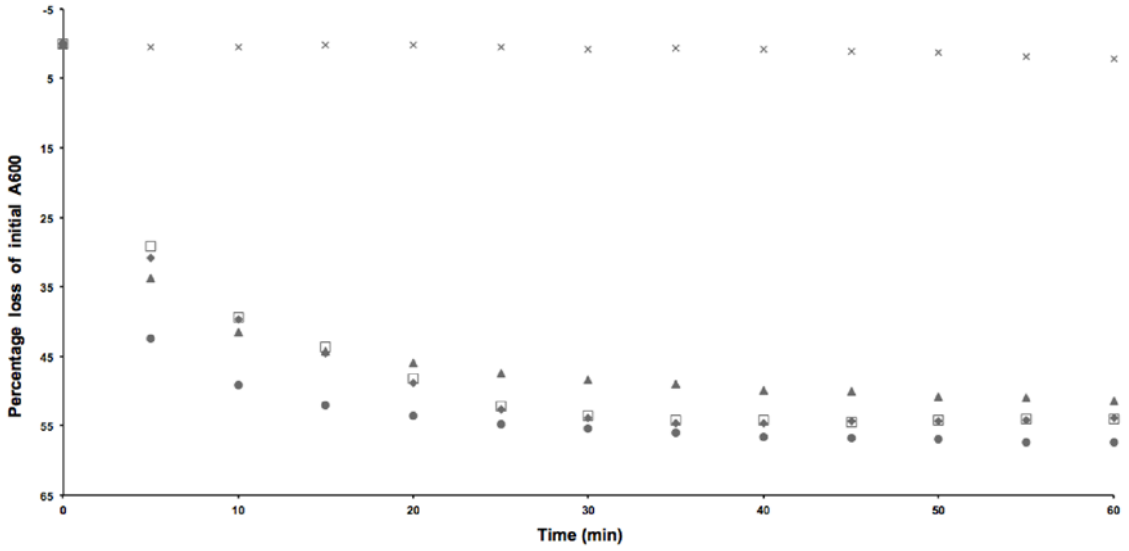


Figure 5.7 Germination of *B. cereus* 14579 strains null for *gerN* and or *gerT* in 10 mM Tris-HCl, pH 7.4 supplemented with 10 mM inosine and 10 mM L-alanine. Key: (×) buffer, (◆) *gerN*, (*) *gerT*, (Δ) *gerN gerT*, (•) wild type.

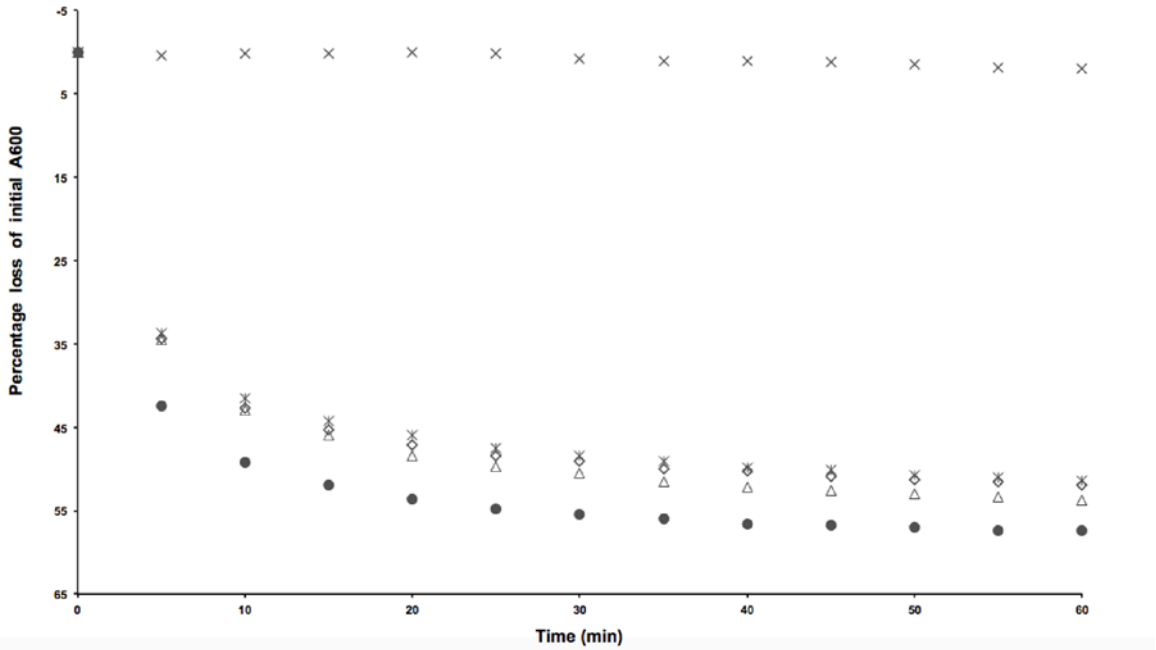


Figure 5.8 Germination of *B. cereus* 14579 strains null for *gerN* and or *gerT* in 10 mM Tris-HCl, pH 7.4 supplemented with 100 mM L-alanine. Key: (×) buffer, (◆) *gerN*, (*) *gerT*, (Δ) *gerN gerT*, (•) wild type.

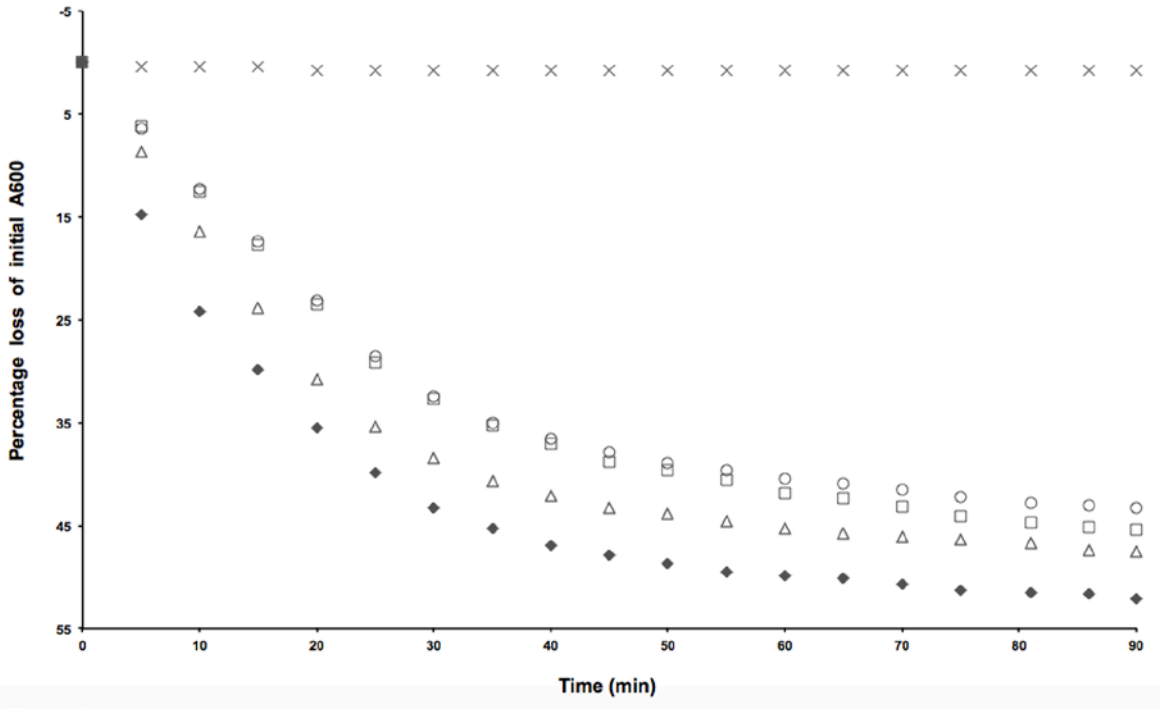


Figure 5.9 Absorbance loss associated with germination of *B. cereus* 14579 strains null for *gerN* and/or *gerT* in 1 mM dodecylamine. Key: (x) buffer, (Δ) *gerN*, (□) *gerT*, (o) *gerN gerT*, (◆) wild type.

5.6 Investigating the role of the GerIA N-terminal domain in spore germination

Results presented above indicate that both the GerN and GerT antiporter proteins are involved in germination that is triggered by the GerI inosine-responsive germinant receptor. The precise details concerning signal transduction events that occur downstream of germinant binding have not been elucidated, although one hypothesis is that the GerI receptor may interact with spore antiporters via the extended 'linker like' N-terminal domain of the GerIA protein. In order to test this hypothesis, first a 14579-derived strain in which the entire *gerI* operon was deleted was constructed (Figure 5.5). Complementation-type experiments were then conducted in this genetic background by constructing strains with (i) a plasmid borne copy of the intact *gerI* operon, and (ii) a variant *gerI* operon in which the N-terminal linker-like sequence of the gene encoding GerIA was deleted. The germination properties of the resultant spores were subsequently tested, as detailed in Figure 5.10.

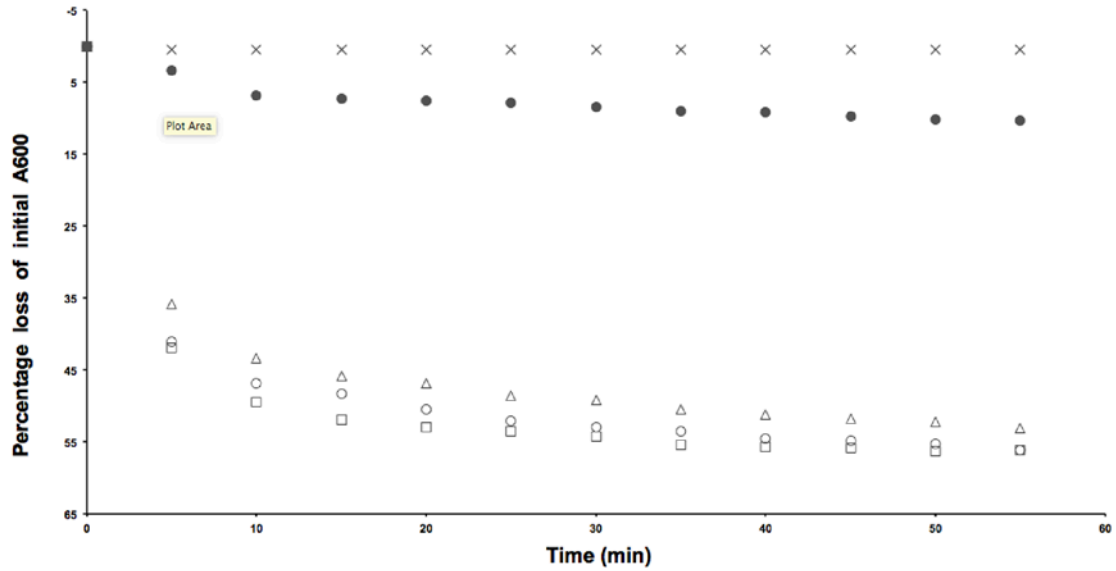


Figure 5.10 Germination of *B. cereus* 14579 *gerI* and *gerIA* variant strains. Spores were incubated in 10 mM Tris-HCl, pH 7.4, plus 10 mM inosine. Key: (×) buffer, (•) *gerI* in 10 mM inosine, (Δ) *gerI* complemented with native *gerI* in 10 mM inosine, (o) *gerI* mutant complemented with *gerI* including variant *gerIA*, (❑) wild type in 10 mM inosine,

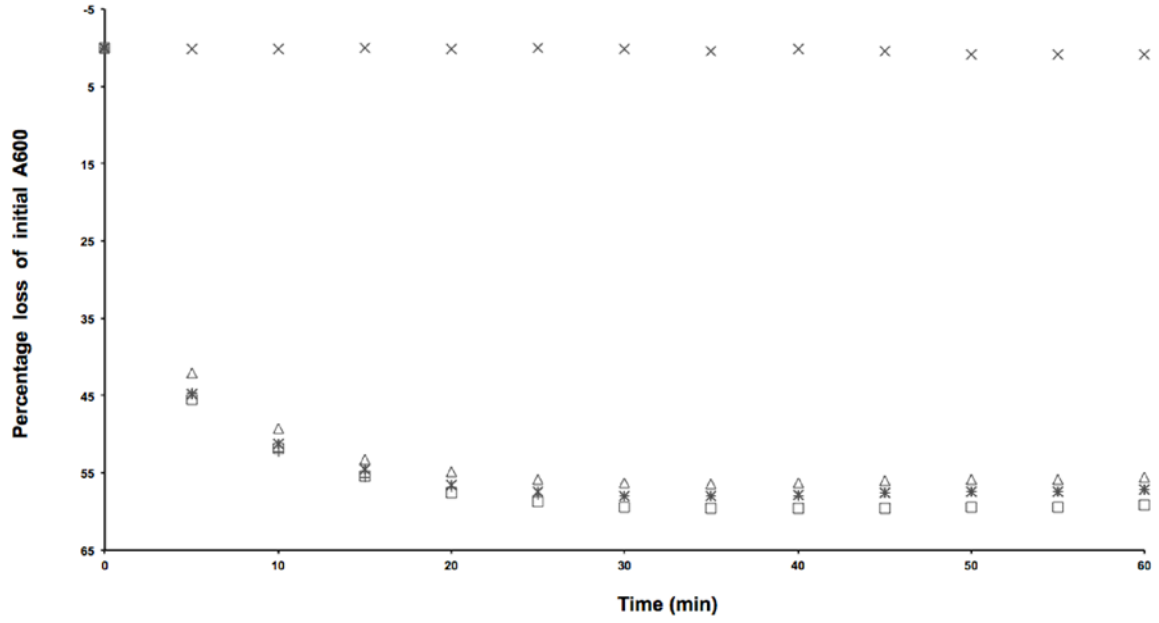


Figure 5.11 Germination of *B. cereus* 14579 *gerI* and *gerIA* variant strains. Spores were incubated in 10 mM Tris-HCl, pH 7.4, plus 100 mM L-alanine. Key: (×) buffer, (Δ) *gerI* mutant, (*) *gerI* variant, (❑) wild type in 100 mM L-alanine,

As shown in Figure 5.10, the *gerI* null spores were seriously defective in their germinative response to inosine, losing less than 20% absorbance after 1 hour (in contrast, the wild type spores have germinated completely within 20 minutes). In both cases, spores of strains complemented

with native or variant *gerI* operons germinated to a level that was comparable to the wild type spores. These data indicate that GerIA's N-terminal 'linker-like' region is not important in the receptor's function, and is presumably therefore not required to mediate hypothetical interactions with the GerN or GerT antiporters. Further *gerI* null spores and the *gerI* variants germinated similar to the germination profile of wild-type strain in L-alanine induced germination suggesting that *gerI* and GerIA's N-terminal 'linker-like' region is not important in the receptor's function.

5.7 Conclusions

The purpose of work conducted in this chapter of the thesis was to characterise the role of the GerN and GerT antiporter proteins in the germinative response of *B. cereus* 14579 spores. In contrast to observations made in the 10876 strain, where only *gerN* spores had a serious germination defect, both the GerN and GerT proteins appear to have a role in inosine mediated germination in spores of the 14579 strain. The defect seems to be associated with germination triggered via the GerI inosine-responsive germinant receptor, since non-nutrient germination was not affected in the mutant strains. However, the 'linker-like' N-terminal domain of the GerIA protein was shown to be dispensable to the GerI receptor function, ruling out any role for this unusual feature in mediating interactions with the GerN and GerT antiporters.

Chapter 6. Characterisation of BC1117 in *Bacillus cereus* 14579

6.1 Introduction

As detailed in the introduction to this thesis, sporulation is a highly sophisticated developmental process adopted by members of the Bacilli and Clostridia as a survival strategy to withstand extreme environmental conditions that normally do not support bacterial growth. Sporulation involves complex gene regulatory processes taking place inside the developing forespore and in the mother cell. The expression level of sporulation genes are fine-tuned by primary and secondary regulator proteins that are under the control of sigma factors, enabling feed forward loops in the regulatory system. The SpoVT protein is an example of such a regulatory protein. SpoVT is expressed in the forespore under the control of σ^G, enhancing the expression of some σ^G -dependent genes while repressing others[153]. A number of previous analyses conducted in *B. subtilis* revealed that disruption to SpoVT resulted in spores with defective spore coats, enhanced rates of nutrient-induced germination, increased sensitivity to UV radiation and a reduced ability for spore outgrowth[153,154].

In the course of this work an opportunity arose to contribute to a project in the Kuipers laboratory examining the role of SpoVT in *B. cereus*. In particular, transcriptomic analyses identified a number of genes, many of unknown function, that were significantly down regulated in the *spoVT* mutant background. These included the gene at locus BC1117, which was down regulated nearly 3,000 fold, and which is predicted to encode a small 50 residue, 50 kDa lysine-rich polypeptide. Hence, the objective of this part of the thesis was to begin to characterise the role of the putative BC1117 protein by constructing a null mutant strain and then examining the properties of the resultant spores.

6.2 Deletion of *B. cereus* BC1117

The pMAD-facilitated procedure outlined elsewhere in this thesis was used to introduce a markerless deletion of the entire open reading frame (minus the start and stop codons) at the BC1117 locus. PCR and sequencing was used subsequently to validate the correct construction of the strain (Figure 6.1).

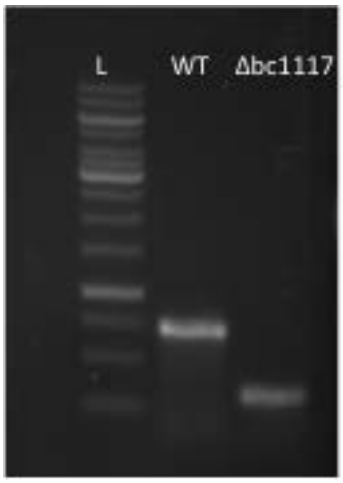


Figure 6.1 Agarose gel showing the products of a diagnostic PCR, conducted with primers that flank the BC1117 operon, indicating successful construction of the *B. cereus* null mutant strain.

6.3 Sporulation and spore properties

In order to ascertain whether deletion of the gene encoded at BC1117 resulted in a sporulation defect, the mutant strain was sporulated by nutrient exhaustion in CCY medium and examined throughout by phase contrast microscopy. The SpoVT null and wild type strains were examined concurrently for control purposes. As shown in Figure 6.2, the BC1117 null strain appeared to sporulate normally, resulting in the release of mature spores to the spent medium. Hence, in contrast to SpoVT – deletion of which results in sporulation that arrests before the formation of phase bright forespores – BC1117 is not an essential sporulation protein. The resultant spores were tested for resistance to heat (80^{o}C for 30 min), chloroform, and lysozyme. In all cases colony recovery on LB medium was similar to wild type spores (data not shown). The germination properties of the BC1117 null spores were also examined (Figure 6.3). The resultant germination profiles in response to inosine and L-alanine, respectively, were again, similar to wild type spores, indicating that BC1117 has no role – directly or otherwise - in spore germination.

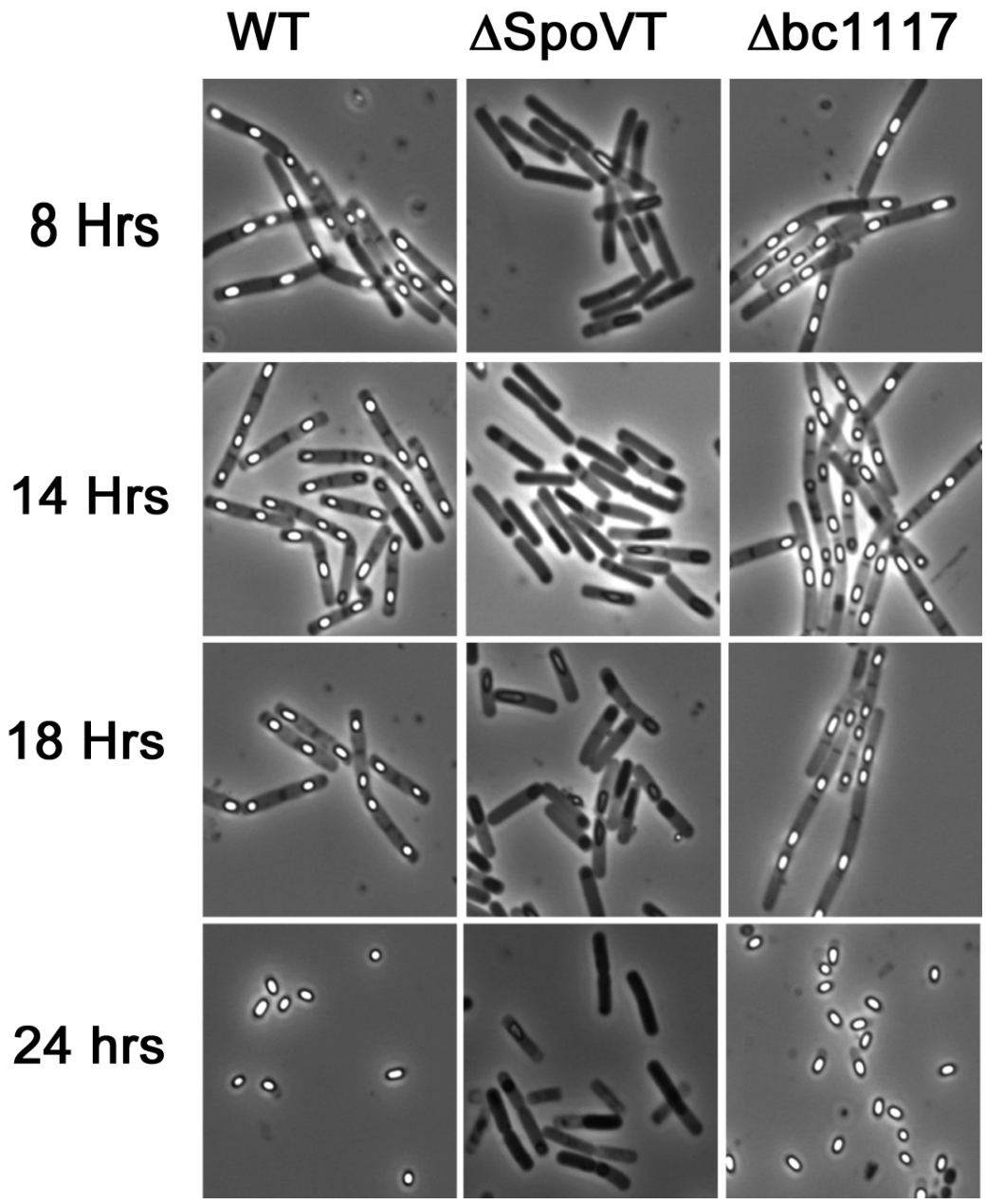


Figure 6.2 Time course analysis of sporulating *B. cereus* wild type, SpoVT and BC1117 null mutant strains in CCY medium.

6.4 Localisation of BC1117

A *B. cereus* strain engineered to express a C-terminal BC1117-GFP fusion protein was created by introducing a pHT315 plasmid borne copy of the fusion construct under control of native regulatory sequences to the 14579 background. The resultant strain was monitored by fluorescence microscopy throughout sporulation. Unfortunately, although

sporulation proceeded as normal, no fluorescent signal could be detected at any stage during growth or in the mature spores (Figure 5.3).

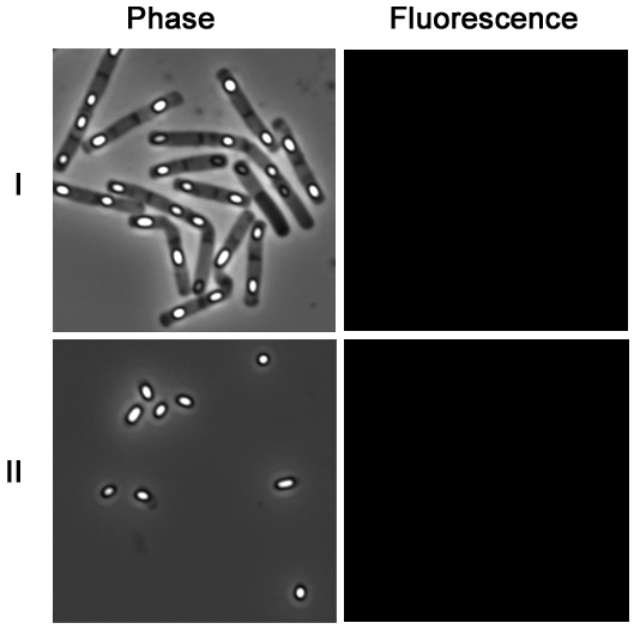


Figure 6.3 Light and fluorescence microscopy analysis of sporulating cells and mature spores of the *B. cereus* BC1117-GFP strain. No fluorescent signal could be detected, precluding attempts at localising the protein within the spore by this method.

6.5 Conclusions

The objective of this chapter was to characterise the protein encoded at the BC1117 locus in *B. cereus* 14579. Expression from this locus is markedly down regulated in the SpoVT null background, in which sporulation arrests before completion, hence it was hypothesised that BC1117 may have a role in sporulation. However, deletion of the ORF had no discernable effect on sporulation or on the properties of the resultant spores. Rather than being a sporulation protein, the small lysine-rich nature of the predicted protein indicates instead that it may be a coat or exosporium protein. However, a GFP-led attempt at localising the protein proved to be unsuccessful.

Chapter 7. Conclusion and Discussion

Understanding the process of germination of bacterial spores remains of the utmost importance due to the ability of a number of pathogenic gram-positive bacteria to cause various foodborne illnesses, nosocomial infections and other pathologies in humans and animals. *B. cereus* spores are a major biological hazard to the food industries. Despite its prominence as a key member of the *Bacilli*, due to this importance as food-borne pathogen and close relation to *B. anthracis*, the causative agent of anthrax, its coat proteins are less extensively studied. Continuous efforts have been made by the researchers across the globe to develop strategies to kill the spores without adversely affecting the food components with very minimal significant achievement to date. Despite intense research for decades, the knowledge of the molecular mechanisms that underpin the process of spore germination remains incomplete. In particular, details of the biochemical and structural mechanisms that are involved at the earliest stages of spore germination remain to be elucidated. However, the past few decades has evidenced an increased momentum in molecular, structural and genetic approaches that has immensely contributed towards our understanding in the overall process of germination. The purpose of the current study was to add to this momentum, with the intention of adding fresh insight to various germination proteins. In this thesis we have employed the molecular-genetic approach for the investigation of a novel exosporium protein (BC1245), GerN proteins, GerIA protein and the six GerP proteins encoded by a hexacistonic operon. This final chapter summarizes the main results obtained and prospects of future research in this area. To paraphrase from the introductory chapter, defined objectives of the study in this thesis were to:

a) Employ a range of bioinformatics and molecular-genetic techniques to characterise the GerP proteins in *B. cereus* ATCC14579.

b) Investigate and characterise the role, if any, of a novel *B. cereus* group-specific spore protein BC1245.

c) Investigate the role of GerN protein, its homologue, GerT and the extended N-terminal of GerIA protein in *B. cereus* 14579.

Considerable progress was made with all three objectives and a number of main conclusions can be drawn from the presented data.

Bacterial spores of all species are presented with a dilemma in that their protective structures must prevent ingress of potentially damaging molecules or chemicals to the cellular protoplast within while permitting access of small molecular germinant molecules to the inner-membrane bound germinant receptors. Additionally, they must be capable of rapidly releasing solutes from the spore core, including CaDPA, to the environment upon spore germination. This is achieved, at least in part, by the presence of a multi-layered coat and in some cases exosporium structures, one function of which is to serve collectively as molecular sieves. The sieving properties vary between species, but in general it seems that moderately sized proteins and other molecules can breach the outermost layers of spores, exemplified by recent work showing transit of the 26 kDa red fluorescent protein through the exosporium of *B. megaterium* spores and of the outer coat of *B. subtilis* spores – but not the inner coat. These observations are consistent with earlier studies, which imply that permeability decreases with progression towards the interior of the spore.

Results of germination experiments conducted in chapter 3 with *B. cereus* 14579 spores that are null for the entire *gerP* operon, or individual genes therein, are similarly consistent with previous studies and with the suggestion that the GerP proteins influence the permeability of the spore coat to small hydrophilic molecules. Defective germinative responses are observed, for example, whether induction is via stimulation of the nutrient germinant receptors by alanine or inosine, or in response to exogenous CaDPA or dodecylamine. The latter is in contrast to results observed in *B. subtilis*, where dodecylamine stimulated a faster germinative response in *gerP* spores compared to wild type spores. Dodecylamine is known to trigger germination by stimulating the opening of inner-membrane located DPA channels that are present in both *B. subtilis* and *B. cereus*, hence it is not clear why deletion of *gerP* should exert differing germinative responses between species. The exception to the above concerns *B. cereus gerPF* null spores, which display essentially wild type germinative responses when stimulated via nutrient or non-nutrient pathways. Presumably the loss of *gerPF* in this case was compensated by either of the two additional *gerPF* homologues encoded elsewhere on the chromosome, as demonstrated previously in closely related *B. anthracis* spores.

A primary objective of this study was to ascertain whether the GerP proteins are structural components of the spore or whether they are involved only in spore assembly.

Fluorescence microscopy of strains expressing GerP-GFP fusion proteins supported the notion that GerP proteins are indeed located in the spore coat, and the images of smooth fluorescent rings appeared suitable for ELM image analysis using a spherical shell model as an approximation to the GerP-GFP location. We then used ELM to more precisely locate the GerP proteins with respect to some other coat and exosporium proteins. These included the BclA exosporial nap protein, coat-localised CotD, and the CLEs CwlJ and SleL. The radial location of each of these benchmark proteins was established using ELM: BclA, as expected, was outermost, followed by CotD, and then SleL and CwlJ. The SleL and CwlJ proteins occupied a similar radial location around 450 nm, which we identify as the position of the inner coat. Overall, we found that all the GerP proteins were also located in the inner coat. Our analysis of GerP locations in mature spores was, unfortunately, complicated by the fact that the spores tended to clump and hence the number of well-separated spores available for analysis was limited. Additionally, even the available images were often poorly-fitted due to adjacent fluorescent material. The high residual fitting errors shown in Figure 3 indicate that the radial locations found for the GerP proteins in mature spores (407 to 454 nm) have limited accuracy and may be biased by image analysis limitations. In order to obtain more accurate estimates of GerP location, additional fluorescence microscopy was conducted with sporulating GerP-GFP cells in which the fluorescent proteins were observed to have completely localised to rings around phase bright forespores, but prior to mother cell lysis. We obtained superior fluorescence brightness with these samples, and much more importantly we found this removed many of the difficulties due to spore clumping and enabled a higher number of cells to be analysed. Subject to the assumption that the GerP protein locations in these specimens are consistent with their locations in mature spores, we believe this method more accurately located the GerP proteins. These results, in which the GerP protein radial locations span a narrower range from 440 nm to 454 nm, indicate that all six proteins remain in the inner coat, but now seem more likely to share proximity with CwlJ (447 nm) and SleL (456 nm) within the spore. This is an important distinction, since the location of CLEs in the inner spore coat could pose a mechanistic problem as to how they access the cortical substrate during germination, especially if the GerP proteins form a proteinaceous layer that lies between the CLEs and the cortex/outer membrane boundary. A future objective will be to improve the resolution of these measurements to definitely ascertain the location of CLEs with respect to the cortex.

The present study has additionally conferred evidence for an apparently limited degree of interdependency between the various GerP proteins for localisation in the developing spore. Indeed, fluorescence microscopy analyses conducted with a series of null mutants and GFP-expressing strains indicate that only the GerPA protein is required for each of the remaining GerP proteins to localise in the developing spore. Equally, we cannot rule out that one or both of the additional GerPF homologues encoded on the chromosome permit localisation of GerPA-GerPE in the *gerPF* null mutant; this is something that will have to be tested in future. Regardless, we can infer from these analyses that the GerPB, GerPC, GerPD and GerPE proteins each have an essential role in maintaining permeability of the inner coat to permit ingress of germinants to their sites of interaction with receptors at the inner membrane. GerPA is also essential, although whether this goes beyond its requirement for localisation of the remaining GerP proteins, or whether it has an additional function relating to spore permeability, has yet to be established.

Overall, the results of the work support the idea that the GerP proteins have a structural role within the spore coat that facilitates the diffusion of small hydrophilic molecules. A number of factors indicate that this may be achieved by the formation of an extended complex that circumscribes the inner spore coat, perhaps with GerPA forming a template that is the site for essential interactions with the remaining GerP proteins. Factors to support this hypothesis include the genetic organisation of the genes as an operon, the shared proximity and strict interdependence on the GerPA protein for localisation of the remaining GerP proteins within the spore, and the requirement for each of the proteins encoded within the operon for maintenance of coat permeability. Preliminary experiments aimed at revealing physical interactions between various GerP proteins using a bacterial-2-hybrid system were unsuccessful, owing in part to the relative insolubility of at least some of the proteins when expressed in *E. coli* (data not shown). Hence, the challenge moving forward will be to identify techniques that enable structural and more precise functional insights to the GerP proteins within *Bacillus* spores.

In terms of the first objective dealt with in Chapter 4, BC1245 is a novel spore protein present only in the members of *B. cereus* family. It is encoded by a monocistronic gene and is highly conserved among the members. Fluorophore-based expression and localisation of the protein where BC1245 was subjected to C-terminal GFP fusion in a WT background showed non-uniform fluorescence along the spore shell with one pole of the

spore having unusual bright fluorescent foci. Another strain of *B. cereus* was constructed with SNAP tag at the C-terminal of BC1245. Fluorescence was observed in this strain as well with non-uniform fluorescence along the spore circumference with one pole of the spore having a bright fluorescent focus. This result is similar to the one observed in BC1245-GFP fusion protein. Anti-GFP assay was conducted on the spores expressing BC1245-GFP fusion protein to check the surface localisation of the BC1245-GFP fusion proteins. The spores tested positive for the surface localisation of the fusion proteins. However, the sonication conducted on the spores expressing the fusion proteins resulted in the shedding of the exosporium. When the sonicated spores were observed under the fluorescence microscope, fluorescence was still detected in the spores suggesting that the BC1245 protein was not localised in the exosporium but the spore coat. The antiGFP assay was showing positive result possibly because the anti-GFP antibodies employed for the assay were able to transverse through the loose exosporium and were able to react with the GFP protein expressed as fusion protein along with BC1245 located on the spore coat. ELM was attempted on the spores expressing the fluorescent fusion proteins but gave unreliable results (data not shown). This could possibly be due to the formation of fluorescent foci on one of the pole of the spores.

Transcriptional analysis to detect the expression of BC1245 gene revealed the expression of BC1245 throughout the sporulation phase (onset of sporulation until free spores were released) but was absent in vegetative cells. This clearly suggests that BC1245 is predominantly a spore protein. Spores of *Δbc1245* DCO mutants were tested with Ca^{2+}-DPA induced germination and also with dodecylamine induced germination, but these treatments did not affect the germination of *Δbc1245* spores, suggesting the lack of role played by BC1245 in non-nutrient based germination. TEMs of the *Δbc1245* did not show any obvious coat defect in the spores.

Studies on GerN protein showed that both GerN and the homologue of GerN called as GerT are involved in inosine-based germination. Mutational analysis on Δ*gerN* revealed a slower germination phenotype with germination to 45% in 4 hours. When *gerT* was deleted in the Δ*gerN* resulting in a double mutant strain and analysed for germination activity, this resulted in a severely disrupted germination phenotype in inosine-induced

germination. The double mutant when tested for germination in a combination of inosine+alanine resulted in a germination of 45% suggesting that alanine helped restore the germination of the double mutant. Also, it suggests that the double mutant strain is defective to only inosine-induced germination and not the alanine-induced germination. Thus GerN and GerT protein should be involved in germination induced by inosine as a sole germinant. The double complemented strain of *gerN* + *gerT* under their native promoters helped restore the germination phenotype and spores germinated to 50%. This clearly depicts the role of *gerN* and *gerT* genes in inosine-induced germination of *B. cereus* spores.

The role of *gerI* operon was further investigated in Δ*gerI* DCO mutant where the mutant showed severely disrupted germination in inosine-based germination. The Δ*gerI* mutant spores germinated normally and similar to WT spores in L-alanine induced germination. Normal germination of the mutant in inosine was restored when the mutant was complemented with the *gerI* operon with the promoter in an episomal plasmid suggesting the role of the *gerI* operon in only inosine-induced germination. GerIA protein possesses an extended N-terminal of unknown function. In order to assess the possible role of this extended N-terminal of GerIA protein in inosine-based germination, Δ*gerI* mutant was transformed with *gerI* operon possessing a truncated *gerIA* under the native promoter in an episomal plasmid and the resulting strain was tested for germination behaviour using inosine as the germinant. The truncated strain germinated similar to the WT and full *gerI* complemented strain suggesting that the extended N-terminal possibly does not play a role in inosine-based germination.

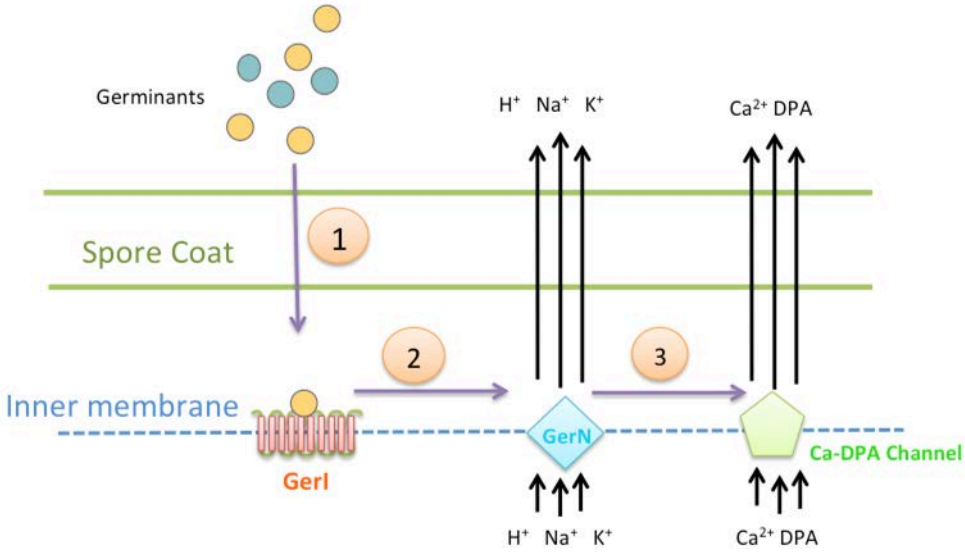


Figure 7.1 Schematic representation of the role of GerI, GerN and GerT proteins in *B. cereus* spore germination. (1) Entry of germinants through the spore coat and binds to cognate receptors located in the inner membrane, (2) Binding of the germinant to GR triggers the GerN to release monovalent ions from the spore core, (3) Release of Ca^{2+}-DPA from the spore core through SpoVA channels.

Results presented in Chapter 6 shows the role of *bc1117* gene in the sporulation of *B. cereus* ATCC 14579 strain. Transcriptomic analysis showed that this gene is severely down regulated in Δ*spoVT* mutant (a protein that is crucial for efficient sporulation of *B. cereus* spores). This chapter aimed to understand the role of *bc1117* gene in sporulation. Mutational analysis conducted in the DCO mutant of this gene revealed no obvious sporulation defect suggesting that the gene might not be directly involved in the process of sporulation. Fusion of GFP to the C-terminal of this protein with an aim to conduct localisation studies were not successful, as the fusion proteins did not express in the *B. cereus* spores engineered to produce the fusion protein.

To conclude, this thesis aimed to characterise several spore-specific proteins, most of which are involved in the process of germination in *B. cereus* ATCC 14579 spores. Several new and exciting findings were observed during this study especially for the GerP proteins and BC1245 protein. Studies were also conducted with an aim to fill-in the gaps in understanding the role of various spore germination proteins such as extended N-terminal of GerIA protein. However, many key questions remain unanswered such as, do the GerP

proteins interact and do they form a structure that affects the permeability of the spore coat. Indeed, the outputs from this work have raised several new lines of enquiry that merit further investigation.

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